DOI 10.1002/pmic.200800840 *Proteomics* 2009, *9*, 2798–2808

RESEARCH ARTICLE

Understanding rice plant resistance to the Brown Planthopper (*Nilaparvata lugens*): A proteomic approach

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Received: October 28, 2008
Revised: December 30, 2008
Accepted: February 3, 2009

Engineering and breeding resistant plant varieties are the most effective and environmentally friendly ways to control agricultural pests and improve crop performance. However, the mechanism of plant resistance to pests is poorly understood. Here we used a quantitative mass-spectrometry-based proteomic approach for comparative analysis of expression profiles of proteins in rice leaf sheaths in responses to infestation by the brown planthopper (*Nilaparvata lugens* Stål, BPH), which is a serious rice crop pest. Proteins involved in multiple pathways showed significant changes in expression in response to BPH feeding, including jasmonic acid synthesis proteins, oxidative stress response proteins, beta-glucanases, protein ;kinases, clathrin protein, glycine cleavage system protein, photosynthesis proteins and aquaporins. The corresponding genes of eight important proteins were further analyzed by quantitative RT-PCR. Proteomic and transcript responses that were related to wounding, oxidative and pathogen stress overlapped considerably between BPH-resistant (carrying the resistance gene *BPH15*) and susceptible rice lines. In contrast, proteins and genes related to callose metabolism remained unchanged and glycine cleavage system protein was up-regulated in the BPH-resistant lines, indicating that they have an efficient and specific defense mechanism. Our results provide new information about the interaction between rice and the BPH.

Keywords:
Brown planthopper / Pest resistance / Quantitative proteomics / Rice plant

1 Introduction

The brown planthopper (*Nilaparvata lugens* Stål, BPH) is a typical vascular feeder and considered one of the most serious pests of all rice herbivores. BPHs cause reductions in leaf area, photosynthetic rate, leaf and stem nitrogen concentrations, chlorophyll contents and organic dry weight, with high infestation ratios, egg numbers, and survival ratios of eggs and nymphs on susceptible rice plants [1]. In the field, feeding by large numbers of BPHs usually causes ''hopperburn,'' *i.e.* drying of the leaves and wilting of the tillers, resulting dramatic reductions in the yield of susceptible rice varieties [2]. The BPH also transmits ''grassy stunt'' and ''ragged'' diseases as a virus vector [3]. Nymph development is generally disrupted, oviposition is severely inhibited and the survival rate of nymphs is significantly lower on resistant rice plants than on susceptible plants. Thus population growth is suppressed, and BPH feeding causes a little or no physiological stress or

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Abbreviations: AOC, allene oxide cyclase; **AOS,** allene oxide synthase; **APX,** ascorbate peroxidase; **BPH,** brown planthopper; **CAT,** catalase; **CRK,** cysteine-rich receptor-like kinase; **FA,** formic acid; **GDC,** glycine decarboxylase complex; **Gns,** glucanase; **JA,** jasmonic acid; **POX,** peroxidase; **RLK,** receptor-like kinase; **TPI,** triose phosphate isomerase

yield loss to crops of resistant plants [4, 5]. For rice crops, growing resistant varieties is the most effective and environmentally friendly way to control the BPH.

Interactions between plants and insects are complex, and many researchers have shown that gene reprogramming can occur when insects invade plants [6]. More specifically, Zhang *et al.* [7] detected changes in the expression levels of genes associated with abiotic stress, pathogen invasion and phytohormone signaling pathways in response to BPH feeding. In addition, genes involved in macromolecule degradation and plant defenses were found to be up-regulated, whereas those involved in photosynthesis and cell growth were down-regulated, following BPH infestation in an investigation of the susceptibility of a rice cultivar by Yuan *et al.* [8]. Further, in an exploration of differences in transcript profiles between susceptible and resistant rice cultivars, using a cDNA microarray containing 1920 suppression subtractive hybridization clones, Wang *et al.* [9] found the expression of 160 unique genes to be significantly affected by BPH feeding. One of the jasmonic acid (JA) biosynthesis genes, *OsLOX*, has been shown to be induced by the BPH and to provide enhanced resistance in transgenic rice [10]. BPH feeding also causes callose synthase genes to be up-regulated, resulting in callose deposition on sieve plates [11]. Furthermore, spindle-like protein bodies in the sieve tubes have been shown to inhibit aphid feeding in broad bean [12]. The formation of barriers in sieve elements prevents the insect from sucking the phloem sap. These insect-affected genes are mainly involved in defense signaling pathways, oxidative stress responses, structural modifications and metabolism.

Proteomics has emerged as a powerful tool for gaining insight into physiological changes at the cellular level, but few attempts have been made to apply this technique to study the response of rice to BPH attack. Relative quantification proteomic analysis, using highly sensitivity mass spectrometry techniques, is becoming increasingly popular, due to the high-throughputs, reproducibility and sensitivity it offers [13]. Currently, isobaric tag-based methodology for relative peptide quantification (iTRAQ), coupled with multidimensional liquid chromatography and tandem mass spectrometry, enables the assessment of protein levels of up to eight samples, which can be compared for their common effects [14]. To investigate the role of defense-responsive proteins in susceptible and resistant rice lines against BPH, we used an iTRAQ proteomic analysis to screen for the pathological levels of proteins in rice. This methodology overcomes limitations of a two-dimensional electrophoresis analysis that we previously reported [15], which was not suitable for analyzing hydrophobic proteins or large proteins. The results of the present study provide useful indications regarding the resistance mechanisms of plants to agricultural pests at a molecular level.

2 Materials and methods

2.1 Plant material and treatments

The susceptible and resistant rice lines used in the experiments have the same genetic history and originate from the variety Taichung Native 1, which is susceptible to BPH, except that the resistant lines bear the BPH resistance gene *Bph15*, a major BPH resistance gene mapped on chromosome 4 [16]. The susceptible near-isogenic lines were represented by nine individuals (w118, w157, w224, w268, w292, w1054, w1060, w1062 and w1058) and the resistant lines by ten individuals (w8, w14, w202, w229, w418, w675, w679, w1237, w1238 and w1246). The rice was grown to the fourth leaf stage in a greenhouse for 3 wk. For feeding experiments, second- and third-instar nymphs of the BPH were introduced at a density of eight insects *per* seedling. A control experiment without the BPH was also conducted. We separated the two outermost layers of leaf sheath surrounding the stem from the plants exposed to BPH for 96 h. The corresponding parts of control plants were collected at the same time. All the collected materials were frozen immediately in liquid nitrogen.

2.2 Protein extraction

Portions of leaf sheaths (5 g) were ground in liquid nitrogen to a fine powder. Total protein was extracted in 10 mL of extraction buffer (66 mM Tris-HCl, pH 6.8, 2% SDS, 2% β-mercaptoethanol), followed by centrifugation for 20 min at 40 000 × *g*. One milliliter of the resulting supernatant was mixed with 8 mL of ice-cold acetone, 1 mL of trichloroacetic acid and 10 μL of β-mercaptoethanol. The precipitation was followed by centrifugation at 18 000 × *g* for 15 min at 4°C and washed twice with 1 mL of ice-cold acetone. The protein pellets were re-suspended in 400 μL of sample preparation buffer (100 mM Tris-HCl, 8 M urea, 0.4% SDS, 5 mM tributylphosphine, pH 8.3) and placed on ice.

2.3 Trypsin digestion and iTRAQ^{TM} assays

Sample mixtures were sonicated with five 20 s pulses (50 W), with 1 min intervals on ice, followed by iodoacetamide alkylation at room temperature for 1 h. Protein concentrations were measured using a MicroBCA protein assay kit (Pierce, Rockford, IL), following the manufacturer's instructions, before reactions were quenched by the addition of DTT. The protein mixtures were then diluted eight-fold in 50 mM Tris-HCl, pH 8.5. Modified trypsin (Sigma, St. Louis, MO) was added to a final substrate to enzyme ratio of 30:1 and the trypsin digests were incubated overnight at 37°C. The peptides from each digest solution were acidified to pH 3.0 with formic acid (FA) and loaded onto a Discovery DSC-18 Cartridge (Sigma). The peptides were desalted (with 5 mL

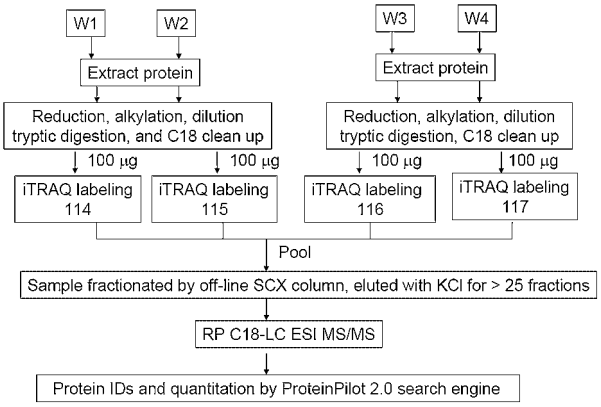

Figure 1. Strategy used for the analysis of protein expression in rice leaf sheaths. W1, leaf sheaths of susceptible control plants; W2, leaf sheaths of susceptible plants infested by BPH; W3, leaf sheaths of resistant control plant; W4, leaf sheaths of resistant plants infested by BPH.

of 0.1% FA) and eluted with 3 mL of a solution composed of 50% ACN with 0.1% FA.

Equal amounts (100 μg) of sample were labeled with 4-plex iTRAQ reagent (Applied Biosystems, Framingham, MA) according to the manufacture's instructions. Briefly, after desalting on a C18 cartridge, the peptide mixtures were lyophilized and re-suspended in 30 μL of 0.5 M triethylammonium bicarbonate, pH 8.5. The appropriate iTRAQ reagent (dissolved in 70 μL ethanol) was added, allowed to react for 1 h at room temperature, and then the reaction was quenched with 10 μL of 1 M Tris, pH 8.5. The entire experiment (including the generation of cell pellets) was performed twice. The general workflow of the iTRAQ experiment is shown in Fig. 1.

2.4 Off-line strong cation exchange chromatography

iTRAQ-labeled peptides were then concentrated, mixed and acidified to a total volume of 2 mL, followed by injection into an Agilent 1100 HPLC system equipped with a Zorbax 300-SCX column (4.6 id × 250 mm) (Agilent, Waldbronn, Germany). Solvent A was 5 mM KH_2PO_4 and 25% ACN (pH 3.0) and solvent B was 350 mM KCl in solvent A. Peptides were eluted from the column with a 40 min mobile phase gradient of solvent B. A total of 30 fractions were collected and samples were dried by a speed-vac prior to LC-MS/MS analysis.

2.5 On-line nano-LC ESI QqTOF MS analysis

A nanobore LC system (Dionex, Sunnyvale, CA), equipped with a Magic C18 100 A pore 75 μm id × 150 mm (Michrom Bioresources, Auburn, CA) column, packed in-house, interfaced to a QSTAR XL QqTOF mass spectrometer with a NanoSpray ion source (Applied Biosystems, Foster City, CA) was used for MS/MS. Peptide mixtures (reconstituted in 125 μL of 5% FA) were injected and eluted from the column with a 90 min mobile phase gradient (5–11% B in 8 min, 11–13% B in 7 min, 13–14% B in 5 min, 14–36% B in 60 min, 36–92% B in 0.5 min and 92% B in 5 min) at a flow rate of 250 nL/min, where solvent B was 85% ACN, 10% isopropanol, 0.1% FA and 0.01% TFA, and the balance was provided by solvent A, consisting of 3% ACN, 0.1% FA and 0.01% TFA. The mass spectrometer was operated in an information-dependent acquisition mode, whereby following the interrogation of MS data (m/z 350–1500) using a 1 s survey scan, ions were selected for MS/MS analysis based on their intensity (>20 cpm) and charge state (+2, +3 and +4). A total of three product ion scans (2, 3 and 3 s) were set from each survey scan. Rolling collision energies were chosen automatically based on the m/z and charge-state of the selected precursor ions.

2.6 Data analysis

Data were processed by a "through" search against a TIGR Rice Genome Annotation Release 5 database (66 710 entries) using the Paragon algorithm [17] within the ProteinPilot v2.01 software with trypsin as the digest agent, cysteine alkylation, an ID focus of biological modifications and other default settings (Applied Biosystems, Framingham, MA). The software calculates a percent confidence, which reflects the probability that the hit is a false positive, so that at the 95% confidence level there is a false–positive identification rate of 5%. This software automatically accepts all peptides with a confidence of identification >1%; therefore, only proteins that had at least one peptide with >95% confidence were initially recorded. However, the software may also support the presence of an identified protein using other peptides. Performing the search against a decoy (reversed) database allowed estimation of a false discovery level of 4%. Using the ProteinPilot software, quantification was based on the signature peak areas (m/z: 114, 115, 116 and 117) and corrected according to the manufacturer's instructions to account for isotopic overlap. Relative quantification ratios of the identified proteins were calculated, averaged and corrected for any systematic errors in the labeling of the iTRAQ™ peptides. The accuracy of each protein ratio is given by a calculated "error factor" in the software, and a p-value to assess whether the protein is significantly expressed. The error factor expresses the 95% uncertainty range (95% confidence error) for a reported ratio, where this 95% confidence error is the weighted standard deviation of the weighted average of log ratios multiplied by the Student t-factor for $n-1$ degrees of freedom, where n is the number of peptides contributing to relative protein quantification. The p-value is determined by calculating the Student t-factor. This was performed by dividing the weighted average of log ratios-log bias by the weighted standard deviation, allowing

determination of the *p*-value, with $n-1$ degrees of freedom, again where n is the number of peptides contributing to relative protein quantification. To be identified as being significantly differently expressed, a protein had to be quantified in at least three spectra (allowing generation of a *p*-value), having a *p*-value of <0.05 and an error factor of <2. These limits were selected on the basis of our previous work with iTRAQ reagents [18, 19]. These proteins that changed significantly in both experiment replicates were taken to investigate the reproducibility of the replicates. Protein expression ratios (115:114 and 117:116) from two experiment replicates were converted to $\log_{10}$ space and plotted. The theoretical slope of the graph should be 1 (slope of 1) and the protein expression ratio in both iTRAQ experiments should be similar, or ideally the same.

2.7 Assignment of proteins to biological processes

For the proteins whose expression changed significantly, the gene ontology classification provided in the TIGR Pseudomolecule release 5 (GOSlim) was used to assign proteins to a hierarchical biological process using the Web Gene Ontology Annotation Plotting tool (WEGO, http://wego.genomics.org.cn) [20]. GOSlim information was available for 89.2 and 87.3% differentially regulated proteins in susceptible lines and resistant lines, respectively.

2.8 Real-time PCR analysis

The primers used for real-time PCR were alpha-DOX (5′-GGAAGAACATCCCAACCTATCA-3′, 5′-CAGTCCCACAAGCCCACCTA-3′), cytochrome P450 (5′-GTCGTCGATCATCCACTCA-3′, 5′-ACACTGTCAGCCCTGTAAAAT-3′), allene oxide cyclase (AOC) (5′-TGCGATGAGCCGATGAACT-3′, 5′-CGTAGTGGCGGTCGTTGTAG-3′), peroxidase (POX) (5′-GTCCGCATCTTCTTCCACG-3′, 5′-GCGTCTGGTTGGGTATCTCAC-3′), cysteine-rich receptor-like kinase 5 (CRK5) (5′-AGTGCTACGCCCGCCTCT-3′, 5′-CCGCTGCTGATGTTGGTG-3′), atypical receptor-like kinase (RLK) (5′-TGGGTATTGTTTTGTTGGATCA-3′, 5′-GGCAGACGATGAAATGAAGGTA-3′), glucanase 5 (Gns5) (5′-TTGCGGCCATTCCTACAGT-3′, 5′-TGGTGAGGGCGATGCTTC-3′), triose phosphate isomerase (TPI) (5′-TGGTGGCAACTGGAAATG-3′, 5′-GACCACGGGAAGGAACAC-3′). The reactions (20 μL) were carried out using the Rotogene 6000TM real-time PCR system (Corbett Research). After the amplification steps, the melting curve was determined for each primer pair to verify that only one specific product had been amplified. The reactions were performed in triplicates and the results were averaged. Relative quantification was performed with the $2C_t$ method [21]. Actin1 (5′-GATCACTGCCTTGGCTCCTA-3′, 5′-GTACTCAGCCTTGGCAATCC-3′) was used as a reference for the mRNA expression.

3 Results and discussion

Expression profiles of proteins in leaf sheaths of both susceptible and resistant plants infected by the BPH were analyzed in two independent iTRAQ experiments. In total, 693 distinct proteins (*p*-value <0.05) were identified (447 proteins from experiment 1 and 574 proteins from experiment 2; for detailed protein and peptide information please see Supporting Information Tables S1 to S4). The expression of 293 (42%) and 258 (37%) of these proteins changed significantly in the susceptible and resistant rice lines, respectively, when they were infested by the BPH. One hundred and sixty-four proteins were regulated in the same direction (70 were up-regulated and 94 were down-regulated) in the susceptible and resistant lines. Interestingly, however, 17 proteins were inversely regulated in the two sets of lines. Three hundred and twenty-eight proteins were in common between both experiments. Among them, 72 proteins changed significantly in both experiments for susceptible lines and 95 for resistant lines. These proteins were taken to investigate the reproducibility of the replicates. Figure 2 shows the high level of reproducibility of the iTRAQ analysis.

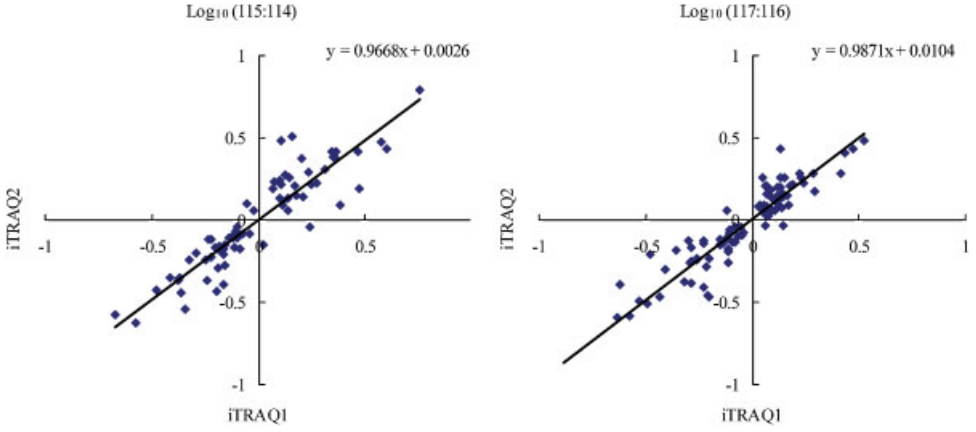

Figure 2. Results of the correlation analysis of the two iTRAQ experimental replicates. Scatter plots of the protein quantitation Log_{10} (115:114) and Log_{10} (117:116) ratios, with regression slopes of 0.97 and 0.99, respectively.

Table 1. List of proteins differentially expressed in susceptible and resistant rice lines infested by BPH

Accession	Protein name	Ratio 115:114[a)]		Ratio 117:116[a)]	
		Experiment 1	Experiment 2	Experiment 1	Experiment 2
Response to biotic stimuli					
LOC_Os01g73170	Peroxidase 12 precursor	**2.25**	**2.43**	**1.74**	**1.68**
LOC_Os01g73200	Peroxidase 12 precursor	2.73	1.02	2.34	**2.16**
LOC_Os03g12500	Cytochrome P450 74A2	**3.97**	**2.72**	**2.70**	**2.58**
LOC_Os03g32314	Allene oxide cyclase 4	2.78	**2.44**	1.18	1.05
LOC_Os04g59150	Peroxidase 12 precursor	**2.94**	**2.63**	**1.68**	**1.91**
LOC_Os04g59190	Peroxidase 2 precursor	**2.22**	**2.60**	**1.69**	**1.80**
LOC_Os07g48020	Peroxidase 2 precursor	1.20	2.58	**0.85**	1.69
LOC_Os07g49400	OsAPx2	**1.16**	**1.56**	**1.08**	**1.22**
LOC_Os12g26290	Alpha-DOX2	**1.19**	**1.72**	**1.12**	**1.83**
Response to stress					
LOC_Os01g55830	Glutathione *S*-transferase GSTF2	**0.68**	0.56	**0.71**	**0.76**
LOC_Os02g02400	Catalase isozyme A	**2.04**	**2.05**	**1.36**	**1.57**
LOC_Os02g18410	DREPP2 protein	0.07	**0.49**	**0.24**	**0.41**
LOC_Os02g41630	Phenylalanine ammonia-lyase	**0.43**	0.54	**0.44**	0.36
LOC_Os03g04240	Glutathione *S*-transferase	**0.60**	**0.60**	**0.51**	**0.56**
LOC_Os03g46770	Glycine-rich RNA-binding protein 2	**0.45**	**0.29**	**0.62**	**0.35**
LOC_Os05g23740	Stromal 70 kDa heat shock-related protein	**1.25**	**1.75**	**1.28**	1.27
LOC_Os06g06290	Alpha-L-fucosidase precursor	**0.38**	**1.45**	**0.55**	**0.58**
LOC_Os06g45820	OsFtsH2-*Oryza sativa* FtsH protease	**1.63**	**1.40**	**1.46**	**1.41**
Protein metabolic and modification processes					
LOC_Os01g42294	Atypical receptor-like kinase MARK	**3.17**	7.32	1.06	2.60
LOC_Os02g02890	Peptidyl-prolyl *cis–trans* isomerase	**0.65**	0.77	**0.83**	**0.73**
LOC_Os02g37862	60S ribosomal protein L6	**2.96**	**1.54**	**1.39**	**0.93**
LOC_Os03g11410	Mitochondrial-processing peptidase beta subunit	**0.68**	**0.62**	0.90	0.99
LOC_Os03g16960	CRK6	**1.82**	2.18	**1.66**	1.11
LOC_Os04g33750	40S ribosomal protein S14	2.24	**2.23**	0.90	1.00
LOC_Os04g56430	CRK5	**5.67**	**6.16**	**2.95**	**2.73**
LOC_Os05g09490	Proteasome subunit beta type 7-A precursor	**0.62**	**0.68**	0.88	0.89
LOC_Os05g49320	50S ribosomal protein L12-1	**0.72**	0.40	**0.62**	**0.34**
LOC_Os12g01390	Clathrin heavy chain	2.55	**2.24**	1.63	1.05
Carbohydrate metabolic processes					
LOC_Os01g52500	NADP-dependent malic enzyme	**1.50**	**1.42**	**1.39**	**1.44**
LOC_Os01g71340	Glucan endo-1,3-beta-glucosidase	**1.59**	2.32	1.01	1.36
LOC_Os01g71670	Glucan endo-1,3-beta-glucosidase GII precursor	0.49	**0.42**	0.37	**0.34**
LOC_Os03g50480	Phosphoglucomutase	0.79	**0.55**	**0.70**	**0.70**
LOC_Os05g31140	Lichenase-2 precursor	7.32	**3.96**	3.3	**2.49**
LOC_Os05g33380	Fructose-bisphosphate aldolase	**0.69**	**0.70**	**0.69**	0.92
LOC_Os06g09450	Sucrose synthase 1	**0.69**	**0.67**	**0.78**	**0.83**
LOC_Os06g40640	Fructose-bisphosphate aldolase	1.29	1.53	**1.95**	**1.51**
LOC_Os08g09250	Lactoylglutathione lyase	**0.82**	**0.67**	0.97	0.88
Amino acid and derivative metabolic processes					
LOC_Os05g04510	*S*-adenosylmethionine synthetase 1	0.63	**0.55**	0.62	**0.44**
LOC_Os06g18670	Anthocyanidin 3-*O*-glucosyltransferase	0.21	**0.26**	**0.23**	**0.25**
LOC_Os08g06100	Quercetin 3-*O*-methyltransferase 1	0.66	**0.44**	0.61	**0.45**
LOC_Os10g37180	Glycine cleavage system H-protein	0.93	0.94	**1.95**	**1.93**
Photosynthesis					
LOC_Os01g05490	Triosephosphate isomerase	0.78	**0.77**	1.03	1.16
LOC_Os02g38920	Glyceraldehyde-3-phosphate dehydrogenase	0.58	**0.74**	0.80	0.91
LOC_Os04g40950	Glyceraldehyde-3-phosphate dehydrogenase	0.51	**0.63**	0.59	**0.75**
LOC_Os05g48630	Expressed protein	0.55	0.80	**1.19**	**1.06**
LOC_Os06g45590	Glyceraldehyde-3-phosphate dehydrogenase	0.65	**0.67**	0.79	**0.83**
Transport					
LOC_Os02g44630	Aquaporin PIP1.1	**0.54**	0.78	**0.60**	**0.72**
LOC_Os04g16450	Aquaporin PIP2.6	**0.47**	**0.57**	**0.39**	**0.51**
LOC_Os06g22960	Aquaporin TIP2.2	**0.75**	**0.78**	**0.78**	**0.88**
LOC_Os07g26690	Aquaporin PIP2.1	**0.65**	**0.65**	**0.52**	**0.56**

a) 114, susceptible lines; 115, BPH-treated susceptible lines; 116, resistant lines; 117, BPH-treated resistant lines. Significant changes (p-value <0.05; error factor <2) are indicated by boldface.

3.1 Protein classification

Differentially regulated rice proteins were sorted according to their biological process by WEGO (Table 1). The BPH response proteins are mainly involved in stress and responses to biotic stimuli, primary metabolic processes, cellular and macromolecule catabolism, and biosynthesis. We conclude that the BPH activated both defense and tolerance responses. Similar responses in gene expression have been reported that genes involved in defense were up-regulated and those involved in photosynthesis were down-regulated following insect infestation [9, 22]. A dramatic functional reorganization could be induced in rice during responses to the BPH, involving the activation of defenses and tolerance proteins, which in turn make large demands on primary metabolism. There was no significant difference in the number of BPH response proteins in each category between the susceptible and resistant lines. In order to obtain a deeper insight into the nature of the proteins that respond to BPH attack, the following section describes a number of notable differentially regulated proteins (with p-value <0.05, error factor <2, in at least one experiment), which are detailed in Table 1.

3.2 Responsive proteins identified in BPH-susceptible and -resistant rice lines

3.2.1 JA biosynthesis proteins

A highly conserved herbivore defense signaling pathway in plant involves JA. Three proteins involved in JA biosynthesis were induced in rice in response to infestation by the BPH: cytochrome P450 (LOC_Os03g12500), AOC 4 (LOC_Os03g32314) and alpha-DOX2 (LOC_Os12g26290) (Table 1). Interestingly, the other two proteins that play important roles in salicylate biosynthesis, phenylalanine ammonia-lyase (LOC_Os02g41630) and isochorismate synthase (LOC_Os09g19734) were down-regulated in response to BPH feeding.

Alpha-DOX2 (LOC_Os12g26290) is a dioxygenase that synthesizes 13-hydroperoxylinolenic acid from linolenic acid in JA biosynthesis. Induction of the expression of the *alpha-DOX* gene has been previously observed during herbivore attacks in tobacco [23]. More recently, Koeduka *et al.* [24] reported that alpha-DOX can be induced by blight bacteria infection, and both oxidative and heavy metal stresses, through the jasmonate signaling pathway in rice seedling leaves. We found that alpha-DOX2 expression significantly increased in both susceptible and resistant rice plants attacked by the BPH.

Cytochrome P450 (LOC_Os03g12500), which is also known as allene oxide synthase (AOS) 2, converts 13-hydroperoxylinolenic acid to 12, 13-epoxylinolenic acid. The reaction catalyzed by AOS is a regulatory point in the jasmonate biosynthesis pathway [25]. AOS2 mRNA accumulates to higher levels in leaves of incompatible rice plants than in compatible plants challenged by the blast pathogen according to Agrawal *et al.* [26], and transgenic rice lines overexpressing the OsAOS2 transgene are believed to have increased resistance to this pathogen [27]. In our experiment, AOS2 increased 2.5- to 3-folds in response to BPH attack in both susceptible and resistant rice lines (Table 1).

AOC4 (LOC_Os03g32314) catalyzes the stereospecific cyclization of an unstable allene oxide to (9S, 13S)-12-oxo-(10, 15Z)-phytodienoic acid, and experiments with the JA-deficient Arabidopsis mutant *opr3* indicate that AOC is the preferential target in the regulation of JA biosynthetic capacity [28]. Since the BPH is a phloem-feeding insect, AOC may have a role in systemic defense signaling. Our iTRAQ results showed that levels of AOC increased approximately 2.5-fold at 96 h in the BPH-treated susceptible lines, compared with the controls, but remained unchanged in the resistant rice lines. Questions remain with respect to the differential response of AOC, which catalyzes the synthesis of the ultimate precursor of JA, with consequent differences in levels of JA in both sets of rice lines. After 96 h of BPH infestation, resistant lines maintained healthy green seedlings nearly equal to those from untreated control rice. On the contrary, susceptible lines showed wilting. JA synthesized from linolenic acid is strongly involved in the induction of defense responses against wounding [29]. The differential response of AOC was probably attributed to the difference in the level of damage inflicted on the susceptible and resistant lines at the time of harvesting.

We suggest that wounding caused by BPH induced subsequent biosynthesis of JA, leading to the expression of defense genes and proteins against insect feeding. Our results showed that BPH feeding on rice led to stimulation of response pathways associated with wounding.

3.2.2 Oxidative stress-related proteins

To prevent oxidative injuries, plants have evolved enzymatic systems that scavenge highly reactive oxygen species. We identified several oxidative stress-related proteins, whose expression increased significantly in response to the BPH, including a catalase (CAT), an ascorbate POX (APX) and five extracellular class III POXs. The activities of these enzymes have high correlations with plant defenses against pathogens.

CAT has been shown to be involved in pathogen defense [30], but Higo and Higo [31] found that the rice CAT isozyme A (LOC_Os02g02400) (CATA) gene did not respond to any tested stress agents, except methyl viologen. In contrast, our iTRAQ data show that CATA was induced in the susceptible lines, but not in the resistant lines, providing new evidence that CATA responds to BPH attack.

In plants, APX is the key enzyme of the H_2O_2-detoxification system. OsAPX2 (LOC_Os07g49400) is one of eight APX genes identified in the rice genome [32]. Transcription of these

genes has been shown to be up-regulated following wounding, attacks by the blast pathogen and exposure to diverse signaling substances, including salicylic acid, ethylene, abscisic acid and hydrogen peroxide [33]. In our study OsAPX2 was induced in both sets of rice lines, indicating that APX plays a role in the protection of rice seedlings against BPH injury.

The five POXs we detected (LOC_Os01g73170, LOC_Os01g73200, LOC_Os04g59150, LOC_Os04g59190 and LOC_Os07g48020), POX2 (LOC_Os07g48020) share 99% sequence similarity to rice POX22.3, which has been shown to be induced in rice leaves challenged by the rice blast fungus [34]. Pathogenesis-related accumulation POX has also been observed in rice vascular systems in accordance with secondary wall thickening of vessels during pathogenic responses [35]. Therefore, in addition to their role in ROS scavenging, POXs may induce the formation of structural barriers that inhibit BPH feeding, such as thickened cell walls. Similar to CATA, four of the five POXs were induced in the susceptible lines, suggesting that BPH injury results in the up-regulation of ROS in susceptible rice lines.

Oxidative stress is one of the first general reactions to the injuries caused by phloem-feeding insects when they penetrate the plant. Our results showed that the BPH triggered a differential modulation of antioxidant proteins in the two lines, possibly due to different degrees of disruption of cell redox homeostasis.

3.2.3 Protein modification and metabolic processes

We identified two CRKs, CRK5 (LOC_Os04g56430) and CRK6 (LOC_Os03g16960). In Arabidopsis, a group of CRK genes has been shown to be induced by pathogen infection and treatment with reactive oxygen species [36]. In addition, overexpression of AtCRK13 has been found to activate cellular and molecular defense markers and enhance the resistance of plants to bacterial pathogens [37]. The rice CRK protein CRK5 can also be up-regulated: in response to blast fungal inoculation in the leaves [34] and when suspension-cultured rice cells are treated with a fungal elicitor [38]. Our iTRAQ results showed that CRK5 and CRK6 expression increased in the leaf sheaths when the BPH were feeding on rice. These observations suggest that the CRK proteins may be involved in plant perception and responses to BPH attack signals.

We also identified atypical RLK (LOC_Os01g42294). Although the atypical RLK contains 11 conserved subdomains of catalytic kinases [39], some of the non-variable and highly conserved amino acids within these subdomains have been substituted in it. For example, the aspartic acid residue in the subdomain VIb has been replaced by asparagine (residue 503) and the glycine within the DFG activation loop has been replaced by cysteine (residues 523). These mutations suggest that atypical RLK has a phosphorylation-independent pathway [40]. It is possible that atypical RLK may be regulated in BPH responses by its intracellular kinase-like domain.

When plants are attacked by insects, they produce a number of defense-related proteins, many of which are synthesized and then secreted to their various destinations within the cell by the Golgi apparatus. Clathrin is a Golgi-associated membrane trafficking protein that is involved in packaging secretory proteins into small vesicles. In our experiments, a heavy chain form of clathrin protein (LOC_Os12g01390) was up-regulated in the susceptible rice lines, but remained unchanged in the resistant rice lines after BPH feeding. Similarly, levels of two biosynthesis proteins, 60S ribosomal protein L6 (LOC_Os02g37862) and 40S ribosomal protein S14 (LOC_Os04g33750), increased approximately two-fold in the susceptible lines, but not in the resistant lines. These results indicate that protein synthesis and secretion patterns change dramatically in the susceptible lines, but not in the resistant lines, when under BPH attack. Previously, it has been reported that the induction of a group of genes including genes encoding clathrin plays an important role in the onset of basal defense responses [41]. Thus, the susceptible rice lines seem to activate their basal defense mechanism under BPH attack, but not other defenses that appear to be induced in the resistant plants.

3.2.4 Carbohydrate metabolic processes

We identified three beta-Gns, which play important roles in plant defense and development. Glucan endo-1,3-beta-glucosidase (Gns4; LOC_Os01g71670) and glucan endo-1,3-beta-glucosidase (Gns5; LOC_Os01g71340) have been classified into defense-related subfamily A and lichenase-2 (Gns1; LOC_Os05g31140) into growth-related subfamily B [42]. Gns5, which catalyzes the hydrolysis of 1,3-beta-glucosyl linkages of 1,3-beta-glucan, such as callose, showed increased expression in the susceptible rice lines, but not in the resistant lines in our experiments. This observation is consistent with a recent report that the Gns5 gene is clearly induced when rice plants are under BPH attack. Gns5 is likely to play an important role in callose decomposition, which ultimately facilitates the ingestion of phloem sap by the BPH in the susceptible rice plants. The absence or limited expression of this gene allows sieve tube occlusions to be maintained in the resistant plants [11]. In contrast to Gns5, Gns4 expression was reduced following BPH attack. Thus, Gns4, which specifically hydrolyzes 1,3;1,6-beta-glucans, is likely to play an important role in antifungal defense, rather than in defense against the BPH [43]. The rice Gns1 hydrolyzes 1,3;1,4-beta-glucosidic linkages in 1,3;1,4-beta-glucan. Besides its functions in development, Gns1 can be induced by ethylene, wounding, salicylic acid and fungal elicitors in rice shoots [44]. In a previous study transgenic rice plants overexpressing Gns1 developed resistant-type lesions on leaves inoculated with virulent blast fungus through activation of the defense-related genes *PR-1* and *PBZ1* [45]. The increased levels of Gns1 we observed in rice on which the BPH was feeding rice may also activate plant defense signals.

3.2.5 Amino acid and derivative metabolic processes

The glycine cleavage system or glycine decarboxylase complex (GDC) is a multienzyme complex composed of four component enzymes, such as the P-protein, H-protein, T-protein and L-protein, and is responsible for the conversion of glycine produced in the peroxisome to serine in the mitochondria, during the operation of the photorespiratory cycle [46]. The H-protein plays a pivotal role in GDC. From an analysis of a mutant of barley deficient in GDC, it was concluded that the biosynthesis and activity of GDC is determined by the biosynthesis of H-protein [47]. In our experiment, H-protein (LOC_Os10g37180) expression approximately doubled in the resistant lines, but remained unchanged in the susceptible lines when challenged by the BPH. The increase of H-protein might cause increase in the activity of GDC in resistant lines. Since GDC also plays an important role in photorespiration, we concluded that the resistant lines might alter the level of photorespiration to cope with the stress caused by BPH at 96 h [48].

3.3 Real-time PCR analysis

We further monitored the expression patterns of the corresponding genes from eight BPH responsive proteins. Total RNA of the susceptible and resistant lines was extracted from 0 to 96 h of BPH feeding, followed by real-time PCR analysis. The expression patterns of the eight genes – *alpha-DOX*, *cytochrome P450*, *AOC*, *POX* (*LOC_Os04g59150*), *CRK5*, *atypical RLK*, *Gns5* and *TPI* (*LOC_Os01g05490*) – are shown in Fig. 3. The eight genes showed different expression patterns between two sets of rice lines. As early as 6 h of BPH infestation, the *alpha-DOX*, *cytochrome P450*, *AOC*, *POX* and *atypical RLK* genes were induced and had a significant higher expression level in susceptible lines compared with resistant lines. The highest expression level of *alpha-DOX*, cytochrome *P450*, *AOC*, *POX* and *Gns5* was found in susceptible lines. These results may reflect the different rates and extent of damage in two lines. The *Gns5* was slightly down-regulated at 6 h in both lines, then increased to a rather high level at 72 h in susceptible lines but maintained at a normal level in resistant lines. The TPI, a photosynthesis-related gene, was down-regulated during 6–96 h in susceptible line but remained relatively constant after a slight decrease at 6 h in resistant lines. This suggested that the resistant lines were capable of sustaining normal level photosynthesis under the BPH stress. In contrast, at 96 h, only the TPI, Gns5 and CRK5 mRNA levels are significantly different between both lines. In some cases (*i.e. AOC*, *POX*, *CRK5* and *atypical RLK*) disparities between mRNA and protein abundance at 96 h were observed. This observation is not unexpected since mRNA abundance is not a good indicator of the levels of the corresponding protein. It is evident from several studies that the proteomic analysis does not necessarily match the transcriptional analysis of the

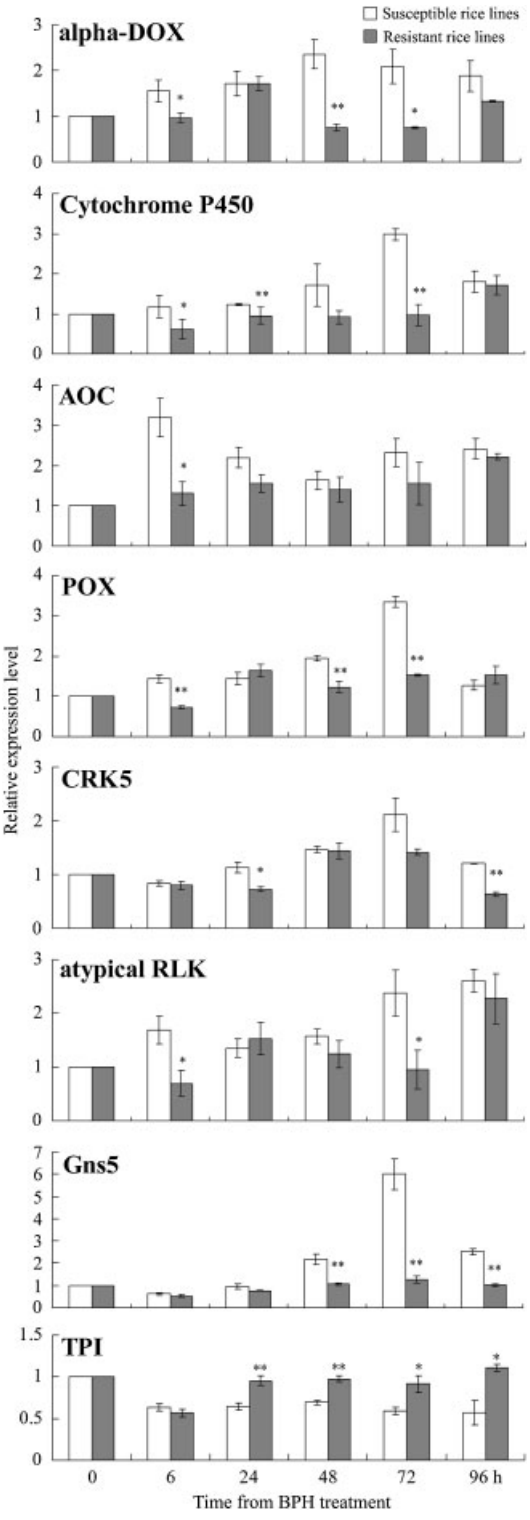

Figure 3. Results of real-time PCR expression analysis of genes for BPH-responsive proteins in susceptible and resistant rice lines. The expression levels of *alpha-DOX*, *cytochrome P450*, *AOC*, *POX* (*LOC_Os04g59150*), *CRK5*, *atypical RLK*, *Gns5* and TPI were quantified relative to the value obtained from 0 (h) samples (BPH-free plants). Significant differences in gene expression are indicated with asterisks: $^{*}p<0.05$ or $^{**}p<0.01$; Student's *t*-test.

same response [49]. The discrepancies could potentially be attributed to mRNA stability, splicing, translational regulation, post-translational processing, control of protein turnover, protein degradation or a combination of these. These results suggest that proteomics data are more relevant to biological responses because proteins, not RNAs, are the functional products of these genes.

4 Concluding remarks

The proteomic data presented here will help to further understand the responses of both susceptible and resistant rice lines to BPH feeding. It is suggested that BPH feeding is accompanied by multiplex stresses similar to wounding, oxidative stress, pathogenesis and insect herbivory. Both sets of rice lines shared the same response. In response to the challenge of stresses caused by pest invasion, plants develop a basal defense, which appeared stronger in the susceptible lines, compared with the resistant lines. For example, expression of the JA synthesis proteins, oxidative stress proteins, Gns1, protein kinases and the clathrin heavy chain protein increased in both lines, but the higher expression levels were seen in the susceptible lines after the BPH treatment. In contrast, Gns5 expression remained unchanged and glycine cleavage system H-protein was up-regulated in only the resistant lines. These differences were probably attributed to a severe difference in the level of damage inflicted on the susceptible and resistant lines, as well as differences in the genotypes between them. The resistant lines carrying the BPH resistance gene *BPH15* might utilize a different defense mechanism, which involves Gns5 and the glycine cleavage system H-protein.

This work was supported by grants from the National Program of High Technology Development (grant no. 2007AA10Z135) and the National Natural Science Foundation of China (grant no. 30730062).

The authors have declared no conflict of interest.

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