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ORIGINAL PAPER

Identification of defense-related genes in rice responding to challenge by *Rhizoctonia solani*

Chang-Jiang Zhao · Ai-Rong Wang · Yu-Jun Shi · Liu-Qing Wang · Wen-De Liu · Zong-Hua Wang · Guo-Dong Lu

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Abstract Rice sheath blight, caused by *Rhizoctonia solani* is one of the major diseases of rice. The pathogen infects rice plants directly through stomata or using lobate appressoria and hyphal masses called infection cushions. The infection structures were normally found at 36 h post-inoculation. During infection, the pathogenesis-related genes, *PR1b* and *PBZ1* were induced in rice plants. To identify rice genes induced early in the defense response, suppression subtractive hybridization (SSH) was used to generate a cDNA library enriched for transcripts differentially expressed during infection by *R. solani*. After differential screening by membrane-based hybridization and subsequent confirmation by reverse Northern blot analysis, selected clones were sequenced. Fifty unique cDNA clones were found and assigned to five different functional categories. Most of the genes were not previously identified as being induced in response to pathogens. We examined expression of 100 rice genes induced by infection with *Magnaporthe grisea*, *Xanthomonas oryzae* pv. *oryze* (*Xoo*) and *X. oryzae* pv. *oryzicola* (*Xooc*). Twenty-five of them were found to be differentially expressed after the sheath blight infection, suggesting overlap of defense responses to different fungal and bacterial pathogens infection.

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Chang-Jiang Zhao and Ai-Rong Wang contributed equally to this work.

C.-J. Zhao · A.-R. Wang · Y.-J. Shi · L.-Q. Wang · W.-D. Liu · Z.-H. Wang (✉) · G.-D. Lu (✉)
The Key Laboratory of Biopesticide and Chemical Biology, The Ministry of Education, and Functional Genomics Center, Fujian Agriculture and Forestry University, Fuzhou 350002, China
e-mail: zonghuaw@163.com

G.-D. Lu
e-mail: lgd@fjau.edu.cn

Introduction

Rice sheath blight, caused by the fungal pathogen *Rhizoctonia solani* Kuhn [Sexual stage: *Thanetophorus cucumeris* (Frank) Donk] is one of the major production constraints in rice-growing countries of the world. Currently, the best method for controlling sheath blight is applying commercial fungicides. Breeding for sheath blight resistant rice plants has not been very successful mainly because of lack of resistant donors in the cultivated varieties of rice (Bonmann et al. 1992).

The most common resistance reaction in plants is the hypersensitive response (HR), which localizes the pathogen at the infection site through cell death. The local responses at the point of infection also trigger a subsequent non-specific resistance that occurs throughout the entire plant; this phenomenon is called systemic acquired resistance (SAR). Typical SAR results in an increase of endogenous salicylic acid (SA) and transcriptional activation of defense-related genes including pathogenesis-related genes *PR1*, *PR2* and *PR5* etc. It finally leads to an enhanced resistance to a broad spectrum of pathogens throughout the plant (Ryals et al. 1996; Durrant and Dong 2004).

The HR typically occurs in the diseases caused by biotrophic pathogens, in which defence signaling is initiated by R protein–Avr protein interactions, and many of these pathways require SA (Denby et al. 2004). However, HR does not protect plants against infection by the necrotrophic pathogens *Botrytis cinerea* and *Sclerotinia sclerotiorum*. In contrast, *B. cinerea* triggered HR facilitates colonization of plants. Hence, these fungi can exploit a host defense

mechanism to promote their pathogenesis (Govrin and Levine 2000). Resistance to necrotrophic pathogens is based on defense responses largely distinct from those considered to be effective against biotrophs. It involves defense mechanisms regulated by the jasmonic acid (JA) and ethylene signaling routes, neutralization of reactive oxygen species (ROS) and cell death control as well as the production of antimicrobial metabolites, such as camalexin in *Arabidopsis* (Van Baarlen et al. 2007; Glazebrook 2005).

Although rice, being the most important food crop for human consumption has attracted great research interest upon the completion of its genome sequence, relatively little is known about the pathways and mechanisms leading to disease resistance. So far, most of research has focused on the "gene-for-gene" interaction diseases, such as rice blast disease and rice bacterial leaf blight disease. It was shown that just as in *Arabidopsis*, a NPR1-mediated signaling similar pathway exists in rice to control resistance to bacterial leaf blight (Chern et al. 2001, 2005a, b). Some signaling components, such as a small GTP-binding protein (OsRac1), MAP kinase cascades, rab-specific GDP-dissociation inhibitors (OsGDIs), a Myb family transcription factor (JAmyb), and the WRKY family of transcriptional activators were found to be associated with rice defense signaling pathway (Kawasaki et al. 1999; Agrawal et al. 2003; Xiong and Yang 2003; Kim et al. 1999; Lee et al. 2001; Liu et al. 2005; Qiu et al. 2007). Many defense-related genes, which were responsive to rice blast (*Magnaporthe grisea*) and/or rice bacterial leaf blight infection (*X. oryzae* pv. *oryzae*), have been isolated using reverse transcription-PCR, suppression subtractive hybridization (SSH), cDNA library differential screening, cDNA array and large-scale EST sequencing methods (Kim et al. 2000; Xiong et al. 2001; Zhou et al. 2002; Lu et al. 2004; Jantasuriyarat et al. 2005).

Rice sheath blight is a necrotrophic disease. Consequently, very little is known about the host–fungus interaction at the molecular level. As a first step to define the defense-related gene expression profile, we first observed the infection process of the pathogen and the corresponding expression pattern of the pathogenesis-related genes, *PR1b* and *PBZ1* (Midoh and Iwata 1996). Our results showed that expression of *PR1b* and *PBZ1* were related with disease development of rice sheath blight. We then applied SSH to identify genes involved in early defense responses in rice. Fifty unique cDNA clones were found and assigned to five different groups according to the putative function of their homologs. To investigate whether there is overlap of rice defense responses to different pathogens, 100 rice ESTs which were previously reported to be induced by *M. grisea*, *X. oryzae* pv. *oryze* and *X. oryzae* pv. *oryzicola* were arrayed to test their expression patterns in rice after challenge by *R. solani*. We found that 25 out of 100 genes were expressed differentially after sheath blight infection. This research represents the first step to understand the response of rice to *R. solani* infection at the molecular level. Results from this study will serve as a foundation for further investigations into rice defense mechanisms against *R. solani* and the breeding of new rice varieties expressing high levels of resistance against the rice sheath blight disease and other rice diseases.

Materials and methods

Plants and inoculation

Rice cultivar 93–11, which was sequenced by the Beijing Genomics Institute (http://www.rice.genomics.org.cn/) was used in this experiment. The fungal pathogen isolate JS of *R. solani* was collected and purified from the breeding field of the Fujian Agriculture and Forestry University. Plants were grown in a greenhouse under natural light. Rice seedlings with 4–6 leaves were inoculated by inserting two small toothpicks, one in the upper part of the first leaf sheath and one in the upper part of the second leaf sheath. The inoculum toothpicks were pre-incubated with *R. solani* isolate JS in potato dextrose agar (PDA) plates for 3 days at 28°C. For mock inoculation, the toothpicks were incubated in PDA plates without the fungus. The inoculated plants were placed in plastic bags for 24 h at 28°C, and subsequently transferred to the growth chamber with a daytime temperature of 28°C and a nighttime temperature of 25°C with 100% relative humidity. Leaf tissue was harvested at 0, 4, 12, 24, 36, 48 and 72 h post-inoculation (hpi) for total RNA extraction and observation of disease development.

RNA isolation and Northern blot analysis

Total RNAs were isolated as described by Lu et al. (2004). For Northern blot analysis, 20 μg of total RNAs were denatured and separated on a 1.2% agarose-formaldehyde gel and transferred to Hybond N+ membranes (Amersham Pharmacia Biotech, UK) according to the manufacturer's instructions. DNA probes were labeled with α-^{32}P-dCTP. Northern blot hybridization was carried out using standard procedures as described in Sambrook et al. (1989), and was repeated twice. Two defense-related genes were used in the Northern analysis. The probes for *PR1b* (accession number U89895) and *PBZ1* (accession number D38170) were amplified from rice genomic DNA. RNA blots were also probed with the rice actin 1 gene (Wang et al.1992) as an internal control.

Observation of the infection process of disease

To observe the *R. solani* infection process, rice samples were prepared as described by Mou et al. (2000) with some

modifications. For light microscopy, leaves were fixed and cleared by soaking in glacial acetic acid and ethanol (1:5, vol/vol) for 48–72 h. Next, samples were stained with an alcoholic lactophenol trypan blue mixture, and destained with chloral hydrate solution. After multiple exchanges of chloral hydrate solution to reduce the background, samples were equilibrated with 50% glycerol, mounted, and observed with a microscope. For electron microscopy, rosette leaf sheaths were fixed with 4% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, and incubated at 4°C overnight, and then dehydrated in a graded series of ethanol. Samples were critical point dried, sputter-coated with platinum, and observed under a scanning electron microscope.

Suppression subtractive library construction and differential screening

Suppression subtractive hybridization was performed using the "PCR Select cDNA Subtraction Kit" from Clontech (Palo Alto, CA, USA). The SSH cDNA library was constructed according to the manufacturer's instructions. For constructing the library, the mixed mRNA samples (equal amounts of RNA from 4, 12, 24, 48 hpi) from inoculated plants were used as the "tester", and the mixed mRNA samples from mock inoculation at the same time point were used as the "driver". The final PCR products were cloned into pGEM-T easy vector (Promega) and transformed by electroporation, into DH10B *Escherichia coli* cells.

Individual clones from the subtractive library were randomly picked and stored in 96-well plates. For differential screening, the clones were transferred to Hybond-N+ nylon membranes (Amersham, Piscataway, NJ, USA) and grown at 37°C for colony hybridization. Duplicate membranes were hybridized with two different probes: the forward-subtracted probe and the reverse-subtracted probe. In the case of the forward-subtracted probe, cDNA was synthesized from mRNA that was obtained from inoculated rice plants as tester and mock inoculated plants as driver. This probe was enriched for transcripts induced during infection by the sheath blight fungus. The reverse-subtracted probe was similarly prepared, except that the tester mRNA was derived from mock inoculated plants and the driver mRNA from inoculated plants. This probe was enriched for transcripts that expressed in uninfected condition. After the first screening, all positive clones showing differential hybridization were picked to new 96-well plates and re-screened using the same set of probes.

cDNA probes prepared from *R. solani* infected leaf tissues

Total RNAs were isolated from *R. solani* infected plants and uninfected plants using the RNeasy Plant Mini Kit from Qiagen (Valencia, CA, USA), and used as templates for cDNA synthesis, using the SMART cDNA construction kit from Clontech. For probes used for reverse Northern blot analysis, cDNAs were labeled withα-^{32}P-dCTP using the Megaprime DNA labeling system (Amersham).

Reverse Northern blot analysis

The cDNA inserts were PCR-amplified individually from positive clones with M13-F and M13-R primers. The PCR products were then loaded, in duplicate, on two 1% agarose gels. After electrophoresis, DNA was transferred to Hybond N+ membranes. The DNA blot contained two control cDNAs, the pathogenesis-related genes (*PR1b*) and a constitutively expressed rice actin1 gene (*Act1*). Duplicate blots were hybridized with ^{32}P-labeled cDNAs from *R. solani* infected and uninfected plants.

Screening of defense response genes triggered by multiple pathogens

Based on published data (Xiong et al. 2001; Rauyaree et al. 2001; Pei et al. 2002; Rao et al. 2002; Zhou et al. 2002; Lu et al. 2004), 169 rice defence response ESTs which were induced by *M. grisea*, *X. oryzae* pv.*oryze* (*Xoo*) and *X. oryzae* pv. *oryzicola* (*Xooc*) were obtained. The software Primer 5.0 was used to design the primers for PCR amplification of these sequences from rice-genomic DNA and 100 gene fragments were amplified successfully. The amplified fragments were denatured by adding 1 μL of the denature buffer (0.4 mol/L NaOH, 100 mmol/L EDTA) to 9 μL PCR products and incubating for 5 min at 90°C. The denatured DNAs were arrayed equally onto two Hybond N$^+$ nylon membranes, the pathogenesis-related gene (*PR1b*) and constitutively active rice actin1 gene (*Act1*) were used as control (indicted in Table 1). After air-drying, the membranes were baked for 2 h in 80°C vacuum oven. To screen the genes induced by *R. solani*, the duplicate filters were hybridized with ^{32}P-labeled cDNA probes derived from infected and uninfected plants.

Analysis of transcript levels by reverse transcriptase-mediated PCR

Reverse transcriptase PCR (RT-PCR) was used to detect the expression patterns of selected genes. Total RNA was reverse-transcribed into first-strand cDNA with oligo-dT primer using the reverse transcription system of Promega, and the cDNA was used as a template for PCR to analyze the selected genes. The primers for the PCR were designed based on the sequences of the genes (Table 2). For the control, the *Act1* gene was amplified using the same cDNA template. The PCR products were separated on a 1.5% agarose gel.

Table 1 100 different ESTs which are known induced by other pathogens were arrayed in nylon membranes

	1	2	3	4	5	6	7	8	9	10
A	*Act1*	BF108313	BF108338	BF108316	CD645562	CD645560	CD645580	CD645545	BF108332	CD645546
B	BF108345	BF108331	CD645551	CD645555	BF108335	BF108336	BF108347	BF108348	BF145170	BF108310
C	BF108312	BF108314	BF145168	BF145213	BF145216	BF145167	BF145206	BF145210	BF145207	BF145176
D	BF108329	BF108334	CD645553	BF108352	BF108364	BF108365	BF145180	BF145182	BF145197	BF145198
E	BF145204	BF108354	BF108351	CD645590	CD645548	BF145163	BF108309	BF108317	BF108325	BF108328
F	BF108321	BF108340	CD645563	CD645582	CD645568	CD645570	CD645573	CD645574	CD645575	CD645576
G	CD645579	CD645580	BF889446	BF108351	CD645567	BF145177	BF145183	BF145184	BF145186	BF145190
H	BF145194	BF889432	BF889436	BF889440	BF889441	BF889444	BF889446	BF889447	BF889449	BF889453
I	BF889464	BF889465	BF889467	BF889478	BF889482	BF889486	AF010579	BE040982	BI796359	AW155114
J	BI798243	BI796809	BI799079	BI797390	BI799917	BI799495	BI795502	BI795001	BI795773	BE607423
K	AC144455	AP002844	AC021893	BF145186	CD645568	CD645558	*PR1b*	*Act1*		

The EST names in the array are accession numbers from NCBI databases. The A7 and G2 (CD645580), E3 and G4 (BF108351), F5 and K5 (CD645568), G3 and H7 (BF889446), G9 and K4 (BF145186) were the repeated ESTs

Table 2 The primers for RT-PCR to confirm differentially regulated genes

Gene	Forward primer	Reverse primer
PR1	5′-CAGGACTACGTGAGGCTCCA-3′	5′-CCTCTGTCCGACGAAGTTGC-3′
PBZ1	5′-ACGCGTCCACTTTGCCCAAG-3′	5′-TTCTCCGGCGACAGTGAGCT-3′
Act1	5′-CTGCTGGAATGTGCTGAGAGAT-3′	5′-CGTCTGCGATAATGGAACTGG-3′
2B4	5′-GCCTTCAACCACATAAACC-3′	5′-CCACCCAAGACCTTAGCAT-3′
1C5	5′-CTTCTTCCATAGAAAATGTT-3′	5′-AGTAGGTTCATAAATTGGTG-3′
4B11	5′-ATGGCTCCCTCGGTGATGG-3′	5′-TTAGTTGCCGCCTGACTCC-3′
4C7	5′-CCAATTCGAGCCGTCAT-3′	5′-CAGCAGCAGTTCTTCATCC-3′
7G3	5′-AGGTGGGCTTTTCGGTGAT-3′	5′-TTGGTCTCGCTTTGGTTGG-3′
8A1	5′-GTGGTCCAATAGAGTGATGC-3′	5′-AGAGACATACCAATTGCAGG-3′
5A5	5′-CCGAGGTACATTAGACA-3′	5′-GGTACTAGGGTAACTACAC-3′
9B12	5′-GATAGTTGTTTCCGACCCC-3′	5′-CCTCGCTCACAATGTTTCT-3′
5J12	5′-GCCTATGAAGATTATGCTC-3′	5′-AATCACCCAAATACTACGG-3′
4D7	5′-ACTGAGAGGCATGAAGGG-3′	5′-ATCGGCATTGCTGAGGAC-3′
5H5	5′-GGCTACTTAGTTCTAGTATACCACC-3′	5′-TACAGACCAACTTCAGAAAGAC-3′
4E12	5′-AAAAGCAACTCAAAATCTAGCATAC-3′	5′-GAACCACTGGCACCTGCTC-3′
7H7	5′–CGTGGTTTGGGTTCCAAGG-3′	5′-CCTAATAAGCTGAAGCCAGCAG-3′
8G7	5′-AAACTGGACAGAAACAATCACACCC-3′	5′-GAGCGGAACATGAGGCAGC-3′
B10	5′-ATGCCTGTTACATCTTCTTG-3′	5′-GTAGTATTGACATTCTGGTTGA-3′
C6	5′-AAAGCCCTTGGAGAGT-3′	5′-ATAAGTGGATAGGTGTGGT-3′
G2	5′-CTTTCTCGGGTCTGCTCTT-3′	5′-CTTCTTTTCCGCTTCCTC-3′
I8	5′-GCGCAGCTTCTCCAAC-3′	5′-TGCTTTCTTAACAACCCATA-3′

Results

Expression profile of pathogenesis-related genes during fungal infection

Northern blot analysis was used to investigate the expression patterns of both *PR1b* and *PBZ1* genes in response to inoculation with *Rhizoctonia*. Even though both genes were induced at 12 hpi, expression of *PR1b* increased gradually from 12 to 72 hpi, while *PBZ1* drastically increased at 48 hpi (Fig. 1). Analysis of the infection process showed that mycelia grew on the plant surface at 12 hpi, and some hyphae were found within the infected tissue at 24 hpi, with plant cell death initiating at 36 hpi. Typical sheath blight lesions were forming in all plants at 48 hpi (data not shown). At 48 hpi, the fungal pathogen formed an infection cushion and penetrated host cuticle directly or through stomata for re-infection (data not shown).

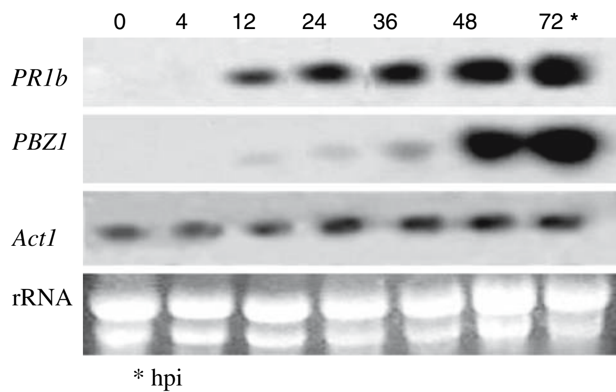

Fig. 1 Northern blot analysis of *PR1b* and *PBZ1* expression during the infection process of rice sheath blight. About 20 μg of total RNA from rice plants at 4, 12, 24, 36, 48, and 72 h after inoculation were used in northern blot analysis. RNA loading of each sample was verified by the *Act1* gene as the internal control and the intensity of ribosomal RNA bands on the agarose gel

cDNA clones differentially screened in the suppression subtractive library

To isolate cDNAs corresponding to genes differentially regulated in interactions with *R. solani*, a subtractive cDNA library was prepared. Two thousand seven hundred and eighty five clones were picked to 29 plates of 96-well microtiter plate for storage at −80°C. The average insert size was approximately 500 bp, ranging from 300 to 700 bp (data not shown). Based on the first differential screening, 800 of the 2,785 clones were identified as showing different hybridization signal between forward-subtracted probe and reverse-subtracted probe. The above clones were re-picked to 96-well plates, and spotted onto nylon membranes for the second screening. Two hundred fifty-six clones with a differential hybridization pattern were found in the screen, and some of them were chosen for sequencing. The sequence results indicated that none of the clones were highly redundant in the library.

Reverse Northern blot analysis and BLAST search of identified clones

To further confirm and characterize the cDNA clones selected from the colony hybridization, “reverse Northern” analysis was used (Xiao et al. 2001). cDNA, synthesized from mRNA of *R. solani* infected plants and uninfected plants, were used as probes in hybridization with the reverse Northern blots containing all 256 genes. Hybridization results showed that most of the selected genes showed increased expression after fungal infection. A representative example indicating the difference of the hybridization patterns with the cDNA probes of fungal infected and uninfected plants is shown in Fig. 2. The clones that showed different hybridization intensity between the two probes were selected for sequencing. We identified 61 unique sequences. A BLASTn search of the rice database (http://www.rice.genomics.org.cn/) revealed that all of them were derived from the rice genome and a total of 50 unique genes from the 61 cDNAs were identified. The 50 unique sequences were submitted to the NCBI and TIGR databases, and the BLASTX program was used to search for known proteins encoded by the genes (Table 3). Among the 50 genes, 48 genes were found to correspond to protein sequences in the TIGR database, though some of them were not present in the NCBI database. Classification of the 48 clones in the rice database KEGG (http://www.genome.jp/kegg/kegg2.html) allowed us to group the genes into five functional categories.

Genes involved in “Environmental Information Processing” were placed in category 1. Eight clones belong to this group, they may be involved in cell rescue and defense response. Among them, clone 7G3 encodes a protein with homology to rice glutathione peroxidase 1 (OsGPX1). *OsGPX1* is a stress-inducible rice gene that protects cells against oxidative stresses (Kang et al. 2004). Clone 8A1 encodes ubiquitin-conjugating enzyme E2, a protein known to be involved in protein proteolysis. It was found previously to be suppressed by rice blast infection (Lu et al.

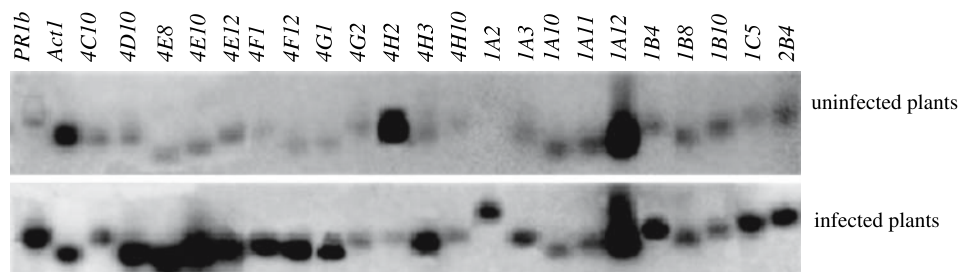

Fig. 2 Reverse Northern blot analysis of cDNA clones selected from membrane hybridization. The cDNAs were amplified using M13 forward and reverse primers and loaded in duplicate on two 1% (w/v) agarose gels. cDNAs synthesized from control and infected plants were labeled with ^{32}P and used as probes for hybridization with reverse Northern blots. *PR1b* (induced in inoculated plants) and *Act1* (a constitutively expressed gene) were used as controls

Table 3 Identified SSH clones and their BLAST search results

Clone	Accession number	Functional annotation of tiger database	Best homologue in NCBI database	*E*-value/sequence identity (%)
Environmental information processing (related with cell rescue/defense)				
7G3	DW246091	Phospholipid hydroperoxide glutathione peroxidase (LOC_Os04g46960)	Glutathione peroxidase 1 [*Oryza sativa*] (AAM47493)	3e-23/96
8A1	DW246085	Ubiquitin-conjugating enzyme E2 W (LOC_Os12g44000)	Os03g0681400 [*Oryza sativa*] (NP_001050906)	3e-62/98
1A3	DW246101	Expressed protein (LOC_Os09g01000)	Putative senescence-associated protein [*Pisum sativum*] (BAB33421)	3e-17/95
1C5	DW246077	Serine/threonine-protein kinase16 (LOC_Os09g06230)	Molybdenum ABC transporter, ATP-binding protein [*Haemophilus ducreyi*](AAP96575)	8.6/51
4E7	DW246096	Disease resistance response protein 206 (LOC_Os07g01660)	Two comoponent transcriptional regulator [*Mariprofundus ferrooxydans* PV-1] (ZP_01452876)	5.0/32
4C7	DW246073	Ethylene-insensitive3-like 1 protein (LOC_Os03g20790)	Ethylene-insensitive3-like 1 protein [*Oryza sativa*] (ABF95697)	5e-29/94
8B2	DW246087	Oxygen-evolving enhancer protein 2 (LOC_Os07g04840)	23 kDa polypeptide of photosystem II [*Oryza sativa*](BAA08564)	3e-04/100
9B12	DW246113	Translationally-controlled tumor protein (LOC_Os11g43900)	Os11g0660500 [*Oryza sativa*](NP_001068405)	2e-22/98
Genetic information processing				
1G8	DW246079	RING zinc finger protein-like (LOC_Os03g31320)	RING zinc finger protein [*Oryza sativa*] (ABF96727)	6e-12/97
2A8	DW246102	Nucleotide-binding protein 1 (LOC_Os02g38900)	Hypothetical protein OsI_007798 [*Oryza sativa*](EAY86565)	2e-28/87
7H7	DW246084	Nucleic acid binding protein (LOC_Os10g35000)	Hypothetical protein OsI_032990 [*Oryza sativa*] (EAY79031)	5e-05/100
5A6	DW246066	Glycine-rich protein A3 (LOC_Os05g02780)	No homology found	
7G5	DW246092	Expressed protein (LOC_Os01g43230)	Hypothetical protein OsI_002841 [*Oryza sativa*] (EAY74994)	0.16/100
4D9	DW246075	60S ribosomal protein L28 (LOC_Os05g46430)	Putative 60S ribosomal L28 protein [*Oryza sativa*] (AAV67824)	9e-24/98
9G8	DW246116	60S ribosomal protein L37a (LOC_Os05g48320)	No homology found	
4E2	DW246095	Elongation factor G (LOC_Os04g45490)	No homology found	
8A4	DW246086	Topoisomerase-like protein (LOC_Os08g02690)	No homology found	
9A9	DW246112	Retrotransposon protein (LOC_Os04g02960)	No homology found	
Metabolism				
4B11	DW246072	Ribulose bisphosphate carboxylase small chain C (LOC_Os12g19470)	Ribulose-1,5-bisphosphate carboxylase/oxygenase small chain C [*Oryza sativa*] (ABA97179)	3e-31/96
4C6	DW246094	Gibberellin 20 oxidase 2 (LOC_Os03g42130)	Oxidoreductase, 2OG-Fe oxygenase family protein [*Oryza sativa*] (ABF97648)	1e-14/100
4D7	DW246074	Nitrogen regulatory protein P-II (LOC_Os05g04220)	PII-like protein [*Oryza sativa*] (BAD88531)	6e-30/100
8E9	DW246081	Transferase, transferring glycosyl groups (LOC_Os02g58139)	No homology found	
8G7	DW246082	Transferase, transferring glycosyl groups (LOC_Os01g43380)	Os01g0622000 [*Oryza sativa*](NP_001043608)	6e-15/98
6D9	DW246088	Hydrolase (LOC_Os10g39090)	Hypothetical protein OsI_033299(EAY79340)	0.007/100
4B2	DW246065	Expressed protein (LOC_Os09g00998)	Glyceraldehyde phosphate dehydrogenase [*Vibrio fischeri*] (AAC80272)	0.014/75
9C4	DW246114	Expressed protein (LOC_Os12g29760)	Hypothetical protein OsI_037076 [*Oryza sativa*](EAY83117)	1e-42/100

Table 3 continued

Clone	Accession number	Functional annotation of tiger database	Best homologue in NCBI database	*E*-value/sequence identity (%)
9G4	DW246115	Cytidine/deoxycytidylate deaminase family protein (LOC_Os07g14150)	Phosphotransferase system cellobiose-specific component IIC [*Pediococcus pentosaceus*] (YP_805061)	3.0/36
4E12	DW246097	ATP-dependent Clp protease (LOC_Os04g32560)	Peptidoglycan glycosyltransferase [*Sphingomonas* sp.] (ZP_01302467)	3.9/47
4G8	DW246099	Beta-fructofuranosidase (LOC_Os01g22900)	Hypothetical protein TVAG_571380 [*Trichomonas vaginalis*] (XP_001290691)	6.7/50
5E5	DW246070	Ribose-5-phosphate isomerase (LOC_Os04g24140)	No homology found	
4G9	DW246100	Ubiquinol-cytochrome c reductase complex ubiquinone-binding protein QP-C (LOC_Os06g07969)	No homology found	
6F10	DW246089	Acetylglucosaminyl transferase/transferase (LOC_Os04g40150)	No homology found	
8G12	DW246111	Nitrilase 4 (LOC_Os02g42350)	Hypothetical protein OsI_008049 [Oryza sativa](EAY86816)	1.3/97
7E8	DW246109	Diphosphomevalonate decarboxylase (LOC_Os02g01920)	No homology found	
5J12	DW246068	Phosphoglycolate phosphatase (LOC_Os02g57100)	Organic solvent tolerance protein [*Zymomonas mobilis*](YP_163046)	9.5/31
Cellular processes and signaling				
5H5	DW246067	F-box domain containing protein (LOC_Os07g13890)	Os07g0242600 [*Oryza sativa*](NP_001059273)	3e-48/100
5A5	DW246069	GAMYB-binding protein (LOC_Os02g16000)	Putative BTB/POZ domain-containing protein [*Oryza sativa*] (BAD20083)	9e-23/96
5A1	DW246105	DNA-binding protein MNB1B (LOC_Os06g51220)	Flagellar hook-associated protein 3 [*Alkalilimnicola ehrlichei*] (YP_741747)	1.8/42
2B7	DW246104	Mitochondrial import inner membrane translocase subunit TIM16 (LOC_Os01g73020)	No homology found	
8A8	DW246110	ADP-ribosylation factor (LOC_Os05g41060)	No homology found	
Unclassified function				
2B4	DW246103	Expressed protein (LOC_Os06g30390)	Unknown protein [*Oryza sativa*](BAD45364)	e-107/97
5H6	DW246071	Expressed protein (LOC_Os08g01110)	Unknown protein [*Oryza sativa*] (BAD03553)	1e-34/100
4G6	DW246098	Expressed protein (LOC_Os05g48340)	Os05g0557200 [*Oryza sativa*](NP_001056287)	1e-24/96
8C4	DW246080	Expressed protein (LOC_Os12g01620)	Expressed protein [*Oryza sativa*](ABG21848)	2e-22/100
4E5	DW246076	Expressed protein (LOC_Os09g00999)	Unnamed protein product [*Kluyveromyces lactis*] (XP_453852)	9e-13/77
7E7	DW246090	Expressed protein (LOC_Os01g68300)	Hypothetical protein OsI_004763 [*Oryza sativa*] (EAY76916)	2e-09/96
1F6	DW246078	Putative homeodomain leucine zipper protein (LOC_Os10g33960)	No homology found	
6D10	DW246106	Expressed protein (LOC_Os03g56610)	No homology found	
No match				
6H5	DW246107	None	No homology found	
6H10	DW246108	None	No homology found	

Genes were grouped according to the function classified in rice database KEGG (http://www.genome.jp/kegg/kegg2.html)

2004). The encoded protein of clone 1A3 has similarity to a putative senescence-associated protein of *Pisum sativum*. This gene was also induced by rice blast fungal infection (Lu et al. 2004). Serine/threonine-protein kinase 16 (1C5) was also found in this group. The serine/threonine protein kinases are a group of enzymes that catalyzes the phosphorylation of serine or threonine residues in proteins. Ser/Thr protein kinases are known to regulate plant resistance pathways, including mitogen-activated protein kinases (MAPKs) (Zhang and Klessig 2001), Ca^{2+}/calmodulin-dependent protein kinase (Romeis et al. 2001) and other kinases involved in resistance, such as Pto (Martin et al. 1993), Pti1 (Zhou et al. 1995), Xa21 (Song et al. 1995), and PBS1 (Swiderski and Innes 2001). Clone 4E7 encodes a homolog of disease resistance response protein 206, a protein induced early in the pea by *F. solani* f. sp. *phaseoli* (Fristensky et al. 1988). However, the roles of ethylene-insensitive-3-like protein (4C7), translationally controlled tumor protein (9B12) and oxygen-evolving enhancer protein 2 (8B2) in response to pathogens are unknown.

The second group contained genes involved in "Genetic Information Processing". Ten clones belong to this group. Although the relationships between these clones to plant defense were unclear, some of them, such as RING zinc finger protein (1G8) and nucleotide-binding protein (2A8 and 7H7) may have roles in rice defense through the function of protein–protein or RNA–protein interaction. Clone 5A6 encodes glycine-rich protein A3. Genes encoding glycine-rich proteins are developmentally regulated and are also induced by physical, chemical and biological factors, suggesting that they participate in important cellular processes (Sachetto-Martins et al. 2000). It is reasonable to find 60S ribosomal L28 protein (4D9), 60S ribosomal protein L37a (9G8) and elongation factor G (4E2), which are involved in protein synthesis in this group, because new protein synthesis is required for plant defense responses. The topoisomerase-like protein is encoded by clone 8A4. Topoisomerases plays an important role in many aspects of DNA metabolism, including transcription, replication, recombination, repair, nucleosome assembly and chromosome segregation. The transcript level of tobacco topoisomerase I was increased following exposure to light, low-temperature stress and abscisic acid (Mudgil et al. 2002).

There were 17 genes in the third category, "Metabolism". The genes in this group encode different enzymatic functions, such as oxygenase, oxidase, transferase, hydrolase, dehydrogenase, nitrilase, deaminase, protease, fructofuranosidase, isomerase, reductase, and decarboxylase. These hint that a wide range of biochemical activities may be involved in rice defense to *R. solani*. Some of them were previously reported to be induced or suppressed by plant pathogens in other studies. For example, the expression of ribulose-1, 5-bisphosphate carboxylase/oxygenase (4B11) was affected by stress and pathogen infection (Conklin and Last 1995; Schenk et al. 2000; Hajduch et al. 2001). It was reported that a putative ATP-dependent Clp protease subunit (4E12) and a putative cytidine deaminase (9G4) were induced in a rice blast resistant mutant upon blast fungus infection (Han et al. 2004). Expression of a putative β-1,4-*N*-acetylglucosaminyltranferase III (6F10) was shown to be induced in cucumber by 10 μM benzothiadiazole (BTH) treatment as well as *Colletotrichum lagenarium* infection (Bovie et al. 2004).

The fourth category was "Cellular Processes and Signaling". Five genes were found in this group. Clone 5H5 encodes an F-box domain containing protein. F-box proteins regulate diverse cellular processes, including cell cycle transition, transcriptional regulation and signal transduction, by playing roles in Skp1p-cullin-F-box protein (SCF) complexes or non-SCF complexes. So far, two F-box proteins were identified to be involved in pathogen defense (Devoto et al. 2003). GAMYB-binding protein was encoded by clone 5A5. GAMYB is a gibberellin (GA)-regulated activator of hydrolase gene expression in the aleurone layer of germinating cereal grains. A GAMYB-binding protein was shown to act as a negative regulator of GAMYB function in aleurone (Woodger et al. 2003). Clone 5A1 encodes DNA-binding protein MNB1B. The MNB1B is an HMG1-like protein, which recognizes an AAGG motif at the MNF1-binding site of maize (Yanagisawa and Izui 1993). Clone 2B7 encodes mitochondrial import inner membrane translocase subunit TIM16, which is an essential component of the PAM complex, a complex required for the translocation of transit peptide-containing proteins from the inner membrane into the mitochondrial matrix in an ATP-dependent manner (Mokranjac and Neupert 2005). ADP-ribosylation factor, encoded by 8A8, has been previously identified to be involved in the defense response to rice blast (Lu et al. 2004; Xiong et al. 2001).

The final group consisted of eight genes with unknown function. No corresponding protein for two additional clones, 6H5 and 6H10, could be found in the NCBI and TIGR databases.

Defense response genes triggered by multiple pathogens

We amplified 100 genes reported previously to be induced by *M. grisea*, *X. oryzae* pv. *oryze* (*Xoo*) and *X. oryzae* pv. *oryzicola* (*Xooc*) (Xiong et al. 2001; Rauyaree et al. 2001; Pei et al. 2002; Rao et al. 2002; Zhou et al. 2002; Lu et al. 2004) and these were arrayed onto duplicate hybond nylon membranes as shown in Table 1. The housekeeping gene *Act1* and pathogenesis-related genes *PR1b* were used as controls in the arrays. To screen the genes also induced by *R. solani*, the duplicate filters were hybridized with ^{32}P-labeled cDNA probes derived from *R. solani* infected and

uninfected plants (Fig. 3). There were 16 genes that showed weak or no hybridization signal to the cDNA probes, and 59 genes displayed the same hybridization level with both the *R. solani* infected and uninfected cDNA probes. Meanwhile, 25 genes showed differential hybridization with *R. solani* infected and uninfected cDNA probes. Among these, 20 genes (A2, B7, B10, C6, D2, D5, E5, E8, F2, F4, G2 (A7), G5, H1, H9, I3, I4, I6, I8, I9 and J8) displayed stronger hybridization with the probe from the *R. solani* infected tissue. Five genes (A10, B9, E2, I7, and K1) were suppressed by infection. The gene IDs and functional annotation of these 25 genes is shown in Table 4. The genes can be grouped into four categories. The first category contained six genes which responded to infection by three different pathogens, *M. grisea, X. oryzae* pv. *oryzae* and *R. solani*. Fourteen genes induced or suppressed by *M. grisea* and *R. solani* were grouped into the second category, and four genes triggered by *X. oryzae* pv. *oryzae* and *R. solani* were grouped into the third category. The fourth category contained only one gene which was regulated by *X. oryzae* pv. *oryzicola* and *R. solani*.

RT-PCR analysis of cDNAs during sheath blight infection

To further confirm the results of the reverse northern blot, and to analyze the expression patterns of these genes during the interaction with *R. solani*, RT-PCR analysis was conducted. Total RNA extracted from rice seedlings at seven time points after *R. solani* inoculation (0, 4, 12, 24, 36, 48, 72 hpi), reverse-transcribed into first-strand cDNA, and used as templates for PCR amplification. Fourteen of the 50 clones from the SSH library (7G3, 4B11, 4C7, 9B12, 5A5, 8A1, 1C5, 2B4, 4E12, 5J12, 5H5, 8G7, 7H7, 4D7) and 4 genes (B10, C6, G2, and I8) from the array were selected for analysis. Our results showed that most of them were induced or repressed early by sheath blight fungus infection (Fig. 4). For example, 7G3, 4C7, 9B12, 2B4, 4E12, B10, C6, G2 and I8 were induced as early as 4 or 12 hpi. For other genes, repression was observed as early as 4 or 12 hpi and expression was restored or even enhanced at later time points (4B11, 4D7, 5A5, 8A1 and 5J12, 5H5, 8G7 and 4D7).

Discussion

Since rice sheath blight is not a typical “gene-for-gene” interaction disease, very little is known about the host–fungus interaction at the molecular level. To our knowledge, this study is the first analysis of gene expression of rice plants responding to challenge by *R. solani*. The characterization of the genes identified in this study will serve as a foundation for future studies to elucidate induced defense mechanisms of rice when infected with *R. solani*.

The behavior of *PR1* and *PBZ1* during the host–fungus interaction

PR1 and *PBZ1* are the well-characterized PR genes in rice. The expression of *PR1* is often used as a marker for SAR development. The *PR1* gene was induced in the resistance reaction to rice blast (Schweizer et al. 1998), whereas

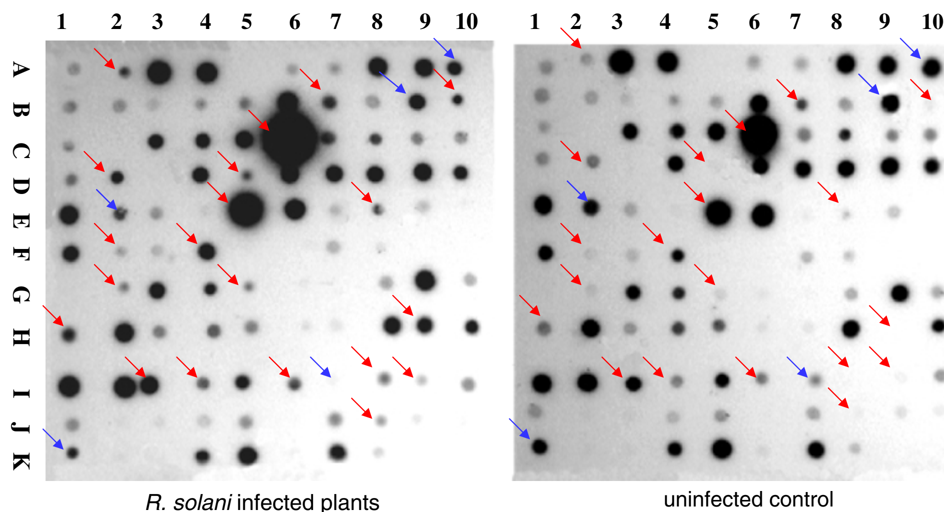

Fig. 3 Reverse Northern blot analysis of EST arrays. 100 ESTs, reported previously induced by *M. grisea*, *X. oryzae* pv. *oryze* (*Xoo*) and *X. oryzae* pv. *oryzicola* (*Xooc*) were amplified successfully by PCR and arrayed equally in two hybond nylon membranes as Table 1. The housekeeping gene *Act1* and pathogenesis-related genes *PR1b* were used as controls in the arrays. The arrays were hybridized with ^{32}P-labeled cDNA probes derived from *R. solani* infected and uninfected plants, respectively. *Arrows* indicate the differentially hybridized clones

Table 4 Defense response genes regulated by multiple pathogens

EST array number	Accession number	Best homologue in NCBI database (annotation from original articles)	Functional annotation of tiger database	Induction[a]
Defense response genes regulated by *M. grisea*, *X. oryzae* pv. *oryzae* and *R. solani*				
A2	BF108313	Translation initiation factor (GOS2) mRNA (AF094774)	Protein translation factor SUI1 (LOC_Os07g34589)	+
B7	BF108347	LIP9 (low temperature induced protein) (AB011367)	G protein-coupled receptor (LOC_Os06g09930)	+
B9	BF145170	Ubiquitin-conjugating enzyme (BAA96583)	Ubiquitin-conjugating enzyme E2 (LOC_Os05g48380)	–
B10	BF108310	Zinc finger protein (T48868)	Zinc finger CCCH type domain-containing protein ZFN-like 2 (LOC_Os01g68860)	+
D5	BF108364	Acrosin precursor (P08001)	Expressed protein (LOC_Os02g15540)	+
I7	AF010579	Glycine-rich protein (AF010579)	Glycine-rich RNA-binding protein 2 (LOC_Os03g46770)	–
Defense response genes regulated by *M. grisea* and *R.solani*				
C6	BF145167	Monogalactosyldiacylglycerol synthase (CAA04005)	MGD2 (LOC_Os02g55910)	+
E5	CD645548	Cytochrome P450 monooxygenase (AJ004810)	Expressed protein (LOC_Os09g01000)	+
E8	BF108317	Putative nonsense-mediated mRNA decay protein (AAD24816)	Nonsense-mediated mRNA decay protein 3 (LOC_Os10g42320)	+
F2	BF108340	Putative integral membrane protein (T34987)	Expressed protein (LOC_Os11g24824)	+
F4	CD645582	Putative senescence-associated protein (AB049723)	Hypothetical protein (LOC_Os03g39370)	+
G2	CD645580	Unkonwn protein (AC010924)	Putative serine incorporator 3 (LOC_Os02g54990)	+
G5	CD645567	Phosphoenolpyruvate carboxykinase 4 (AF136163)	Phosphoenolpyruvate carboxykinase (LOC_Os03g15050)	+
H1	BF145194	Unknown protein (BAB10177)	lysM domain containing protein (LOC_Os10g38040)	+
H9	BF889449	Putative receptor-like protein kinase (AL163527)	Brassinosteroid insensitive 1-associated receptor kinase 1 precursor (LOC_Os01g44110)	+
I3	BF889467	Putative ankyrin-containing protein (AC016661)	Protein binding protein (LOC_Os01g61990)	+
I4	BF889478	No homology	Expressed protein (LOC_Os07g26930)	+
I6	BF889486	Salt stress-induced protein (SalT) (AF001395)	Salt stress-induced protein (LOC_Os01g24710)	+
J8	BI795001	Calcium-dependent protein kinase (H005E08)	Serine/threonine-protein phosphatase PP1 (LOC_Os03g16110)	+
A10	CD645546	s-Adenosyl-L-methionine:carboxyl methyltransferase-like protein (NM_120520)	Benzoate carboxyl methyltransferase (LOC_Os11g15040)	–
Defense response genes regulated by *X. oryzae* pv. *oryzae* and *R. solani*				
D2	BF108334	Putative S-adenosylmethionine: 2-demethylmenaquinone methyltransferase (BAB84438)	Regulator of ribonuclease activity A (LOC_Os02g52450)	+
E2	BF108354	Metallothionein-like protein mRNA (AF009959)	Metallothionein-like protein type 3 (LOC_Os05g11320)	–
I8	BE040982	MAP kinase kinase 4 (BE040982)	OsMKK4 (LOC_Os02g54600)	+
I9	BI796359	Metallothionein-like protein (H044C02)	Expressed protein (LOC_Os01g10400)	+
Defense response genes regulated by *X. oryzae* pv. *Oryzicola* and *R. solani*				
K1	AC144455	Putative histone deacetylase HD2 (AAW57802)	Putative histone deacetylase 2b (LOC_Os05g51830)	–

[a] "+" means the gene was induced by *R. solani* in the array, "–" means the gene was suppressed by *R. solani* in the array

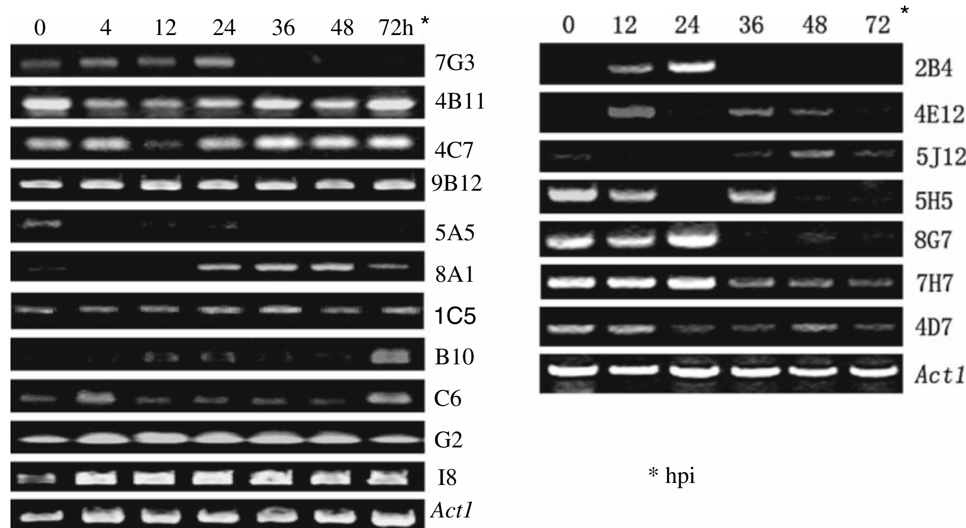

Fig. 4 Semi-quantitative RT-PCR analysis of some selected cDNA clones to show the typical expression pattern in rice plants after challenged by *R. solani*. The total RNA from rice plants at 4, 12, 24, 36, 48, and 72 h after inoculations with *R. solani* were reverse-transcribed into first-strand cDNA with oligonucleotide dT primer. The equal cDNAs were used as template for PCR reaction. Fourteen of 50 clones from the SSH library (7G3, 4B11, 4C7, 9B12, 5A5, 8A1, 1C5, 2B4, 4E12, 5J12, 5H5, 8G7, 7H7, 4D7) and 4 genes (B10, C6, G2, and I8) from the EST array were selected randomly to design primers for RT-PCR analysis. The *Act1* gene was amplified as the internal control. The number of RT-PCR cycles was 28 and the PCR products were separated on a 1.5% agarose/EtBr gel

PBZ1, an intracellular protein, was induced by probenazole (3-allyloxy-1, 2-benzisothiazole-1, 1-dioxide), an effective chemical inducer of host resistance against rice blast infection (Midoh and Iwata 1996). These two PR genes were activated in rice lesion mimic mutants at different levels during lesion development (Takahashi et al. 1999; Yin et al. 2000). Our results showed that expression of the *PR1b* and *PBZ1* genes was also induced by *R. solani* infection. Northern blot analysis showed that both *PR1b* and *PBZ1* genes were detected at 12 hpi. At this time point, the fungal mycelium grew on the surface of the plant and we suspect that the host defense response was triggered by the pathogen. The expression of the *PR* genes increased during disease development. *PR1b* increased gradually from 12 to 72 hpi, while *PBZ1* increased to its maximum level at 48 hpi. In the meantime, a few lesions started to develop at 36 hpi, and typical lesions were found in all plants at 48 hpi. Obviously, 48 hpi was a critical time point for disease development and *PBZ1* expression. Combined with observations of infection and colonization of the host, we conclude that the expression of *PR1b* and *PBZ1* is related with disease development.

Defense responsive gene expression in rice infected with *R. solani*

SSH is a powerful tool to study gene expression and has been used previously to study the interaction between rice and its pathogens, especially with respect to rice blast (Xiong et al. 2001; Lu et al. 2004). In this study, we used SSH to isolate 50 genes that displayed differential expression patterns after challenge by the sheath blight fungus. Among the 50 genes, 48 genes matched to sequences in the Tiger rice database and were assigned to different functional groups in the rice KEGG database. The clones in the "Environmental information processing" group were related with cell rescue and defense. They matched with several known pathogenesis-related proteins. The clone 7G3 encodes rice glutathione peroxidase1 (OsGPX1), which is a phospholipid hydroperoxide glutathione peroxidase, belonging to glutathione peroxidase family that protects cells against both metabolic and environmental oxidative stresses. It was reported that a tomato phospholipid hydroperoxide glutathione peroxidase (LePHGPx) inhibits not only oxidative stress induced cell death in yeast but also inhibits salt, heat, and Bax induced PCD in tobacco plants (Chen et al. 2004). The transcription of *OsGPX1* gene was induced in the seedlings within one hour of exposure to salt stress and was also gradually increased by cold and drought stress (Kang et al.2004). Although a variety of reactive oxygen species (ROS) removing enzymes are induced during plant defense responses, this is the first time that *OsGPX1* was found to be induced by pathogen infection. The *OsGPX1* was induced by *R. solani* from 4 hpi to maximum at 24 hpi in our test (Fig. 4). It is interesting to note that 8A1 encodes a putative ubiquitin-conjugating enzyme E2. The ubiquitin/proteasome pathway is the major selective protein degradation system in eukaryotes. Growing evidence indicates that this pathway plays an important role in plant defense responses (Dreher and Callis 2007;

Gonzalez-Lamothe et al. 2006; Zeng et al. 2006, 2004; Austin et al. 2002; Kim et al. 2002). However, few evidences have demonstrated a direct or indirect role for ubiquitin-conjugating enzymes in the plant defense system. The EST matching to a ubiquitin-conjugating enzyme gene was found to be suppressed by rice blast infection (Lu et al. 2004). In this test, the repression of 8A1 could begin as early as 4 hpi by sheath blight and was recovered or even enhanced after 24 hpi (Fig. 4). Further characterization of this gene may shed light on the relationship between the ubiquitination pathway and the defense response in plants. The clone 1A3 encodes a protein matching to putative senescence-associated protein of *Pisum sativum*. This same gene was also found induced in the rice blast infection (Lu et al. 2004). In *Arabidopsis*, some leaf senescence associated genes are known to be pathogen-induced and participate in the HR (Quirino et al. 1999; Gepstein et al. 2003), suggesting that there is an overlap between pathways leading to cell death in both leaf senescence and HR.

A serine/threonine-protein kinase was encoded by 1C5. As the mentioned above, a variety of Ser/Thr protein kinases are known to regulate plant defense. In *Arabidopsis* ethylene signaling pathway, CTR1, a serine-threonine kinase, is a negative regulator of ethylene signaling and ethylene-insensitive 2 (EIN2) is a positive regulator of the pathway. EIN2 acts on downstream of CTR1. A mitogen-activated protein kinase (MAPK) module may act between CTR1 and EIN2. The EIN2 protein activates the transcriptional activator EIN3 and several other EIN3-like proteins (EILs). Subsequently these proteins activate other transcription factors, thereby regulating the expression of genes involved in the response to ethylene. Two F-box-containing proteins, EBF1 and EBF2, target EIN3 for proteosome-mediated degradation (Potuschak et al. 2003; Stepanova and Alonso 2005). In Table 3, three cDNAs were found, which respectively encode EIN3 (4C7), Ser/Thr protein kinase 16 (1C5) and F-box domain containing protein (5H5). That gives a clue that ethylene signaling pathway may be involved in rice defense response to *R. solani*. Actually, ethylene production by infected plants is an early resistance response leading to activation of plant defense pathways (Chang and Shockey 1999; Thomma et al. 1999; Etheridge et al. 2005).

The disease resistance response protein 206 (4E7) is a nonhost resistance protein in pea. Its transcript rapidly accumulated to high, sustained levels in the successful resistance response to bean pathogen *F. solani* f. sp. *phaseoli*, but expressed only transiently to low levels in the unsuccessful resistance response to pea pathogen *Fusarium solani* f. sp. *pisi* (Culley et al. 1995). It will be worth to know the function of this protein in rice defense against sheath blight. The clone 9B12 displays similarity to the translationally controlled tumor protein (TCTP). TCTP is a highly conserved, abundantly expressed protein found in a wide range of organisms. Previous reports have characterized TCTP as a tubulin-binding protein, a histamine-releasing factor, and a calcium binding protein, and TCTP also acts as a guanine nucleotide dissociation inhibitor (MacDonald et al.1995; Arcuri et al. 2005; Cans et al. 2003). In plant, the TCTP was found to be induced by dark and *Xanthomonas campestris* pv. *vesicatoria* infection (Sage-Ono et al. 1998; Jung and Hwang. 2000). The oxygen-evolving enhancer protein 2 (OEE2) was found in this group (8B2). The protein is a chloroplast protein involved in the regulation of photosystem II. In *Arabidopsis*, the OEE2 acts as the substrate for WAK1 in the AtGRP-3/WAK1 signaling pathway, and is possibly involved in defense signaling (Yang et al. 2003). WAK1 is a wall-associated receptor kinase with a cytoplasmic Ser/Thr kinase domain, and AtGRP-3 is a glycine-rich extracellular protein. AtGRP-3 regulates WAK1 function through binding to the cell wall domain of WAK1 and the interaction of WAK1 with AtGRP-3 occurs in a pathogenesis-related process in planta (Park et al. 2001). Interestingly, a cDNA encoding glycine-rich protein was found in "Genetic Information Processing" category (5A6), and as the mentioned before, 1C5 encodes a Ser/Thr protein kinase. It will be interesting to determine if there is an AtGRP-3/WAK1/OEE2-like signaling pathway in rice to play a role in defense against sheath blight.

In addition to the clones in the "Environmental information processing" group, several other types of cDNA clones are also potentially related to defense response. Most of these genes were related to genetic information, cellular processes and signaling. A RING zinc finger protein is encoded by 1G8. The RING finger is a small zinc-binding domain found in many functionally diverse proteins. Because of this diversity, the biochemical function of RING finger proteins had been unclear for a long time. However, recent studies have revealed that RING finger proteins can specifically interact with the ubiquitin conjugating enzyme (E2) as ubiquitin ligase E3, thereby promoting ubiquitination of target proteins (Freemont 2000; Takai et al. 2002). For example, EL5, a rice elicitor-responsive RING-H2 finger protein, is a ubiquitin ligase which functions in vitro in co-operation with an elicitor-responsive ubiquitin-conjugating enzyme, OsUBC5b. The EL5 and OsUBC5b have roles in plant defense response through the turnover of protein(s) via the ubiquitin/proteasome system (Takai et al. 2002). Clone 2A8 and 7H7 encode nucleotide binding protein, the diverse proteins which are common in various prokaryotic and eukaryotic genomes. For example, the superfamily of GTP-binding proteins includes signal-transducing proteins such as the small GTP-binding proteins and the G-protein family. The majority of plant disease resistance genes (R-genes) encode a nucleotide binding site (NBS) region attached to a C-terminal leucine-rich repeat (LRR) (Meyers et al. 1999). The transcript of

7H7 was suppressed by sheath blight pathogen at 36 hpi (Fig. 4). 8A8 encodes ADP-ribosylation factor (ARF). The ARF proteins are members of the RAS family of small GTP-binding proteins that regulate various cellular functions in eukaryotes, such as vesicle formation and intracellular vesicle transport (McElver et al. 2000). The protein has been previously identified to be involved in the defense response to rice blast (Lu et al. 2004; Xiong et al. 2001). Recently, a cDNA encoding RARF1 (rice ADP-ribosylation factor 1) was isolated from fungal elicitor-treated rice suspension culture cells. RARF1 transcripts accumulated in response to H_2O_2 and SA and rapidly in cells inoculated with an avirulent pathogen. Constitutively over-expressed RARF1 in tobacco plants triggered spontaneous induction of lesion mimics, induced an array of pathogenesis-related (PR) genes, reduced susceptibility to a fungal pathogen, and caused accumulation of SA. The results indicated that RARF1 might be a component of various plant defense-signaling pathways involved in inducing the expression of a subset of PR genes (Lee et al. 2003).

Some enzymes in the "Metabolism" group may also be involved in plant defense. The clone 4B11 encodes rice ribulose-1, 5-bisphosphate carboxylase/oxygenase small subunit (rbcS), a photosynthetic protein. Gesch et al. (1998) reported that carbohydrates may be important in the regulation of rbcS expression. However, rbcS could be affected by stress such as ozone, salt, metal and pathogen infection (Mouly et al. 1992; Conklin and Last 1995; Schenk et al. 2000; Hajduch et al. 2001). In recent rice proteomic research, ribulose-1, 5-bisphosphate carboxylase/oxygenase proteins were reported to increase in the rice leaf sheath by wound stress (Shen et al. 2003). In Fig. 4, the gene of 4B11 was repressed at 4 hpi by sheath blight and recovered after 24 hpi. Clones 4B2 and 9C4 encode enzymes that have potential roles in removing of ROS in response to oxidative burst. During the HR, it is known that plants rapidly produce and accumulate reactive oxygen species in the early stages after pathogen attack (Dangl and Jones 2001). The clone 4B2 encodes a protein with 75% identity to glyceraldehyde phosphate dehydrogenase (GAPDH) of *Vibrio fischeri*. GAPDH plays an important role in glycolysis and gluconeogenesis and is known to be induced by anaerobic conditions in rice and other plants (Umeda and Uchimiya 1994; Ricard et al. 1989; Sachs et al. 1996). It was reported that *GADPH* was induced in potato leaves and stems by infection of the late-blight pathogen *Phytophthora infestans* (Laxalt et al. 1996). The clone 9C4 encodes a hypothetical protein of rice, which matches to galactose dehydrogenase of *Actinidia deliciosa* with high fidelity. The galactose dehydrogenase is involved in ascorbate biosynthesis in plants (Gatzek et al. 2002). It is clear that ascorbate has a number of roles in stress resistance, growth and control of hydrogen peroxide levels. A β-fructofuranosidase is encoded by 4G8. The enzyme is an invertase, which catalyzes the hydrolysis of sucrose to glucose and fructose in plant. Lots of evidences indicated that invertases are not only involved in regulating carbohydrate partitioning, but also in regulating plant developmental processes, hormone responses and biotic and abiotic interactions (Roitsch and Gonza´lez 2004). It was reported that a putative β-1,4-*N*-acetylglucosaminyltranferase III gene in cucumber was shown to be induced by BTH treatment and fungal infection, suggesting a potential role of the gene in the SAR pathway or in the state of resistance (Bovie et al. 2004). Clone 6F10 encodes a acetylglucosaminyl tranferase. Whether the enzyme plays any role in rice defense against the pathogen requires further investigation.

Common defense genes in different diseases response

The DNA microarray-based expression profiling work revealed similarities and distinctions between different defense signaling pathways, and cross talk (both overlap and interference) between pathogenesis-related responses and plant responses to other stresses (Wan et al. 2002). Our previous experiments also suggested that a minimum overlapping of defense pathways exist in the responses to rice blast and bacterial leaf blight at an early stage of infection (Lu et al. 2004). However, it is still unclear whether infection from sheath blight and other pathogens trigger a similar set of defense response. Rice blast (*M. grisea*), sheath blight (*R. solani*), bacterial leaf blight (*X. oryzae* pv. *oryze*) and bacterial leaf streak (*X. oryzae* pv. o*ryzicola*) are the most important rice pathogens in China. A hundred genes induced by *M. grisea*, *X. oryzae* pv. *oryze* and *X. oryzae* pv. *oryzicola* in previous studies were arrayed on filters. Among them, 20 were induced and 5 were suppressed by *R. solani* infection. This result suggested that 25 genes on this array were involved in rice defense responses to multiple pathogens. Specially, six genes either induced or suppressed by three different pathogens (Table 4). Manipulation of these genes may generate broad-spectrum resistant rice plants to multiple rice diseases.

In summary, the SSH method has allowed us to generate a differential cDNA library highly enriched for defense-related genes from rice seedlings during sheath blight disease development. From the library, 50 cDNAs were found to be involved in rice/sheath blight fungus interaction. By screening a cDNA array, 25 genes were shown to be involved in the interaction between rice and several pathogens, including *M. grisea*, *X. oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola* and *R. solani*. This is the first report of genes that are induced or repressed during sheath blight infection. Further characterization and functional analysis of these genes will facilitate our understanding of the defense response mechanisms in rice plants.

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