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ORIGINAL ARTICLE

Fengming Song · Robert M. Goodman

OsBIMK1, a rice MAP kinase gene involved in disease resistance responses

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Abstract The activation of mitogen-activated protein kinases (MAPKs) has been previously implicated in signal transduction during plant responses to pathogen attack as well as to various environmental stresses. We have isolated from rice a new MAPK cDNA, *OsBIMK1* (O*ryza sativa* L. BTH-induced MAPK 1), which encodes a 369-amino-acid protein with moderate to high nucleotide sequence similarity to previously reported plant MAPK genes. OsBIMK1 contains all 11 of the MAPK conserved subdomains and the phosphorylation-activation motif, TEY. We analyzed in detail the expression of *OsBIMK1* upon treatment with various chemical and biological inducers of resistance responses in rice and in both incompatible and compatible interactions between rice and *Magnaporthe grisea*. Expression of *OsBIMK1* was activated rapidly upon treatment with benzothiadiazole (BTH) as well as with dichloroisonicotinic acid, probenazole, jasmonic acid and its methyl ester, *Pseudomonas syringae* pv. *syringae*, or wounding. Expression of *OsBIMK1* was induced rapidly during the first 36 h after inoculation with *M. grisea* in BTH-treated rice seedlings and in an incompatible interaction between *M. grisea* and a blast-resistant rice genotype. BTH treatment induced a systemic activation of *OsBIMK1* expression. These results suggest that *OsBIMK1* plays an important role in rice disease resistance.

The nucleotide sequence data of the *OsBIMK1* gene have been deposited in the GenBank database under accession number AF332873

F. Song · R.M. Goodman (✉)
Department of Plant Pathology, Russell Laboratories,
Room 689, 1630 Linden Drive, University of Wisconsin,
Madison, WI 53706, USA
E-mail: rgoodman@facstaff.wisc.edu
Fax: +1-608-2628643

Present address: F. Song
Department of Plant Protection,
College of Agriculture and Biotechnology,
Zhejiang University-Huajiachi Campus,
Hangzhou, Zhejiang 310029, P.R. China

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Abbreviations BTH: benzothiadiazole · HR: hypersensitive response · INA: dichloroisonicotinic acid · JA: jasmonic acid · MAPK: mitogen-activated protein kinase · MeJA: methyl jasmonate · *OsBIMK1*: *Oryza sativa* L. BTH-induced MAPK 1 · SA: salicylic acid · SAR: systemic acquired resistance

Introduction

Biochemical and physiological studies have produced a large body of evidence defining numerous early signaling events in plant defense responses, including the oxidative burst, calcium fluxes, ion channel fluxes, G-proteins, nitric oxide production, and phosphorylation and dephosphorylation of unknown proteins (Blumwald et al. 1998; Durner and Klessig 1999). The fact that the disease-resistance genes *Pto* and *Xa21* contain kinase domains or have kinase activity (Martin et al. 1993; Song et al. 1995) suggests a key role for protein phosphorylation in perception of pathogen signals and the subsequent signaling pathways in plants. Phosphorylation of the downstream effectors has been demonstrated to be a major mechanism in the *Pto*-mediated signaling pathway of tomato (Zhou et al. 1995). Reversible protein phosphorylation, catalyzed by protein kinases and phosphatases, has also been implicated, through the use of their inhibitors, in the generation of reactive oxygen species, activation of defense responses and development of the hypersensitive response (HR; Adam et al. 1997; Suzuki et al. 1999).

The mitogen-activated protein kinase (MAPK) cascade is a universal signal transduction pathway found in both unicellular and multicellular eukaryotes (Roberts et al. 2000). MAPK cascades usually comprise three protein kinases. The activation of serine/threonine MAPK occurs via phosphorylation of conserved threonine and tyrosine residues in the catalytic

subdomain VIII (TEY) and is affected by dual specific MAPK kinases (MAP2Ks), which in turn are activated by serine/threonine MAP2K kinases (MAP3Ks). MAPK cascades have been shown to function in various signal transduction pathways, including stress responses in animals and yeast, where the cascades have also been elucidated (Kyriakis and Avruch 1996; Roberts et al. 2000).

MAPK cascades are present in higher plants and play an important role in signal transduction in response to hormones and biological signals as well as in environmental stresses such as wounding, cold, salt, drought, oxidative stress and ozone (Hirt 1997; Stratmann and Ryan 1997; Berberich et al. 1999; Seo et al. 1999; Ichimura et al. 2000; Mikolajczyk et al. 2000; Mockaitis and Howell 2000; Samuel et al. 2000; Yuasa et al. 2001). Evidence is now accumulating that MAPK cascades are also involved in signal transduction leading to activation of plant defense responses. Two MAPKs, a 48-kDa salicylic acid (SA)-induced protein kinase (SIPK) and a 44-kDa wound-induced protein kinase (WIPK) were found to be activated in tobacco during infection with tobacco mosaic virus (TMV; Zhang and Klessig 1998a) and in tobacco suspension cells induced by treatment with the Avr9 peptide elicitor to express tomato *Cf-9* (Romeis et al. 1999). These results indicate that MAPK cascades may play a role in *R* gene-mediated signal transduction pathways. Not only the activity but also the expression of the gene for WIPK was activated upon induction with some defense-inducing treatments such as SA, ethylene, jasmonate and nitric oxide in tobacco suspension cells (Zhang and Klessig 1997, 1998b; Kumar and Klessig 2000; Zhang et al. 2000). Moreover, fungal, microbial and chemical elicitors were also shown to activate MAPK in *Arabidopsis*, parsley and tobacco (Ligterink et al. 1997; Takezawa 1999; Cardinale et al. 2000; Nuhse et al. 2000; Desikan et al. 2001).

Recently, two MAPK kinase (NtMEK2 and SIPKK) genes have been cloned from tobacco (Liu et al. 2000; Yang et al. 2001). Expression of a constitutively active mutant of MAPK kinases induces HR-like cell death and activates defense-related gene expression (Ren et al. 2001; Xing et al. 2001; Yang et al. 2001; Zhang and Liu 2001). On the other hand, it has also been found that MAPK cascades may regulate negatively the defense responses and disease resistance in plants (Petersen et al. 2000; Frye et al. 2001).

Recently, an MAPK gene, *BWMK1*, was cloned and identified in rice and its expression was found to be induced as early as 4 h after infection with *Magnaporthe grisea*, the causal agent of rice blast (He et al. 1999). Here we report the cloning of a novel MAPK gene, *OsBIMK1* [O*ryza* s*ativa* L. benzothiadiazole (BTH)-induced MAPK 1], from rice and its expression in defense responses induced by chemical and biological inducers, as well as in rice–*M. grisea* interactions. Expression of *OsBIMK1* was rapidly activated by various inducers and in the incompatible interaction between rice and *M. grisea*. Our results suggest that *OsBIMK1* may play an important role in signaling pathways leading to disease resistance in rice.

Materials and methods

Treatment of rice seedlings with inducers and inoculation with *M. grisea*

Rice (*Oryza sativa* L. cv. Yuanfengzao; kindly provided by Dr. Zheng Zhong, Zhejiang University, China) seedlings were grown in a growth chamber (16 h light daily, 244 μmol photons m^{-2} s^{-1}; 22 °C in darkness, 27 °C in light). Three-week-old seedlings were treated by spraying with solutions of benzothiadiazole (BTH; Novartis Crop Protection Inc., Research Triangle Park, N.C., USA), dichloroisonicotinic acid (INA; Novartis Crop Protection Inc.), salicylic acid (SA; Sigma; adjusted to pH 6.5), probenazole and jasmonic acid (JA; Sigma) in water or with sterile water as a control. Treatment with methyl jasmonate (MeJA; Sigma) was carried out by placing the seedlings in a sealed glass container and pipetting pure MeJA onto a cotton ball inside the container (approx. 0.5 μl/l air). The third and the fourth leaves were collected for analysis of gene expression.

Pseudomonas syringae pv. *syringae* DC3000 was grown overnight in Luria Bertani broth (10 g/l tryptone, 5 g/l yeast extracts, 5 g/l NaCl, pH 7.2) at 28°C with shaking. The bacteria were collected by centrifugation and resuspended in 10 mM $MgSO_4$ to a concentration of 5×10^8 colony-forming units (cfu)/ml. The fourth leaves of 4-week-old rice seedlings were treated by infiltration at five locations with 20 μl of the bacterial suspension or 10 mM $MgSO_4$ using a 1-ml syringe without a needle. Wounds were applied to the fourth leaf by squeezing the leaf blade with forceps at 10 locations. For analysis of the systemic effect of BTH on gene expression, the fourth leaves of 4-week-old seedlings were injected at five locations with 20 μl of 300 μM BTH solution or water. In these cases, the treated fourth leaves and the untreated fifth leaves were collected separately.

A pair of near-isogenic lines, H8R and H8S, was used for analysis of gene expression in the incompatible and compatible interactions between rice and *M. grisea*. Three-week-old seedlings were inoculated with *M. grisea* and the fourth leaves were collected for Northern blot analysis.

Three days after the treatments described above the rice seedlings were inoculated with spore suspension (5×10^5 spores/ml in 0.05% Tween 20) of *M. grisea* (race ZB1, isolate 85-14B1) or with sterile water containing 0.05% Tween 20. The inoculated and the uninoculated rice seedlings were kept in a plastic box with 100% relative humidity in darkness for 36 h; thereafter, the seedlings were incubated in the growth chamber under the conditions described above.

Extraction of total RNA

Total RNA was extracted from rice leaf tissues by a hot-phenol method (Verwoerd et al. 1989). Leaf tissues were homogenized in liquid nitrogen and extracted in a 1:1 mixture of phenol and extraction buffer [100 mM LiCl, 100 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% SDS] at 80 °C. Chloroform (0.5 volume) was added, mixed by vortexing, and centrifuged. RNA was precipitated overnight at 4 °C in 2 M LiCl. After centrifugation, the RNA pellet was dissolved in diethyl pyrocarbonate (DEPC)-treated water, precipitated in 0.1 volume of sodium acetate (pH 5.2) and two volumes of ethanol, and resuspended in DEPC-water.

Suppression subtractive hybridization (SSH)

SSH was performed using the PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, Calif., USA) according to the manufacturer's recommendations. Briefly, two populations of double-stranded cDNAs (tester cDNA and driver cDNA) were synthesized and

digested with *Rsa*I. The digested cDNAs were divided into two samples and ligated with two different adaptors, followed by two rounds of hybridization. An excess of driver cDNA was added to the diluted adaptor 1-ligated tester or adaptor 2R-ligated tester cDNA and allowed to hybridize for 9 h at 68 °C. These two samples were then mixed in the presence of freshly denatured driver cDNA and hybridized for 20 h at 68 °C. Two rounds of PCR were performed to amplify exponentially the differentially expressed sequences, and the PCR products were cloned into pGEM T-Easy vector (Promega, Madison, Wis., USA) by T/A cloning. These libraries of differentially expressed cDNAs were subsequently screened by two rounds of differential screening using the PCR-Select Differential Screening Kit (Clontech) and a third round of reverse Northern analysis. The remaining clones were sequenced and similarity searches were performed as described below.

Construction and screening of rice cDNA library

Rice leaf samples were harvested from uninoculated BTH-treated and untreated rice seedlings 4 days after treatment and from inoculated BTH-treated and untreated rice seedlings 1 day after inoculation. One milligram of total RNA from each leaf sample was pooled and used for poly(A)$^+$RNA isolation with Promega's PolyATtract mRNA Isolation System. A cDNA library was constructed using Stratagene's ZAP Express cDNA Construction Kit according to the manufacturer's recommendations.

Screening of the cDNA library was carried out to isolate the full-length cDNA encoded by the putative MAP kinase gene. The 515-bp insert of the SSH clone BIHI-n36, which showed similarity to wheat MAPK gene *WCK-1* (Takezawa 1999), was amplified with the nested primers provided by Clontech's PCR-Select Subtraction Kit and digested with *Rsa*I, *Eag*I and *Sma*I to remove the adaptors from both ends of the cDNA. The digested PCR products were purified with QiaQuick PCR Purification Kit (Qiagen) and labeled with digoxigenin (DIG)-dUTP using a random primer labeling system (Boehringer Mannheim).

Plaque lifts were prepared following Stratagene's protocol. Prehybridization was carried out in 10 ml of aqueous hybridization solution [0.5 M $NaHPO_4$ (pH 7.2), 1% BSA, 1 mM EDTA and 7% SDS] containing 50 µl of blocking solution (10 mg/ml sheared salmon sperm DNA) for 2 h at 65 °C. The hybridization was performed overnight with approx. 20 ng/ml DIG-labeled probe at 65 °C. The membranes were washed with 10 ml 2×SSC plus 0.5% SDS (4×20 min; 1×SSC = 0.15 mM NaCl, 0.015 mM Na-citrate, pH 7.0) and then with 10 ml 0.2×SSC plus 0.5% SDS (2×20 min) at 65 °C. Immunodetection of DIG-labeled probes was done by the chemiluminescence method using CSPD according to the manufacturer's instructions (Boehringer Mannheim). The cDNA inserts of the positive clones were excised in vivo as phagemids following Stratagene's protocol.

DNA sequencing and sequence analysis

DNA sequencing was performed on both strands using the BigDye Sequencing Reagent by PCR with the phagemid DNA as template and with T3 and M13r primers. Sequencing reactions were analyzed by the ABI PRISM 377 Automatic Sequencer (Perkin-Elmer, Foster City, Calif., USA) at the University of Wisconsin Biotechnology Center. Nucleotide sequence similarity searches were carried out at the National Center for Biotechnology Information using the BLAST network service (Altschul et al. 1990). Protein sequences of plant MAPKs were retrieved from GenBank and alignments were performed using DNAStar software (DNAStar, Madison, Wis., USA). A phylogenetic tree based on the genetic distance of the protein sequences was constructed by the Clustal method using DNAStar software.

Northern analysis

Twenty micrograms of total RNA was fractionated on a 1.2% agarose-formaldehyde gel and transferred by capillary action overnight to MagnaGraph nylon membranes (Micron Separation Inc., Westboro, Mass., USA) using 10×SSC. After transfer, the RNA was fixed by baking at 80 °C for 2 h. The purified 515-bp insert cDNA was labeled with α-[^{32}P]dCTP (1.11×10^{14} Bq/mmol) by a random priming labeling system (Amersham Pharmacia). Pre-hybridization was performed at 42 °C for 1 h in UltrHyb hybridization buffer (Ambion) and hybridization was carried out overnight at 42 °C in the same hybridization buffer with the ^{32}P-labeled probe. After hybridization, the blots were washed four times with 2×SSC, 0.1% SDS and 0.2×SSC, 0.1% SDS for 10 min each at room temperature. All Northern analysis experiments were repeated at least twice using independent samples and representative results are presented.

Results

Cloning of *OsBIMK1*, a cDNA encoding a rice MAPK

It was previously shown that BTH could induce systemic acquired resistance (SAR) in several dicot plants as well as in maize and wheat (Gorlach et al. 1996; Lawton et al. 1996; Morris et al. 1998). We found that pre-treatment of rice seedlings by spraying or soil-drenching with BTH induced resistance against blast disease (data not shown). In an effort to understand the molecular biology of disease resistance in rice, we employed suppression subtractive hybridization and identified 276 differentially expressed cDNAs that may be associated with BTH-induced SAR in rice. One clone from a library constructed with cDNA from BTH-treated and pathogen-inoculated leaf samples subtracted with cDNA from water-treated and inoculated samples contained a 515-bp insert and showed 91% identity at the nucleotide sequence level to a wheat MAPK gene, *WCK-1* (GenBank AF079318; Takezawa 1999). To obtain the full-length cDNA of this putative MAPK gene, a rice cDNA library was screened using a DIG-labeled insert as probe. Approximately 6×10^6 plaques were screened and three positive clones were obtained with 0.9-, 1.1- and 1.4-kb inserts, respectively. Sequencing of the longest clone, designated as *OsBIMK1* for O*ryza sativa* L. BTH-Induced MAPK 1, revealed that it contained 1,424 nucleotides with an 1,110-bp open reading frame (ORF), flanked by 5′- and 3′-non-coding sequences of 98 bp and 216 bp, respectively (Fig. 1).

The ORF of *OsBIMK1* encodes a 369-amino-acid polypeptide (Fig. 1) with a calculated molecular weight of 43 kDa and isoelectric point of 5.59. Alignment of the deduced protein sequence with other MAPKs from plants indicated that OsBIMK1 contains all 11 conserved subdomains that are characteristic of serine/threonine protein kinases (Fig. 2; Hanks and Hunter 1995) and the TEY motif (Fig. 1), which is the putative phosphorylation site. A phylogenetic tree based on the amino acid sequences of OsBIMK1 and other plant MAPKs (Fig. 3) indicated that OsBIMK1 is most closely related to oat AsMAP1 (91.9%), wheat WCK-1 (91.6%; Takezawa 1999) and maize ZmMAPK4 (91.4%; Berberich et al. 1999). OsBIMK1 shows only approx. 47–53% identity at the amino acid level with

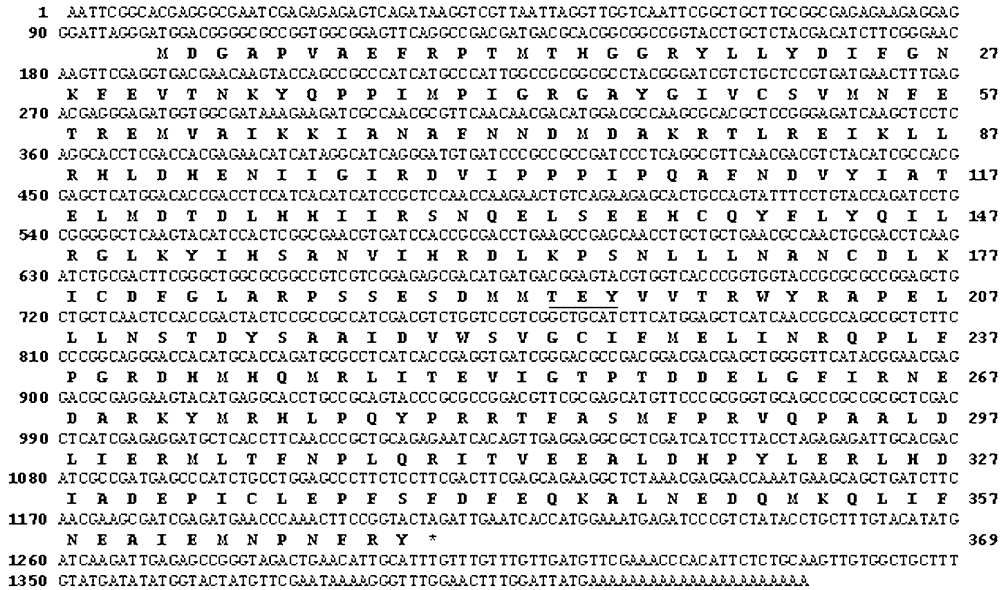

Fig. 1. Nucleotide and deduced amino acid sequences of the rice (*Oryza sativa*) *OsBIMK1* cDNA (GenBank accession number AF332873). The nucleotides and amino acids are numbered on the *left* and *right*, respectively. The conserved motif TEY is *underlined*

previously reported MAPKs from rice. These results indicate that *OsBIMK1* is a novel MAPK in rice.

Expression of *OsBIMK1* is activated by various chemical and biological inducers of disease resistance responses

We first analyzed whether expression of *OsBIMK1* could be activated by BTH induction. In a time-course experiment, expression of *OsBIMK1* was detected as early as 1 day after treatment by spraying 3-week-old rice seedlings with 0.3 mM BTH; the steady-state level of mRNA detected remained unchanged over the experimental period (Fig. 4b). The expression of *OsBIMK1* induced by BTH was found to be BTH dose-dependent (Fig. 4a).

SA, INA, probenazole and *P. syringae* pv. *syringae* have been shown to induce resistance against blast disease (Smith and Metraux 1991; Schweizer et al. 1997; Sakamoto et al. 1999). Therefore, we tested whether expression of *OsBIMK1* is induced by these inducers. As shown in Fig. 5, treatments with INA, probenazole, JA, and MeJA induced expression of *OsBIMK1*. Expression of *OsBIMK1* was increased further in JA- or MeJA-treated but not in INA- or probenazole-treated seedlings after infection with the blast fungus. SA treatment did not induce an appreciable increase in *OsBIMK1* gene expression; but, compared with that in the control, expression of *OsBIMK1* in SA-treated seedlings was increased substantially after infection by the fungus. Infiltration of rice leaves with DC3000 did not activate *OsBIMK1* expression, but pathogen infection substantially increased expression of *OsBIMK1*, both in the infiltrated and the upper non-infiltrated leaves (Fig. 5b). These results indicate that the *OsBIMK1* gene is expressed in response to various chemical and biological SAR inducers, which can induce resistance in rice to blast disease.

OsBIMK1 is wound-inducible

Wounding has been shown to activate MAPK cascades. In rice, wounding was found to induce disease resistance and activate expression of BWMK1 (Schweizer et al. 1998; He et al. 1999). We found that mechanical wounding alone activated expression of *OsBIMK1* in the wounded leaf but not in the upper unwounded leaves and that infection with *M. grisea* induced both local and systemic expression of *OsBIMK1* in the wounded rice seedlings (Fig. 5d), as compared with those in the unwounded control seedlings with or without pathogen inoculation (Fig. 5c). Moreover, JA and MeJA, which are implicated in wound-mediated signal transduction, also activated expression of *OsBIMK1* (Fig. 5c).

OsBIMK1 is expressed specifically in resistance responses

To determine whether induced expression of *OsBIMK1* is associated with the disease resistance induced by BTH, we analyzed the expression kinetics in BTH-treated rice seedlings after infection with *M. grisea* and compared the results with those from control seedlings (Fig. 6). Since the basal level and the induced level of *OsBIMK1* gene expression seem to be very low, the difference in expression level at zero time in Fig. 6 and Fig. 4 may be due to the detection limit. In BTH-treated rice seedlings, infection with *M. grisea* rapidly activated expression of *OsBIMK1*. Expression of *OsBIMK1* was detected as early as 6 h after inoculation with the fungus and peaked at 18–24 h. Relatively high levels of expression were maintained for 36 h after inoculation and thereafter returned to the basal level (Fig. 6a). Conversely, no appreciable expression of *OsBIMK1* was detected in water-treated rice seedlings during the early stage (6–30 h) after inoculation with the blast fungus; an

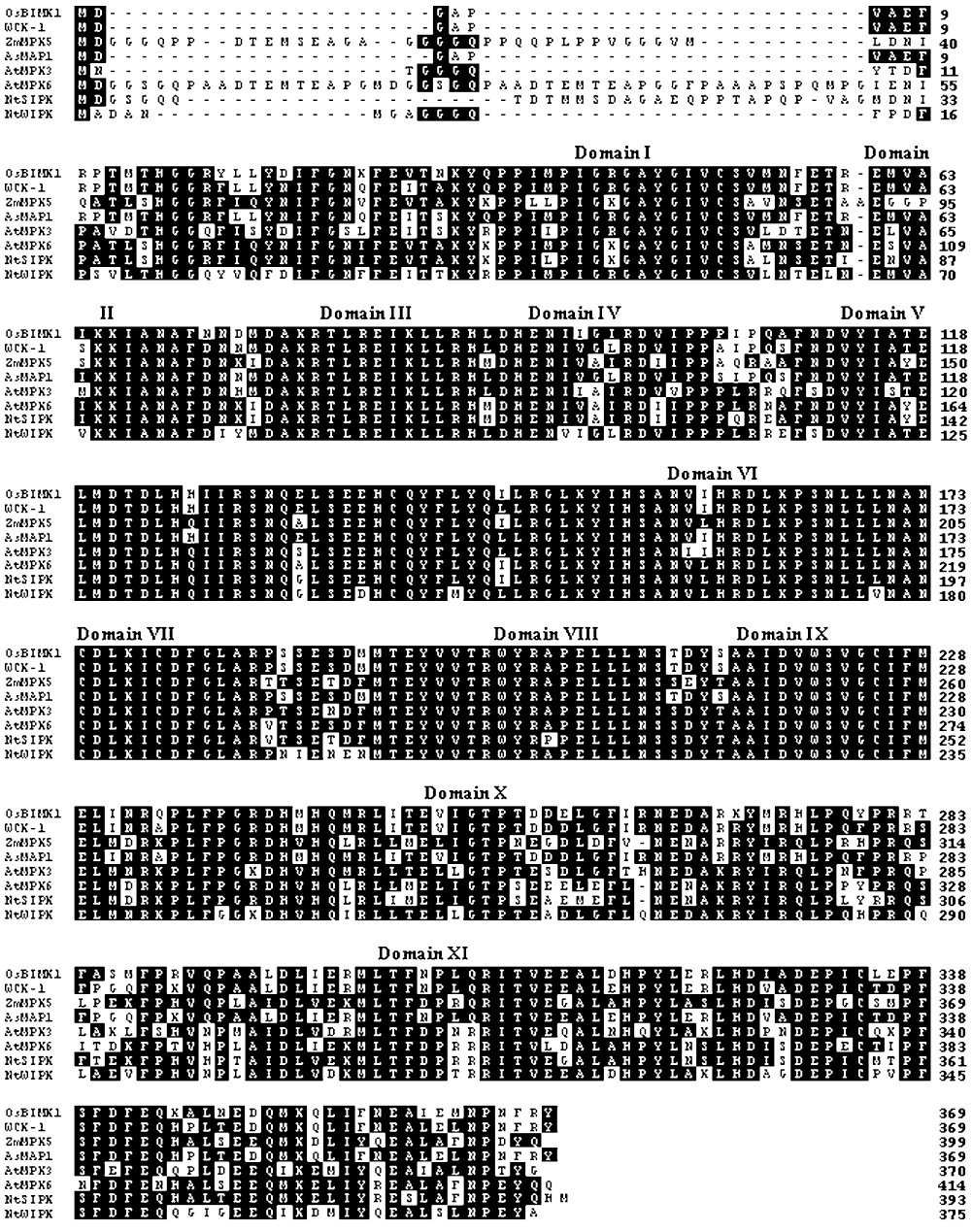

Fig. 2. Alignment of the rice OsBIMK1 with MAPKs from other plant species. The plant MAPKs used for alignment are: maize (*Zea mays*) ZmMPK5 (AB016802); wheat (*Triticum aestivum*) WCK-1 (AF079318); oat (*Avena sativa*) AsMAP1 (X79993); *Arabidopsis thaliana* AtMPK6 (D21842) and AtMPK3 (D21839); and tobacco (*Nicotiana tabacum*) NtSIPK (U94192) and NtWIPK (D61377)

increased level of expression was observed during the late stage (36–60 h).

We then analyzed the expression kinetics of *OsBIMK1* in rice seedlings using a pair of near-isogenic lines (H8R and H8S) to see whether *OsBIMK1* is specifically expressed in the incompatible interaction between blast-resistant rice and *M. grisea*. When infected with strain 85-14B1 of *M. grisea*, HR-like small dark-brown lesions were observed in H8R seedlings approx. 2–3 days after inoculation, whereas typical blast lesions appeared on the H8S leaves approx. 5–7 days after inoculation (data not shown). The disease responses of H8R and H8S represent incompatible and compatible interactions, respectively. Expression of *OsBIMK1* in H8R leaves was induced rapidly by infection with the blast fungus (Fig. 6b). As seen in the BTH-treated rice seedlings, expression of *OsBIMK1* was detected as early as 6 h after inoculation, and remained at a high level during the first 36 h. Compared with the expression in H8R, very low levels of expression were observed in H8S leaves during the first 36 h after inoculation. Thus, *OsBIMK1* is only expressed during a resistance response and/or an incompatible interaction.

Expression of *OsBIMK1* is activated systemically by BTH

To define a role for *OsBIMK1* in systemic activation of defense-related genes, we infiltrated BTH solution into the intracellular spaces of the fourth leaf, and then analyzed the expression levels of *OsBIMK1* in the upper

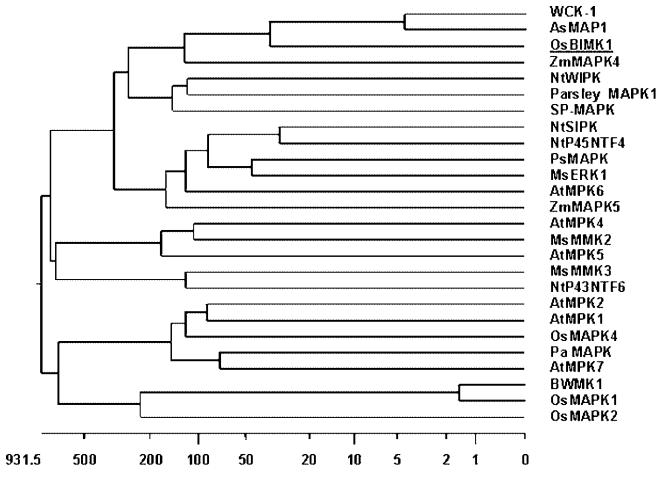

Fig. 3. The phylogenetic relationship of rice OsBIMK1 with other MAPKs from plant species. A phylogenetic tree based on the genetic distance of the protein sequences was constructed by the Clustal method using DNAStar software. The protein sequences of the MAPKs used for construction of the tree are listed in the GenBank database under the following accession numbers: oat AsMAP1 (S56638); *Arabidopsis* AtMPK2 (D14714); *Arabidopsis* AtMPK4 (D21840); *Arabidopsis* AtMPK5 (D21841); *Arabidopsis* AtMPK6 (D21842); *Arabidopsis* AtMPK7 (D21843); rice BWMK1 (AF177392); alfalfa (*Medicago sativa*) MsERK1 (L07042); alfalfa MsMMK2 (X82268); alfalfa MsMMK3 (AJ224336); tobacco NPK2 (D31964); tobacco NtP43NTF6 (X83879); tobacco NtP45NTF4 (X83880); tobacco NtSIPK (U94192); tobacco NtWIPK (D61377); sweet potato (*Ipomoea batatas*) SP-MAPK (AF149424); rice OsMAPK1 (AF194415); rice OsMAPK2 (AF194416); rice OsMAPK4 (AJ251330); apricot (*Prunus armeniaca*) PaMAPK (AF134730); parsley (*Petroselenium crispum*) MAPK1 (Y12785); pea (*Pisum sativum*) PsMAPK (X70703); wheat WCK-1 (AF079318); maize ZmMPK4 (AB016801); maize ZmMPK5 (AB016802)

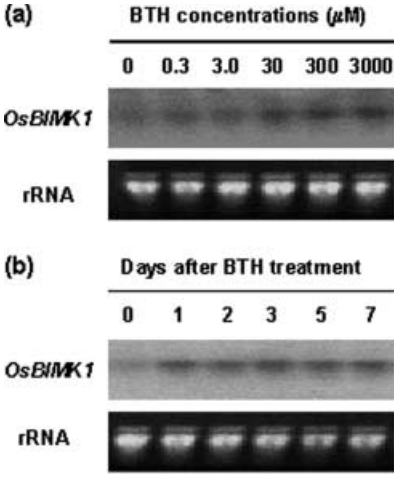

Fig. 4a, b. Induction of *OsBIMK1* expression by BTH. **a** Rice seedlings were treated by spraying with different concentrations of BTH solution and leaf samples were collected 1 day after treatment. **b** Rice seedlings were treated by spraying with 0.3 mM of BTH solution and leaf samples were collected at time points as indicated

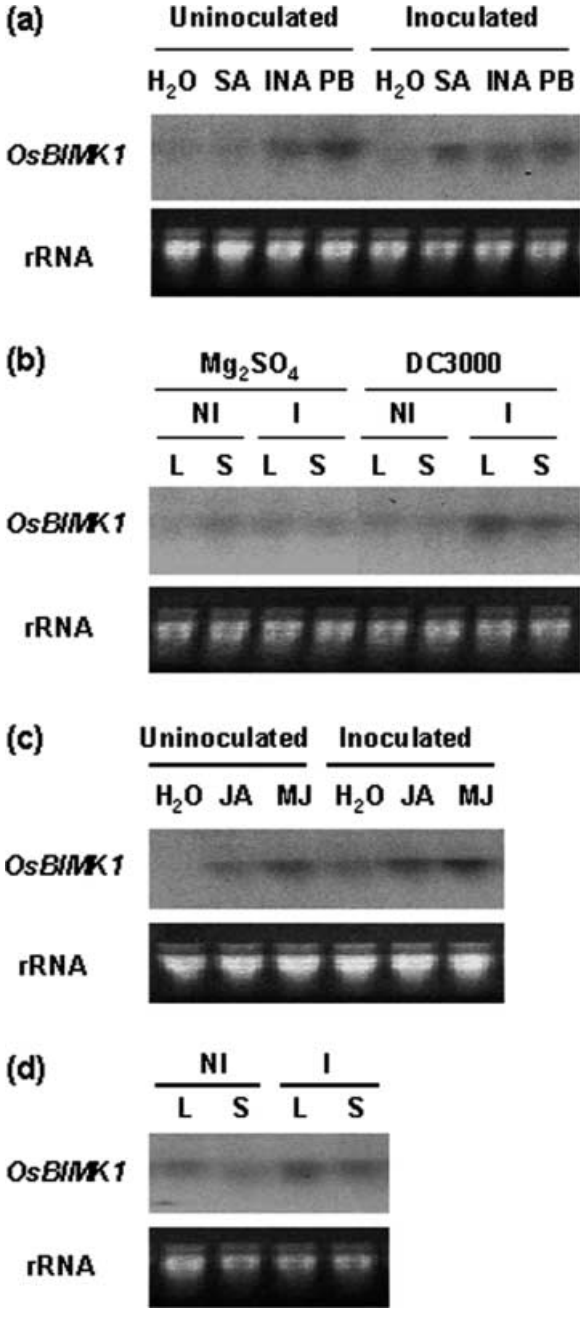

Fig. 5a–d. Induction of *OsBIMK1* expression by chemical and biological inducers. **a** Rice seedlings were treated by spraying with 2 mM SA, 0.3 mM INA, 0.5 mM probenazole (*PB*) or water. **b** The fourth leaves of 4-week-old rice seedlings were infiltrated with *Pseudomonas syringae* pv. *syringae* DC3000 in 10 mM $MgSO_4$ (5×10^8 cfu/ml) or 10 mM $MgSO_4$. **c** JA (1 mM) and MeJA (*MJ*; 0.3 μl/l air in a container) were applied to rice seedlings as described in *Materials and methods*. **d** The fourth leaves of 4-week-old rice seedlings were wounded by squeezing with blunt-ended forceps at 10 locations. The unwounded controls are the same as in **c**. The fourth and the fifth leaves were collected separately as local and systemic samples, respectively. Rice seedlings were inoculated with spores of *Magnaporthe grisea* 3 days after treatments and leaf samples were collected 1 day after inoculation. Twenty micrograms of total RNA was fractionated on a 1.2% agarose-formaldehyde gel and hybridized with ^{32}P-labeled probe. *L* Local – the fourth leaf, *S* systemic – the fifth leaf, *NI* not inoculated, *I* inoculated

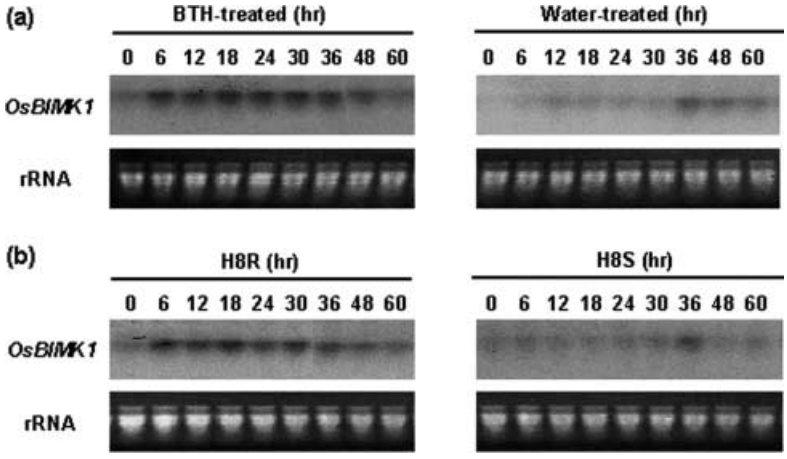

Fig. 6a, b. Induction of *OsBIMK1* expression is associated with resistance responses. **a** Rice seedlings were treated by spraying with 0.3 mM BTH solution or water, and inoculated with *M. grisea* 3 days after treatment. **b** Three-week-old rice seedlings of H8R and H8S were inoculated with *M. grisea*. Leaf samples were collected at each time point (hr) as indicated. Twenty micrograms of total RNA was fractionated on a 1.2% agarose-formaldehyde gel and hybridized with ^{32}P-labeled probe

fifth leaves. Infiltration of water did not induce expression of *OsBIMK1* in upper leaves in uninoculated rice seedlings or in inoculated seedlings (Fig. 7). Infiltration of the fourth leaves with BTH induced expression of *OsBIMK1* in the fifth leaves, and the expression level increased further when plants were infected with the fungus (Fig. 7). This result suggests that expression of *OsBIMK1* is activated systemically by BTH.

Discussion

OsBIMK1 shows >90% identity at the amino acid level to oat AsMAP1, wheat WCK-1 and maize ZmMAPK4 (Berberich et al. 1999; Takezawa 1999) but only 47–53% identity to rice MAPKs previously reported (He et al. 1999) or deposited in GenBank. We, therefore, conclude that *OsBIMK1* is a novel MAPK gene in rice. Our results provide several lines of evidence that *OsBIMK1* plays an important role in rice disease resistance. *OsBIMK1* was activated rapidly upon treatments with BTH as well as with other chemical and biological inducers that can induce resistance in rice against blast disease (Figs. 4, 5). Expression of *OsBIMK1* is specifically activated in the resistance response and/or in the incompatible interaction between rice and *M. grisea* (Fig. 6), and is induced systemically upon BTH treatment (Fig. 7).

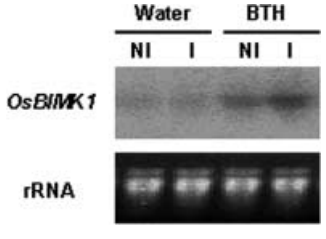

Fig. 7. Systemic induction by BTH of *OsBIMK1* in rice seedlings. The fourth leaves of 4-week-old rice seedlings were infiltrated at five locations with 20 µl of BTH solution (0.3 mM) or water. The rice seedlings were inoculated with spores of *M. grisea* or with water 3 days after infiltration and the fifth leaves were collected 1 day after inoculation. Twenty micrograms of total RNA was loaded in each lane and hybridized with ^{32}P-labeled probe. *NI* Not inoculated, *I* inoculated

Expression of *OsBIMK1* was rapidly induced by treatment with BTH, even at very low concentrations of BTH (Fig. 4), suggesting that an efficient system for perception of BTH or other signals exists in rice. Activation of *OsBIMK1* expression was only observed during the early stage in BTH-treated rice seedlings but not in control seedlings after infection with the blast fungus (Fig. 6a). Moreover, BTH treatment also activated the systemic expression of *OsBIMK1*. Infection with the tobacco mosaic virus was found to activate expression of the *WIPK* gene in tobacco (Zhang and Klessig 1998a). Based on these results, we hypothesized that expression of *OsBIMK1* is likely involved in the activation of defense responses. This interpretation is strengthened by results showing that other SAR inducers (INA and probenazole) also induce expression of *OsBIMK1*.

Treatment of rice with *P. syringae* or its elicitor, syringolin, induced resistance to blast disease and activated defense responses (Smith and Metraux 1991; Wäspi et al. 1998). In our study, SA and *P. syringae* alone did not induce *OsBIMK1* expression (Fig. 5a, b); however, expression of *OsBIMK1* was noticeably activated in SA- or *P. syringae*-treated rice seedlings after infection with the fungus, suggesting that SA and *P. syringae* may potentiate rice seedlings to rapidly activate *OsBIMK1* upon infection. Recently, it was shown that BTH and *P. syringae* activated expression of different sets of defense-related genes in rice, and that the signaling pathways involved in BTH- or *P. syringae*-induced defense responses might be different (Schweizer et al. 1999).

Induction of another rice MAPK gene, *BWMK1*, is independent of the rice blast resistance gene, and resistance or susceptibility responses (He et al. 1999). Unlike the case with *BWMK1*, expression of *OsBIMK1* was activated rapidly during early stages of the incompatible interaction but not in the compatible interaction between rice and the blast fungus (Fig. 6b), suggesting that *OsBIMK1* also plays a role in disease resistance gene-mediated signaling pathways.

The mechanisms for the activation of MAPKs in response to extracellular stimuli differ. In yeast and mammals, activation of the MAPKs is mediated solely by a mechanism of post-translational phosphorylation (Widmann et al. 1999). This mechanism is also used for activation of the MAPKs in higher plants; for example, the activity of SIPK in tobacco was found to be regulated post-translationally (Zhang and Klessig 1998a). Interestingly, however, activation of the tobacco WIPK has been shown to be regulated both transcriptionally and post-translationally (Zhang et al. 2000). Increases in WIPK transcripts have been shown to precede WIPK activation in tobacco resisting against TMV infection, and activation of WIPK gene expression is required for regulation of its enzymatic activity (Zhang and Klessig 1998a; Zhang et al. 2000). Indeed, expression of genes for *Arabidopsis* AtMPK3, parsley ERMK and alfalfa MMK4 is also activated by various stresses (Ligterink et al. 1997). In our study, we found that expression of the rice *OsBIMK1* gene was activated by chemical and biological SAR inducers as well as by pathogen infection. Expression of another MAPK gene, *BWMK1*, was also found to be activated by infection with blast fungus (He et al. 1999). However, the biochemical mechanism for activation of OsBIMK1 enzymatic activity in the defense response and whether induction of *OsBIMK1* gene expression is required for activation of its activity need to be studied further. Comparative study of the activation of OsBIMK1 activity and gene expression in the defense response will be helpful in addressing these questions.

Since expression of *OsBIMK1* was activated upon induction by diverse chemical and biological inducers and wounding, as well as in the incompatible interaction between rice and *M. grisea*, it is a reasonable hypothesis that a putative *OsBIMK1*-mediated MAPK cascade is an early common signaling event that is shared by various signaling pathways leading to activation of defense responses in rice. However, the mechanisms by which this putative *OsBIMK1*-mediated cascade plays its function in the signaling pathways leading to disease resistance need to be elucidated.

The amino acid sequence of the OsBIMK1 shows a high level of identity to the wheat WCK-1, whose expression is activated by Ca^{2+} (Takezawa 1999). More recently, it was demonstrated that H_2O_2, a major component of the oxidative burst that is required for activation of defense responses, activated an MAPK cascade in *Arabidopsis* and soybean (Kovtun et al. 2000; Taylor et al. 2001). In another experiment, we found that two differentially expressed cDNAs, showing a high level of similarity to the calmodulin- and plasma membrane-bound NADPH oxidase that is involved in generation of the oxidative burst, were activated in rice by BTH treatment (data not shown), implying that Ca^{2+} signaling and the oxidative burst may be involved in the defense response induced by BTH. Therefore, the relationships of the *OsBIMK1*-mediated MAPK cascade with the Ca^{2+} signaling and oxidative burst are worthy of further study.

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