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Cloning and Characterization of a Probenazole-Inducible Gene for an Intracellular Pathogenesis-Related Protein in Rice

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Probenazole (3-allyloxy-1,2-benzisothiazole-1,1-dioxide) induces disease resistance in rice against rice blast fungus. To investigate the molecular mechanism of probenazole-induced resistance, we isolated and characterized a cDNA clone of a probenazole-inducible gene in rice, which encoded a protein designated PBZ1. Sequence analysis revealed that significant homology at the amino acid level exists between the predicted PBZ1 protein and intracellular pathogenesis-related (IPR) proteins. Accumulation of *PBZ1* mRNA was not induced by wounding, but markedly induced by inoculation with rice blast fungus. In addition, it was induced sooner by inoculation with an incompatible race than that with a compatible race. On the other hand, when the accumulation of the *PBZ1* mRNA was examined after treatment with probenazole-related compounds, it was not fully correlated with anti-rice blast activity. However, it was induced after treatment with N-cyanomethyl-2-chloro-isonicotinamide (NCI), which belongs to another group of compounds known to induce disease resistance. Thus, although the accumulation of the *PBZ1* mRNA was not fully correlated with anti-rice blast activity, our findings suggest that the *PBZ1* gene has an important function during the disease resistance response in rice.

Key words: Disease resistance — Pathogenesis-related protein — Probenazole — Rice (*Oryza sativa*) — Rice blast fungus (*Pyricularia oryzae*).

Probenazole (Oryzemate®, 3-allyloxy-1,2-benzisothiazole-1,1-dioxide) is known as an effective agricultural chemical against rice blast disease (Watanabe et al. 1977). Although probenazole has only a weak effect on isolated rice blast fungus, it is very effective in protecting rice plants from infection of the fungus (Watanabe 1977). This was suggested to be the result of induced disease resistance in the host plant (Watanabe et al. 1979). In probenazole-treated rice plants, the activities of defense-related enzymes, such as peroxidase, polyphenoloxidase, phenylalanine ammonia-lyase, tyrosine ammonia-lyase and catechol-*O*-methyltransferase, show marked increases upon infection (Iwata et al. 1980). In addition, anticonidial germination substances, such as α-linolenic acid and some hydroxy unsaturated fatty acids, are produced upon infection (Sekizawa et al. 1981, Shimura et al. 1981, 1983). Furthermore, the maximum rate of respiration occurs at an earlier time upon infection (Sekizawa et al. 1985). However, despite these extensive studies, the primary site of action of probenazole remains unknown.

Some chemicals, such as 2,6-dichloro-isonicotinic acid (INA) and N-cyanomethyl-2-chloro-isonicotinamide (NCI), have been shown to induce disease resistance similarly to probenazole (Metraux et al. 1991, Yoshida et al. 1990, Seguchi et al. 1992a, b). Among these chemicals, INA has been investigated in terms of the relationship between disease resistance and the expression of particular genes. INA is an effective agent against bacterial, fungal and viral diseases and the disease resistance induced by INA is correlated with the expression of pathogenesis-related (PR) genes (Ward et al. 1991, Uknes et al. 1992, 1993). Therefore, it is suggested that the induced disease resistance results, at least partly, from the coordinate expression of PR genes.

It was reported recently that the accumulation of at least five specific transcripts was induced after treatment with probenazole in rice, and a cDNA clone (designated pPB-1) was isolated from probenazole-treated rice plants (Minami and Ando 1994). Accumulation of pPB-1 mRNA was induced in the green tissues of rice plants, especially in leaves, after treatment with probenazole or light exposure. However, the function of the corresponding gene is unknown and other probenazole-inducible genes have not been reported.

Here, we describe the isolation of a cDNA clone of a probenazole-inducible gene in rice. The encoded protein was designated PBZ1. We show that the predicted PBZ1 protein is homologous to intracellular pathogenesis-related (IPR) proteins and that the accumulation of the *PBZ1* mRNA is significantly induced after inoculation of rice leaves with rice blast fungus. In addition, the relationship

Abbreviations: BIT, 1,2-benzisothiazole-3(2H)-one 1,1-dioxide; 2-butyl BIT, 2-butyl-1,2-benzisothiazole-3(2H)-one 1,1-dioxide; CTAB, cetyltrimethylammonium bromide; 2-ethoxycarbonylmethyl BIT, 2-ethoxycarbonylmethyl-1,2-benzisothiazole-3(2H)-one 1,1-dioxide; INA, 2,6-dichloro-isonicotinic acid; IPR protein (gene), intracellular pathogenesis-related protein (gene); NAA, 2-(1-naphthyl)acetic acid; NCI, N-cyanomethyl-2-chloro-isonicotinamide; PR protein (gene), pathogenesis-related protein (gene).

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between disease resistance and the *PBZ1* gene is discussed.

Materials and Methods

Plant material—Rice plants (*Oryza sativa* L. cv. Jikkoku, *Pia*) were grown in a greenhouse supplemented with artificial light at 20–25°C, 13 h day/11 h night.

Chemicals—Probenazole, 2-butyl-1,2-benzisothiazole-3(2H)-one 1,1-dioxide (2-butyl BIT), allyl *O*-sulfamoylbenzoate, 2-ethoxycarbonylmethyl-1,2-benzisothiazole-3(2H)-one 1,1-dioxide (2-ethoxycarbonylmethyl BIT) and NCI were synthesized in the chemical laboratory of the Pharmaceutical Research Center of Meiji Seika Kaisha, Ltd. Other chemicals were purchased from commercial suppliers.

RNA isolation—For the construction of a cDNA library, total RNA was isolated from frozen rice leaves by the guanidine thiocyanate/CsCl method (Maniatis et al. 1982) and poly(A)$^+$-RNA was obtained using a Oligotex-dT30 (Nippon Roche) according to the supplier's protocol.

For the synthesis of labeled cDNA probes for differential screening, total RNA was isolated from frozen rice leaves by the guanidine thiocyanate/CsCl method (Maniatis et al. 1982).

For Northern blot hybridization, total RNA was isolated from frozen rice leaves by phenol/chloroform extraction followed by lithium chloride precipitation as described by Watanabe and Price (1982).

Construction of a cDNA library—The pots of two-week-old rice seedlings were submerged in a solution of 100 mg liter^{-1} probenazole containing 0.05% Tween 20 and 1% acetone (submerged application) for 10 days. Tween 20 and acetone were used to facilitate dispersion of the chemical. Then the leaves were harvested and poly(A)$^+$RNA was prepared as indicated above. cDNA was synthesized from the poly(A)$^+$RNA using a TimeSaver cDNA synthesis kit (Pharmacia) according to the supplier's protocol. Then, the cDNA was ligated into a λExCell phage vector (Pharmacia) and the phage was packaged using a Gigapack II packaging extract (Stratagene) according to the supplier's protocol. The resulting cDNA library contained 3.5×10^3 pfu μl^{-1}.

Synthesis of labeled cDNA probes for differential screening—Two-week-old rice seedlings were treated with a solution of 100 mg liter^{-1} probenazole containing 0.05% Tween 20 and 1% acetone or with 100 mg liter^{-1} 2-butyl BIT by submerged application for 6 days. Then the leaves were harvested and total RNA was prepared as indicated above. ^{32}P-labeled cDNAs were synthesized from 20 μg of total RNA derived from probenazole-treated and from 2-butyl BIT-treated rice leaves with 25 units of AMV reverse transcriptase and 4.5 MBq [α-^{32}P]dCTP in a total volume of 50 μl of AMV reverse transcriptase buffer [50 mM Tris-HCl, 50 mM KCl, 4 mM dithiothreitol, 10 mM $MgCl_2$, 3 mM dATP, dGTP and dTTP, 0.1 μg μl^{-1} poly(dT)$_{16}$, 4 mM sodium pyrophosphate, 50 units RNase inhibitor, pH 8.3]. The mixture was incubated at 41°C for 2 h and then the reaction was terminated by adding 3 μl of 0.5 M EDTA (pH 8.0) and 50 μl of 0.3 M NaOH. The labeled cDNAs were heated to 100°C for 5 min to hydrolyze the RNA. After adding 15 μl of 1 M Tris-HCl (pH 8.0), the cDNAs were extracted once with 50 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)-saturated phenol. Then the cDNAs were precipitated twice with 0.5 volume of 7.5 M ammonium acetate and 2.5 volumes of ethanol to remove unincorporated labeled nucleotides. Finally, the pellet was resuspended in 200 μl of TE. The labeled cDNAs were used as probes for differential screening.

Differential screening—A total of about 40,000 phage plaques was plated at a density of about 4,000 plaques per plate (11 × 14 cm) and transferred onto a set of duplicate membranes (Hybond-N+; Amersham). After fixing the phage DNA to the membranes, one set was allowed to hybridize with the ^{32}P-labeled cDNA probe derived from probenazole-treated rice leaves and the other set was allowed to hybridize with the ^{32}P-labeled cDNA probe derived from 2-butyl BIT-treated rice leaves. Hybridization was carried out using hybridization buffer prepared from Hybridization buffer tablets (Amersham) at 65°C overnight. The membranes were washed in 2 × SSC (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate), 0.1% SDS at room temperature for 15 min and then in 1 × SSC, 0.1% SDS at 65°C for 1 h and finally in 0.1 × SSC, 0.1% SDS at 65°C for 1 h. Autoradiography was carried out using intensifying screens (Hyperscreen; Amersham) at −80°C for 1–3 days. Differentially hybridizing plaques were isolated and repurified by further round of differential screening. The inserts of positive clones were excised in vivo from the λExCell phage vector for subcloning into the pExCell plasmid vector according to the supplier's protocol.

Sequence analysis—Restriction maps were constructed by a single or multiple restriction enzyme digestion of the cDNA clones. After subcloning the insert into pBluescript II KS+ (Stratagene), various overlapping subclones were made by standard procedures (Maniatis et al. 1982). Single-stranded templates were prepared by phagemid rescue according to the supplier's protocol. DNA sequencing was carried out using a Dye primer cycle sequencing kit (Applied Biosystems) and a 373A DNA sequencer (Applied Biosystems) according to the manufacturer's instructions. Homology searches were carried out using Macvector software (Eastman Kodak Company) to search the Entrez database. Amino acid sequences were aligned using the Geneworks program (IntelliGenetics).

Northern blot hybridization—Ten μg aliquots of total RNA were separated by electrophoresis through a formamide agarose gel (1.3% agarose) and blotted onto a nylon membrane (Hybond-N+; Amersham) according to the supplier's protocol. Ethidium bromide was included in the running buffer at 50 μg liter^{-1}, which allowed photography under UV light after electrophoresis to confirm equal sample loading.

To examine the induction of the accumulation of *PBZ1* mRNA by inoculation with a pathogen or by wounding and the time course experiment after inoculation, as well, the whole *PBZ1* cDNA insert was labeled with ^{32}P using a Ready To Go DNA labeling kit (Pharmacia) according to the supplier's protocol and used as a probe. Hybridization was carried out with the denatured probe using hybridization buffer prepared from Hybridization buffer tablets (Amersham) at 68°C overnight. The membrane was washed in 2 × SSC, 0.1% SDS at room temperature for 15 min and then in 1 × SSC, 0.1% SDS at 50°C for 1 h and finally in 0.1 × SSC, 0.1% SDS at 50°C for 1 h. Autoradiography was carried out using intensifying screens (Hyperscreen; Amersham) at −80°C overnight.

For the other experiments, hybridization and washing were carried out using ECL direct nucleic acid labelling and detection systems (Amersham) according to the supplier's protocol. In these experiments, the whole *PBZ1* cDNA insert was used as the probe.

Time course experiment after treatment with probenazole—Two-week-old rice seedlings were treated by submerged application with a solvent only (control; 0.05% Tween 20, 1% acetone) or with the same solution containing 100 mg liter^{-1} probenazole. At each time point, leaves were harvested, frozen immediately in liquid nitrogen and then stored at −80°C prior to RNA isolation.

Inoculation—Conidia of rice blast fungus were prepared as

described by Watanabe et al. (1977).

For the inoculation experiment (Fig. 5), two-week-old rice seedlings were inoculated by spraying the conidial suspension (1×10^7 conidia ml^{-1}) of compatible rice blast fungus (*Pyricularia oryzae* Cavara, race No. 007) onto the leaves. The inoculated seedlings were incubated in a dark, moist chamber at 25°C for 30 h and then grown in a greenhouse. Total RNA was isolated from the leaves 6 days after inoculation.

For the time course experiment (Fig. 6), two-week-old rice seedlings were inoculated by spraying the conidial suspension (2×10^6 conidia ml^{-1}) of compatible rice blast fungus (*Pyricularia oryzae* Cavara, race No. 007) or of incompatible rice blast fungus (*Pyricularia oryzae* Cavara, race No. 101, MAFF 235007) onto the leaves. The inoculated seedlings were incubated in a dark, moist chamber at 25°C for 36 h and then grown in a greenhouse. At each time point, leaves were harvested, frozen immediately in liquid nitrogen and then stored at −80°C prior to RNA isolation.

Wounding—The leaves of two-week-old rice seedlings were wounded by tapping the leaves rather vigorously with a brush and then the seedlings were grown in a greenhouse. Total RNA was isolated from the leaves 6 days after wounding.

Effects of various chemicals and probenazole-related compounds—The leaves of two-week-old rice seedlings were sprayed with a solution of 100 mg $liter^{-1}$ ethephon (2-chloroethylphosphonic acid), 100 mg $liter^{-1}$ 2-(1-naphthyl)acetic acid (NAA) or 5,000 mg $liter^{-1}$ sodium salicylate (foliar application). Alternatively, seedlings were treated with a solution of 1,000 mg $liter^{-1}$ NaCl, 1,000 mg $liter^{-1}$ mannitol, 100 mg $liter^{-1}$ probenazole, 100 mg $liter^{-1}$ 1,2-benzisothiazole-3(2H)-one 1,1-dioxide (BIT), 100 mg $liter^{-1}$ allyl *O*-sulfamoylbenzoate, 100 mg $liter^{-1}$ 2-ethoxycarbonylmethyl BIT, 100 mg $liter^{-1}$ 2-butyl BIT or 100 mg $liter^{-1}$ NCI by submerged application. All solutions including the control test in these experiments contained 0.05% Tween 20 and 1% acetone to maintain comparable conditions. The leaves were harvested 6 days after treatment and total RNA was isolated as indicated above.

DNA isolation and Southern blot hybridization—Genomic DNA was isolated by the cetyltrimethylammonium bromide (CTAB) precipitation method as described by Saghai-Maroof et al. (1984). Three μg aliquots of DNA were digested with restriction enzymes, separated by electrophoresis through a 0.8% agarose gel and blotted onto a nylon membrane (Hybond-N+; Amersham) according to the supplier's protocol. The whole *PBZ1* cDNA insert was labeled with ^{32}P using a Ready To Go DNA labeling kit (Pharmacia) according to the supplier's protocol and used as the probe. Hybridization was carried out with the denatured probe using hybridization buffer prepared from Hybridization buffer tablets (Amersham) at 65°C overnight. The membrane was washed in 2×SSC, 0.1% SDS at room temperature for 15 min and then in 1×SSC, 0.1% SDS at 65°C for 1 h and finally in 0.1× SSC, 0.1% SDS at 65°C for 1 h. Autoradiography was carried out using intensifying screens (Hyperscreen; Amersham) at −80°C for 3 days.

Results

Isolation of a cDNA for a probenazole-inducible gene—To investigate the molecular mechanism of anti-rice blast activity in rice induced by probenazole, we attempted to isolate cDNA clones of probenazole-inducible genes by differential screening of a cDNA library constructed from mRNA of probenazole-treated rice leaves. In the differential screening procedure, we used cDNA probes derived from probenazole-treated and, as a control, 2-butyl BIT-treated rice leaves (the structures of these compounds are shown in Figure 1). 2-Butyl BIT is a probenazole-related compound but hardly induces disease resistance in rice. Therefore, we expected to isolate cDNA clones of probenazole-inducible genes whose mRNA accumulation was not induced as a simple response to chemical stimulation. Of about 40,000 recombinant clones, two clones were found to hybridize more strongly to the cDNA probe derived from probenazole-treated rice leaves than from those treated with 2-butyl BIT. These two clones hybridized to each other. Therefore, we analyzed the cDNA clone with the longer insert and its encoded protein was designated as PBZ1. The *PBZ1* mRNA was estimated to be about 850 bp by RNA blot hybridization (data not shown), so the cloned cDNA appeared to be nearly full-length.

Sequence analysis—The *PBZ1* cDNA sequence is shown in Figure 2. The *PBZ1* cDNA was 833 bp long and contained a major open reading frame of 474 bp that encoded a putative protein of 158 amino acids with a predicted mol wt of 16,687 and a pI of 4.73. A putative polyadenylation signal (AATAAA) was found in the 3′ non-coding region.

Sequence comparison—The predicted PBZ1 amino acid sequence was used to search the Entrez database. Significant sequence homology was found between the predicted PBZ1 protein and IPR proteins. IPR proteins do not have a signal sequence, in contrast with other PR proteins, and have been suggested to be intracellular (Somssich et al. 1988, Allaire and Hadwiger 1994). In addition, they are comparatively small (155–161 amino acid residues) acidic proteins and the IPR genes form a multigene family (Somssich et al. 1988, Chiang and Hadwiger 1990, Walter et al. 1990, Crowell et al. 1992, Breiteneder et al. 1993, Iturriaga et al. 1994). However, the function of these genes has

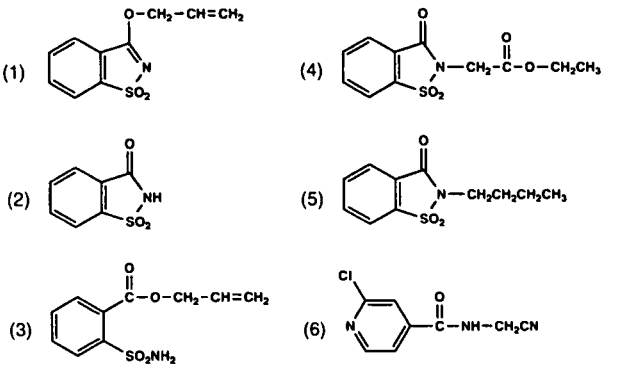

Fig. 1 Chemical structures of probenazole, probenazole-related compounds and NCI. (1), probenazole; (2), BIT; (3), allyl *O*-sulfamoylbenzoate; (4), 2-ethoxycarbonylmethyl BIT; (5), 2-butyl BIT; (6), NCI. See list of abbreviations for full names of compounds.

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        10         20         30         40         50         60
CAGCTCTAGCTAGCTACAGGCATCAGTGGTCAGTAGAGTGATCAGTTGCAACTAGCTAGC

        70         80         90        100        110        120
TAGTTAGATTATATCTTCAGTGATGGCTCCGGCCTGCGTCTCCGACGAGCACGCCGTCGC
                       M  A  P  A  C  V  S  D  E  H  A  V  A

       130        140        150        160        170        180
GGTGTCGGCGGAGCGGCTGTGGAAGGCGTTCATGGACGCGTCCACTTTGCCCAAGGCCTG
  V  S  A  E  R  L  W  K  A  F  M  D  A  S  T  L  P  K  A  C

       190        200        210        220        230        240
CGCCGGCTTGGTCGACGACATTGCGGTCGAGGGGAACGGTGGTCCGGGCACCATCTACAC
  A  G  L  V  D  D  I  A  V  E  G  N  G  G  P  G  T  I  Y  T

       250        260        270        280        290        300
CATGAAGCTTAACCCTGCCGCGGGTGTGGGAAGCACATACAAGACCCGGGTGGCGGTGTG
  M  K  L  N  P  A  A  G  V  G  S  T  Y  K  T  R  V  A  V  C

       310        320        330        340        350        360
CGACGCCGCAAGTCATGTCCTAAAGTCGGATGTGCTCGAGGCAGAAAGCAAGGTGGGGAA
  D  A  A  S  H  V  L  K  S  D  V  L  E  A  E  S  K  V  G  K

       370        380        390        400        410        420
GCTCAAGTCACACTCGACGGAGACGAAGCTTGAGGCCACCGGCGATGGCTCCTGTGTGGC
  L  K  S  H  S  T  E  T  K  L  E  A  T  G  D  G  S  C  V  A

       430        440        450        460        470        480
CAAGCTCAAGGTGGAGTACGAGCTCGAGGACGGCAGCTCACTGTCGCCGGAGAAGGAGAA
  K  L  K  V  E  Y  E  L  E  D  G  S  S  L  S  P  E  K  E  K

       490        500        510        520        530        540
GGACATCGTGGATGGCTACTATGGCATGCTCAAGATGATCGAGGACTACCTCGTCGCTCA
  D  I  V  D  G  Y  Y  G  M  L  K  M  I  E  D  Y  L  V  A  H

       550        560        570        580        590        600
CCCTGCCGAATACGCCTAAGATGAAGAGGAATACTGCCTCTATCCAGTATATCCCACCTA
  P  A  E  Y  A  *

       610        620        630        640        650        660
GAGTGAGTGATAATTAAATAATGAGAGCCGCAGAAATGTCCAAATTCTCGTGGCGTTTGA

       670        680        690        700        710        720
GTCCGTGAGAGTAATTTCGTGCTTTAAGTTTGTGGTTGTGTTTATGTGCCTTTCTATGGT

       730        740        750        760        770        780
CGTATTCAGTGTTAAAGTTATCATTTTGCTTCATCAATGGGTGAATAAAGAGAGGCAAGT

       790        800        810        820        830        840
CTGAATGTGTTCTGCTATGGTTTGGAGGTTAATATGGAAGATTGAAAATCAAA.......
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Fig. 2 Nucleotide and deduced amino acid sequence of *PBZ1* cDNA. A putative polyadenylation signal is underlined. The nucleotide sequence data has been submitted to the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the accession number D38170.

not yet been elucidated. This class of proteins includes pea disease resistance response protein pI49 (30% identity; Fristensky et al. 1988), birch allergenic protein BetvI (29% identity; Breiteneder et al. 1989), asparagus wound-induced protein AoPR1 (29% identity; Warner et al. 1992), soybean stress-induced protein SAM22 (28% identity; Crowell et al. 1992), hazel allergenic protein CoraI (27% identity; Breiteneder et al. 1993), potato elicitor-induced protein STH-2 (27% identity; Matton and Brisson 1989), pea ABA-responsive protein ABR17 (26% identity; Iturriaga et al. 1994), parsley PR protein PcPR1-1 (25% identity; Somssich et al. 1988) and bean elicitor-induced protein PvPR1 (24% identity; Walter et al. 1990). Alignment of the amino acid sequences of these IPR proteins is shown in

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PBZ1      MAPACVSDEH AVAVSAERLW KA-FMDASTL -PKACAGLV- DDIAVEGNGG     47
pI49      *GVFN*E**I TSV*APAI*Y **LVT**DN* T**VI-DAIK SIEI******     49
BetvI     *GVFNYET*T TSVIP*A**F **FIL*GDN* F**VAPQAIS SVENI******     50
AoPR1     *SSGSW*H*V **N*A*G*MF **AML*WHN* G**IVPDFIA GGSV*S*D*S     50
SAM22     *GVFTFE**I NSP*APAT*Y **LVT**DNV I***L-DSFK SVEN******     49
CorAI     *GVFNYEA*T TSVIP*A**F *SYVL*GDK* I**VAPQAIT SVEN******     50
STH-2     *GVTSYTH*T TTPIAPT**F **LVV*SDN* I**LMPQV-- --KNI*AE*D     46
ABR17     *GVFVFD**Y VST*APPK*Y **LAK**DEI V**VIKEA-Q GVEII******     49
PcPR1-1   *GVQKSEV*T TSS****K*F *GLCL*ID** L*QVLP*AIK SSETL**D**     50
PvPR1     *GVFTFE*QT TSP*APAT*Y **VAK**D*I F***LPDSFK SVEI*******     50

PBZ1      PGTIYTMKLN PAAGVGSTYK TRVAVCDAAS HVLKSDVLEA ESKVGKLKSH     97
pI49      A***KKLTFV EDG-ETKHVL HK*ELV*V*N LAYNYSIVGG VGFPDTVEKI     98
BetvI     ****KKISFP EGF-PFKYV* D**DEV*HTN FKYNYS*I*G GPIGDT*EKI     99
AoPR1     V***REI*I* NP*IPF*YV* E*LDFV*HDK FEV*QTLV*G GGLGKMFECA    100
SAM22     ****KKITFL EDG-ETKFVL HKIESI*E*N LGYSYS*VGG AALPDTAEKI     98
CorAI     ****KNITFG EGS-RYKYV* E**DEV*NTN FTYSYT*I*G DVLGD**EKV     99
STH-2     -*S*KK*NFV E-GSPIKYL* HKIH*V*DKN L*T*YSMI*G DVLGD**E*I     94
ABR17     ****KKLSIL EDG-KTNYVL HKLDAV*E*N FGYNYSLVGG PGLHES*EKV     98
PcPR1-1   V**VKLVH*G D*S-PFK*M* QK*DAI*K*T FTYSYSIIDG DILL*FIE*I     99
PvPR1     ****KKISFV EDG-ETKFVL HKIESI*E*N LGYSYSIVGG VALPETAEKI     99

PBZ1      STETKLEATG DGSCVAKLKV EYELEDGSSL SPEKEKDIVD GYYGMLKMIE    147
pI49      *F*A**S*GP N*GSI***S* K*FTKGDAAP *E*QL*TDKA KGD*LF*AL*    148
BetvI     *N*I*IV**P **GSIL*ISN K*HTKGDHEV KA*QV*ASKE MGETL*RAV*    149
AoPR1     T*HF*F*PSS N*G*LV*VTA S*KILP*VAD ESA*A*EGIT NHM---*AT*    147
SAM22     TFDS**V*GP N*GSAG**T* K**TKGDAEP NQDEL*TGKA KADALF*A**    148
CorAI     CH*L*IV*AP G*GSIL*ISS KFHAKGDHEI NA*EM*GAKE MAEKL*RAV*    149
STH-2     *YDL*F**H* N*G**C*SIT **HTKGDYV* KD*EHNEGQK QGMELF*IV*    144
ABR17     AF**IIL*GS **GSIV*IS* K*HTKGDAA* *DAVRDETKA KGT*LI*A**    148
PcPR1-1   NNHFTAVPNA **G*TV*STI IFNTKGDAVV PE*NI*FAN* QNLTIF*AV*    149
PvPR1     TFDS**SDGP N*GSLI**SI T*HSKGDAPP NEDEL*AGKA KSDSLF*AV*    149

PBZ1      DYLVAHPAEY A                                              158
pI49      G*CL***D-* N                                              158
BetvI     S**L**SDA* N                                              160
AoPR1     A**L*N*TA* V                                              158
SAM22     A**L***D-* N                                              158
CorAI     T**L**S*** N                                              160
STH-2     A**L*N*SV* *                                              155
ABR17     G*VL*N*GY- -                                              157
PcPR1-1   A**I*N---- -                                              155
PvPR1     A**L*N*--- -                                              156
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Fig. 3 Alignment of the amino acid sequences of PBZ1 and other IPR homologues. The predicted amino acid sequence of the PBZ1 protein is compared with that of pea pI49 protein, birch BetvI allergenic protein, asparagus AoPR1 protein, soybean SAM22 protein, hazel CorAI allergenic protein, potato STH-2 protein, pea ABR17 protein, parsley PcPR1-1 protein and bean PvPR1 protein. Amino acids in the individual proteins that are identical to residues in the PBZ1 protein are indicated by asterisks. Gaps, introduced to maximize homology, are shown by hyphens. Conserved amino acid residues are boxed.

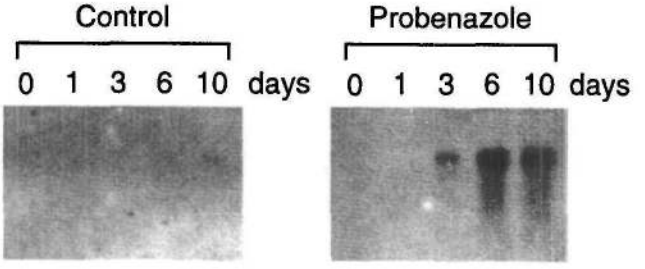

Fig. 4 Accumulation with time of *PBZ1* mRNA after treatment with probenazole. Total RNA was isolated from rice leaves after treatment for the indicated times with a solvent only (0.05% Tween 20, 1% acetone; control) or a solvent containing 100 mg liter^{-1} probenazole by submerged application. Ten μg of total RNA were applied to each lane in a formaldehyde agarose gel. After electrophoresis, RNA was transferred onto a nylon membrane and allowed to hybridized with *PBZ1* cDNA as probe.

Figure 3. No conserved domains were found but conserved residues were found scattered throughout the sequence of the PBZ1 protein.

Accumulation of PBZ1 mRNA in response to probenazole—We examined the time course of the accumulation of *PBZ1* mRNA in rice after treatment with probenazole by submerged application (Fig. 4). Accumulation of the *PBZ1* mRNA was not induced in the control plants at least over the experimental period. However, the accumulation of *PBZ1* mRNA was induced within 3 days after treatment with probenazole, reached the maximum level at 6 days and remained at an elevated level with only a slight decrease until at least 10 days. Although the extent of accumulation was several-fold lower, the same accumulation pattern was observed after treatment with 2-butyl BIT (data not shown). In the greenhouse, the anti-rice blast activity was maximal after treatment with probenazole by submerged application 6 to 10 days before inoculation (Meiji Seika Kaisha, Ltd., unpublished results). This result suggests that the time course of the accumulation of the *PBZ1* mRNA after treatment with probenazole corresponds to that of the development of anti-rice blast activity.

Accumulation of PBZ1 mRNA by inoculation with a pathogen or by wounding—The predicted PBZ1 amino acid sequence exhibited significant homology to IPR proteins. Therefore, we examined the accumulation of *PBZ1* mRNA by inoculation with a pathogen or by wounding. As shown in Figure 5, accumulation of *PBZ1* mRNA was significantly induced by inoculation with rice blast fungus but not at all by wounding, indicating that the PBZ1 protein is a PR protein.

The accumulation of some PR proteins is known to be induced more rapidly after inoculation in an incompatible than in a compatible interaction (Bell et al. 1986, Constabel and Brisson 1992). Such a differential pattern of accumulation is considered to be important in disease resistance. If the *PBZ1* gene is related to disease resistance, its expression would thus be induced sooner after inoculation in an incompatible than in a compatible interaction. To investigate this possibility, we examined the time course of the accumulation of *PBZ1* mRNA in rice after inoculation in both types of interaction. As shown in Figure 6, the accumulation of the *PBZ1* mRNA was induced 36 h after inoculation in an incompatible interaction, whereas 60 h were required in a compatible interaction. This result indicates that the *PBZ1* gene is somewhat related to disease resistance. On the other hand, necrotic lesions appeared 60 h after inoculation in both compatible and incompatible interactions, and thereafter spread only in a compatible interaction in this experiment. Thus, the appearance of necrotic lesions seemed not to be correlated with the accumulation of *PBZ1* mRNA.

Accumulation of PBZ1 mRNA after treatment with various chemicals and probenazole-related compounds—To investigate whether simple chemical stimulation could also induce the accumulation of *PBZ1* mRNA, we examined the levels of this transcript after treatment with various agents. As shown in Figure 7, no accumulation of *PBZ1* mRNA was induced after treatment with ethephon (ethylene releasing agent), NAA, sodium salicylate, NaCl

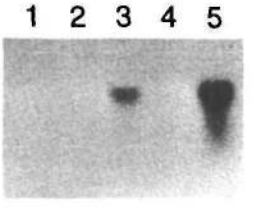

Fig. 5 Accumulation of *PBZ1* mRNA by inoculation with a pathogen or by wounding. Total RNA was isolated from rice leaves 6 days after treatment. Lane 1, no treatment; lane 2, mock inoculation; lane 3, inoculation with compatible rice blast fungus; lane 4, wounding; lane 5, 100 mg liter^{-1} probenazole by submerged application. Ten μg of total RNA were analyzed in each lane with *PBZ1* cDNA as probe.

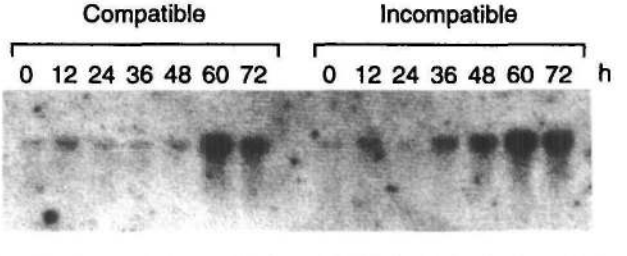

Fig. 6 Accumulation with time of *PBZ1* mRNA by inoculation with a compatible race or an incompatible race of rice blast fungus. Total RNA was isolated from rice leaves after inoculation with a compatible race or an incompatible race of rice blast fungus. Ten μg of total RNA were analyzed in each lane with *PBZ1* cDNA as probe.

or mannitol. This result indicates that the expression of the *PBZ1* gene is not a simple nonspecific response to chemical stimulation.

Next, to investigate the relationship between the accumulation of *PBZ1* mRNA and the anti-rice blast activity of probenazole-related compounds, we examined the transcript levels after treatment with probenazole-related compounds (Fig. 8). The structures of these compounds are shown in Figure 1. BIT and allyl *O*-sulfamoylbenzoate are metabolites of probenazole in rice (Uchiyama et al. 1973) and the anti-rice blast activity of these compounds is comparatively strong. By contrast, N-substitution of BIT decreases its anti-rice blast activity dramatically and the activities of 2-butyl BIT and 2-ethoxycarbonylmethyl BIT are very weak. In this experiment, we also used NCI, which belongs to another group of compounds showing strong anti-rice blast activity and inducing activity of disease resistance in rice similarly to probenazole (Seguchi et al. 1992a, b). As shown in Figure 8, accumulation of *PBZ1* mRNA was induced at high level after treatment with probenazole or allyl *O*-sulfamoylbenzoate, at a lower level after treatment with NCI and at further low level after treatment with 2-butyl BIT but not after treatment with BIT or 2-ethoxycarbonylmethyl BIT. Thus, the accumulation of *PBZ1* mRNA seemed to be correlated with anti-rice blast activity after treatment with probenazole, with allyl *O*-sulfamoylbenzoate, with 2-ethoxycarbonylmethyl BIT and with NCI but not after treatment with BIT or with 2-butyl BIT. Although BIT has strong anti-rice blast activity, this compound did not induce the accumulation of the *PBZ1* mRNA. Thus, the accumulation of *PBZ1* mRNA after treatment with probenazole-related compounds was not fully correlated with anti-rice blast activity.

Organization of genes similar to PBZ1—To further characterize the *PBZ1* gene, we performed Southern blot analysis of rice DNA using *PBZ1* cDNA as probe. We used

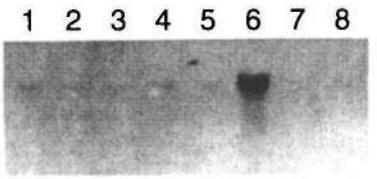

Fig. 7 Accumulation of *PBZ1* mRNA after treatment with various chemicals. Total RNA was isolated from rice leaves 6 days after treatment with a solvent only (0.05% Tween 20, 1% acetone; control, lane 1), or a solution of 100 mg liter^{-1} ethephon (lane 2), 100 mg liter^{-1} NAA (lane 3) or 5,000 mg liter^{-1} sodium salicylate (lane 4) by foliar application or from rice leaves 6 days after the start of treatment with a solution of 1,000 mg liter^{-1} NaCl (lane 5), 100 mg liter^{-1} probenazole (lane 6), 1,000 mg liter^{-1} mannitol (lane 7) or a solvent only (control, lane 8) by submerged application. Ten μg of total RNA were analyzed in each lane with *PBZ1* cDNA as probe.

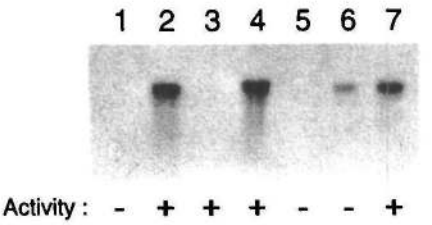

Fig. 8 Accumulation of *PBZ1* mRNA after treatment with probenazole-related compounds or NCI. Total RNA was isolated from rice leaves 6 days after the start of treatment with a solvent only (0.05% Tween 20, 1% acetone; control, lane 1), or a solution of 100 mg liter^{-1} probenazole (lane 2), 100 mg liter^{-1} BIT (lane 3), 100 mg liter^{-1} allyl *O*-sulfamoylbenzoate (lane 4), 100 mg liter^{-1} 2-ethoxycarbonylmethyl BIT (lane 5), 100 mg liter^{-1} 2-butyl BIT (lane 6) or 100 mg liter^{-1} NCI (lane 7) by submerged application. Ten μg of total RNA were analyzed in each lane with *PBZ1* cDNA as probe. Anti-rice blast activity is indicated under each lane as inactive (−) or active (+).

*Bam*HI, *Eco*RI and *Xba*I to digest rice DNA. These restriction enzymes do not cut within the *PBZ1* cDNA. As shown in Figure 9, one intense band appeared in each lane. In addition, three other bands of much weaker intensity appeared in the case of the *Bam*HI digest of rice DNA and two other bands of much weaker intensity appeared in the case of the *Eco*RI digest and the *Xba*I digest. These data suggest that the *PBZ1* gene is part of a small multigene family with at

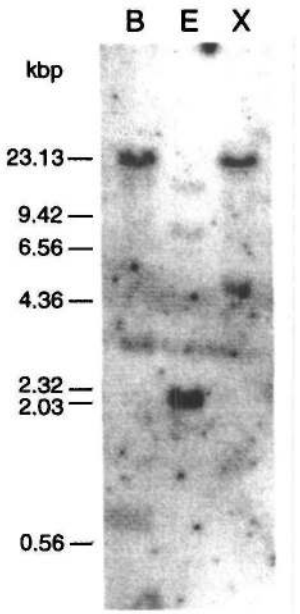

Fig. 9 Southern blot analysis of the *PBZ1* sequence in the rice genome. Three μg of DNA were digested with *Bam*HI (B), *Eco*RI (E) or *Xba*I (X) and applied to the indicated lane in an agarose gel. After electrophoresis, DNA was transferred onto a nylon membrane and allowed to hybridize with *PBZ1* cDNA as probe.

least three members in rice.

Discussion

Probenazole is an effective agricultural chemical against not only rice blast disease but also some bacterial diseases. Although it has been used for about 20 years in Japan, the appearance of resistant strains has not been reported to date. This might be attributed to its mechanism of induction of disease resistance. Extensive studies have been conducted concerning the induction of disease resistance by probenazole (Watanabe et al. 1979, Iwata et al. 1980, Sekizawa et al. 1981, 1985, Shimura et al. 1981, 1983). However, its primary site of action has not yet been elucidated. Therefore, to investigate the mechanism of action of probenazole, we attempted to isolate cDNA clones of probenazole-inducible genes.

Recently, it was reported that the accumulation of at least five transcripts was induced after treatment with probenazole, and a probenazole-responsive cDNA clone, pPB-1, was isolated (Minami and Ando 1994). We described here the isolation of another cDNA clone, which encoded a protein designated PBZ1, by differential screening of a cDNA library constructed from mRNA of probenazole-treated rice leaves. However, we failed to isolate pPB-1 in our screening, perhaps because of differences in the screening methods used in these two studies. To isolate the cDNA clones of probenazole-inducible genes, we used cDNA probes derived from probenazole-treated and 2-butyl BIT-treated rice leaves. By contrast, for the isolation of pPB-1, Minami and Ando used cDNA probes derived from probenazole-treated and non-treated rice leaves. If the accumulation of pPB-1 mRNA is induced at a high level after treatment with 2-butyl BIT, we would obviously have failed to isolate pPB-1 cDNA by our method. In addition, we did not isolate other cDNA clones of probenazole-inducible genes, which might indicate that such transcripts represent only a minor population of the total mRNA. However, we believe that the expression of several genes is necessary for probenazole to induce disease resistance in plants, and the isolation of other genes should be possible by more extensive screening.

The predicted PBZ1 protein showed significant structural similarity to IPR proteins. However, the extent of identity at the amino acid level between the predicted PBZ1 protein and IPR proteins of both dicotyledonous and monocotyledonous plants was low (about 30% in both cases). IPR proteins do not have a signal sequence and are comparatively small, acidic proteins. In addition, IPR genes form a multigene family (Somssich et al. 1988, Chiang and Hadwiger 1990, Walter et al. 1990, Crowell et al. 1992, Breiteneder et al. 1993, Iturriaga et al. 1994). However, their functions are currently unknown.

The accumulation of *PBZ1* mRNA was induced after inoculation with rice blast fungus (Fig. 5). Furthermore, it was induced sooner by inoculation with an incompatible race than that with a compatible race (Fig. 6). These results indicate that at least one of the probenazole-inducible genes is a PR gene and the *PBZ1* gene might be involved, at least to some extent, in anti-rice blast activity. In addition, these results also suggest that the mechanism of action of probenazole might be consistent with that of INA, which induces disease resistance in plants by inducing the coordinate expression of several PR genes.

We examined the accumulation of *PBZ1* mRNA after treatment with various chemicals and found that it was not induced after treatment with ethephon or NAA (Fig. 7), both of which are known to induce disease resistance in rice (Matsumoto et al. 1980, Iwata et al. 1981). In addition, ethylene has been suggested to act as a signal molecule in the resistance response and NAA to induce disease resistance by inducing ethylene production (Sekizawa and Mase 1981). If the *PBZ1* gene is important in disease resistance, induction of the expression of this gene might be an upstream event in ethylene production or there might exist another signal transduction pathway that does not require ethylene. On the other hand, the accumulation of *PBZ1* mRNA was not induced after treatment with sodium salicylate (Fig. 7). Salicylic acid is an endogenous signal molecule required for the induction of PR genes (Malamy et al. 1990, Metraux et al. 1990, Gaffney et al. 1993) and induces disease resistance in various plant species (White 1979, Millis and Wood 1984, Pennazio et al. 1987, Rasmussen et al. 1991, Walters et al. 1993). In addition, some IPR proteins, such as SAM22 and AoPR1, are known to be induced after treatment with salicylic acid (Crowell et al. 1992, Warner et al. 1994). However, it is not necessarily surprising that the expression of the *PBZ1* gene was not induced by salicylic acid in rice since this compound has been shown to be present in rice at high levels under normal conditions (Raskin et al. 1990) and no salicylic acid-binding activity has been detected in rice (Sanchez-Casas and Klessig 1994). Therefore, salicylic acid seems unlikely to act as an endogenous signal molecule in rice. It seems likely that probenazole and/or its metabolites might work as agonists of the endogenous signal molecule(s) required for the induction of PR genes in rice.

When we examined the accumulation of *PBZ1* mRNA after treatment with probenazole-related compounds, we found that it was not fully correlated with anti-rice blast activity (Fig. 8). Although BIT has comparatively strong anti-rice blast activity, this compound did not induce the accumulation of *PBZ1* mRNA. This finding was unexpected since BIT has been considered to be an active form of probenazole in plants. BIT is a major metabolite of probenazole in plant tissue (Uchiyama et al. 1973). Furthermore, in BIT-treated rice leaves, the rapid generation of superoxide anions, as occurs in an incompatible interaction, has been

observed in a compatible interaction (Sekizawa et al. 1987) and the release of α-linolenic acid is activated after elicitor stimulation (Kanoh et al. 1993a, b). However, we showed here that at least one differential response related to disease resistance exists between probenazole and BIT. If the induced disease resistance results from the coordinate expression of PR genes, BIT might evoke disease resistance by inducing the expression of PR genes but not of some other genes, such as PBZ1, by an as yet unknown mechanism. In addition, this might explain why the anti-rice blast activity of BIT is slightly lower than that of probenazole (Watanabe et al. 1977). To investigate whether BIT-inducible genes exist in rice, we attempted to isolate cDNA clones of BIT-inducible genes by differential screening of a cDNA library constructed from the mRNA of BIT-treated rice leaves. However, we failed to isolate any cDNA clones of BIT-inducible genes. Perhaps such transcripts represent a very minor population among the total mRNA. However, the isolation of BIT-inducible genes should be possible by further, more extensive screening. By contrast, the accumulation of *PBZ1* mRNA was induced by NCI (Fig. 8), which belongs to another group of compounds known to induce disease resistance in rice similarly to probenazole (Seguchi et al. 1992a, b). This finding supports our hypothesis that the *PBZ1* gene is related to disease resistance.

Although the PBZ1 protein appears to be related to disease resistance, the function of this protein remains unknown. In this study, the accumulation of *PBZ1* mRNA was not fully correlated with anti-rice blast activity after treatment with probenazole-related compounds (Fig. 8). Furthermore, induction of the accumulation of *PBZ1* mRNA by inoculation with an incompatible race required rather a long lag period (36 h; Fig. 6) as compared with the resistance response: the growth of invading hyphae stops within 22 h (Koga 1994). Therefore, we can assume that the PBZ1 protein is not related to the primary defense response but is, rather, related to the secondary response. In other words, the PBZ1 protein might be needed, for example, to restore certain functions of damaged cells after infection.

Constabel et al. (1993) reported that transgenic potato plants expressing the STH-2 gene remained susceptible against a compatible race of *Phytophthora infestans* and potato virus X. By contrast, Chang et al. (1993) reported that transgenic potato plants expressing the pea DRR49 (pI49) gene had higher yields of tuber than those of control plants when grown on potato early dying-infected soil. In addition, such plants developed fewer and smaller lesions than the control plants after inoculation with *Verticillium dahliae*. Although these reports on IPR proteins are conflicting, they suggest that the introduction of only a single gene for an IPR protein can increase disease resistance against specific pathogens. Constitutive expression of the *PBZ1* gene in plants might also confer disease resistance against certain pathogens and an investigation of this possibility is currently in progress in our laboratory.

In this paper, we reported that probenazole induced the expression of a member of the family of IPR genes, which encoded a putative protein designated as PBZ1. Although the function of the putative PBZ1 protein remains to be determined, elucidation of its function might help us to understand not only the mechanism of action of probenazole but also some aspects of disease resistance in plants.

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