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Physiological and molecular alterations promoted by *Schizotetranychus oryzae* mite infestation in rice leaves

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Abstract

Infestation of phytophagous mite *Schizotetranychus oryzae* in rice causes critical yield losses. To better understand this interaction, we employed Multidimensional Protein Identification Technology (MudPIT) approach to identify differentially expressed proteins. We detected 18 unique proteins in control and 872 in infested leaves, respectively, along with 32 proteins more abundant in control leaves. *S. oryzae* infestation caused decreased abundance of proteins related to photosynthesis (mostly photosystem II-related), carbon assimilation and energy production, chloroplast detoxification, defense, fatty acid and gibberellin synthesis. On the other hand, infestation caused increased abundance of proteins involved in protein modification and degradation, gene expression at the translation level, protein partitioning to different organelles, lipid metabolism, actin cytoskeleton remodeling, and synthesis of jasmonate, amino acid and molecular chaperones. Our results also suggest that *S. oryzae* infestation promotes cell wall remodeling and interferes with ethylene biosynthesis in rice leaves. Proteomic data were positively

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Supporting Information Available

Table S-1. Gene-specific PCR primers used for RT-qPCR analyses. Table S-2. List of unique and differentially expressed proteins obtained from the MudPIT experiments. Files S3–8. DTASelect output files listing all identified proteins and peptide sequences along with their associated scores in “control and mite-infested leaves” experiments replicates 01, 02 and 03.

correlated with enzymatic assays and RT-qPCR analysis. Our findings describe the protein expression patterns of infested rice leaves, and suggest that the acceptor side of PSII is probably the major damaged target in the photosynthetic apparatus. These data will be useful in future biotechnological approaches aiming to induce phytophagous mite resistance in rice.

Keywords

MudPIT; photosynthesis; phytophagous mite; rice infestation; *Schizotetranychus oryzae*; shotgun proteomics

Introduction

Rice is a staple food for more than 50% of the world's population, making it one of the most important crop plants on Earth¹. It is cultivated on approximately 150 million hectares of land and its annual yield is close to 610 million tons (<http://beta.irri.org/>). However, rice yield is reduced by different biotic stresses. Potential worldwide losses caused by animal pests in rice are estimated to be around 25%². One of the most significant losses in rice production is caused by phytophagous mite infestation³, which can harm rice plants during their entire development, based on the mite species and number.

Phytophagous mites of the Tarsonemidae and Tetranychidae families are observed in rice plantations all over the world causing drastic yield reductions of up to 90%⁴. *Schizotetranychus oryzae* Rossi de Simons, a spider mite (Acari: Tetranychidae) has been reported in several South American countries. Rice leaves infested by *S. oryzae* may present small yellowish-white elongated areas visible on the upper side of the leaf. These areas correspond to small mite colonies in different stages of development, usually localized on the lower surface of the leaves. Infested leaves also exhibit chlorotic areas at both the abaxial and adaxial surfaces^{3,5}.

When attacked by phytophagous mites, the growth of plants is inhibited and their photosynthetic structures and storage organs are damaged⁶. The primary metabolism of infested plants becomes disturbed. This affects the metabolism of amino acids and carbohydrates, and changes the hydric potential of their cells. Increased levels of reactive oxygen species and lipid peroxidation and decreased levels of soluble protein and sugars are also common in plants after mite infestation^{7,8}.

Plants developed several defense strategies (chemical and physical) against herbivore arthropods. They can synthesize toxic metabolites followed by defense proteins that will, in the long term, create physical defenses, such as an increase in the density of trichomes, waxes, and thorns, reducing the access of the phytophagous to vegetal tissues and interfering with their feeding^{9,10}. Chemical defenses also include the release of volatile organic compounds that attract predatory mites^{11,12} and the activation of systemic resistance mechanisms, triggering the defense mechanisms of the plant systemically, and not only at the site of induction¹³. Gaseous phytohormones, such as ethylene and a series of terpenes, as well as jasmonic acid (JA) and salicylic acid (SA)¹⁴ are released by plants and regulate

several defense signaling mechanisms¹². Genes involved in cellular detoxification may also be up-regulated, signaling for the production of antioxidant enzymes¹⁵.

There is a growing interest in the development of plants that efficiently resist to phytophagous mite infestation. However, the information available about changes in the host plants caused by phytophagous mites are typically limited to the visual effects of the infestation. Meanwhile, the molecular and physiological modifications of the plants remain largely uncharacterized. The aim of this work is to identify proteins that are significantly differentially expressed in rice leaves infested with the phytophagous mite *Schizotetranychus oryzae*. To our knowledge, this is the first study that investigates rice responses to phytophagous mites using a high-throughput proteomic analysis. Our findings will contribute to a better understanding of the molecular and physiological mechanisms involved in rice response to *S. oryzae* infestation and could be helpful for future biotechnological and molecular breeding efforts.

Experimental section

Plant growth conditions and mite infestation

Seeds of rice (*Oryza sativa* L. ssp. *indica*) from BR-IRGA424 cultivar were surface sterilized and germinated for four days in an incubator (28 °C) on paper soaked with distilled water. After germination, plantlets were transferred to vermiculite/soil mix (1:3) for 14 days in greenhouse conditions, and then transferred to plastic buckets containing soil and water. Infestation of rice leaves with *S. oryzae* was performed by proximity (direct contact) using highly infested rice plants (kindly provided by Instituto Rio-Grandense do Arroz, IRGA, Cachoeirinha, RS) in the middle of several plastic buckets containing healthy rice plants. Control plants (without *S. oryzae* infestation) were maintained isolated to prevent mite infestation. In all the experiments, we analyzed control (no infestation) and early-infested leaves (EI, containing about 18.5 ± 1.8 mites per leaf) (Figure 1).

Protein extraction and Rubisco depletion

Three samples (approximately 250 mg), each containing three leaves from three different plants, were subjected to protein extraction using Plant Total Protein Extraction Kit (Sigma-Aldrich). Protein concentrations were determined by BCA assay using bovine serum albumin as standard (Thermo Scientific, Rockford, IL). Depletion of Rubisco proteins was performed using the method of Krishnan and Natarajan¹⁶.

Sample preparation for mass spectrometry

Approximately 100 µg of Rubisco depleted protein extracts were suspended in digestion buffer (8 M urea, 100 mM Tris-HCl pH 8.5). Proteins were reduced with 5 mM tris-2-carboxyethyl-phosphine (TCEP) at room temperature for 20 min and alkylated with 10 mM iodoacetamide at room temperature in the dark for 15 min. After the addition of 1 mM CaCl₂ (final concentration), the proteins were digested with 2 µg of trypsin (Promega, Madison, WI, USA) by incubation at 37 °C during 16 h. Proteolysis was stopped by adding formic acid to a final concentration of 5%. Samples were centrifuged at 14,000 rpm for 20 min, and the supernatant was collected and stored at -80 °C.

MudPIT and mass spectrometry

The protein digest was pressure-loaded into a 250 μm i.d. capillary packed with 2.5 cm of 5 μm Partisphere strong cation exchanger (SCX) (Whatman, USA), followed by 2 cm of 3 μm Aqua C18 reversed phase (RP) (Phenomenex, USA) with a 1 μm frit. The column was washed with a buffer containing 95% water, 5% acetonitrile, and 0.1% formic acid. After washing, a 100 μm i.d. capillary with a 5 μm pulled tip packed with 11 cm of 3 μm Aqua C18 resin (Phenomenex, USA) was attached via a union. The entire split-column was placed in line with an Eksigent quaternary HPLC and analyzed using a modified 10-step separation. The buffer solutions used were 5% acetonitrile/0.1% formic acid (Buffer A), 80% acetonitrile/0.1% formic acid (Buffer B), and 500 mM ammonium acetate, 5% acetonitrile, and 0.1% formic acid (Buffer C). Step 1 consisted of a 60 min gradient from 0–100% (v/v) of buffer B. Also, a 10 steps (120-min) MudPIT was run with salt pulses of 10, 20, 30, 40, 50, 60, 70, 80, and 100% of buffer C and 90% buffer C/10% buffer B. Three replicates were performed for both control and early-infested samples prepared as described above. Peptides eluting from the microcapillary column were electrosprayed directly into an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA) with the application of a distal 2.5 kV spray voltage. A cycle consisted of one full scan of the mass range (MS) (400–2000 m/z , resolution of 60,000) followed by five data-dependent collision induced dissociation (CID) MS/MS spectra in the LTQ. Dynamic exclusion was enabled with a repeat count of 1, a repeat duration of 30 s, an exclusion list size of 150, and exclusion duration of 180 s. Also, the mass window for precursor ion selection was set to 400–1600, unassigned and charge 1 was rejected, and the normalized collision energy for CID was 35. Mass spectrometer scan functions and HPLC solvent gradients were controlled through the XCalibur data system.

Protein identification and quantification analyses were done using the Integrated Proteomics Pipeline (IP2, Integrated Proteomics Applications, Inc., www.integratedproteomics.com/). MS/MS spectra were extracted from RAW files into MS2 files using RawExtract 1.9.9¹⁷ and were searched against rice proteins sequences of the Reference Sequence Database from the National Center for Biotechnology Information (NCBI) using the ProLuCID algorithm¹⁸. The 28,673 protein sequences were downloaded on May 25, 2014 using *Oryza sativa* as query and plants as additional filter (<http://www.ncbi.nlm.nih.gov/protein>). Peptide mass search tolerance was set to 3 Da, and carboxymethylation (+57.02146 Da) of cysteine was allowed as a static modification. ProLuCID results were assembled and filtered using the DTASelect program¹⁹ resulting in a dataset with a false discovery rate of 1% for protein. Also, the search was made using the following parameters: the protein must contain at least 1 peptide (minimum of 6 amino acids), 1 tryptic end per peptide, the subset proteins were included, and the precursor delta mass cut-off in ppm was set to 10. The NCBI protein sequence database used for protein identification is comprehensive, but it only provides a small number of protein annotations. To address this issue, all identified protein sequences were blasted against the TIGR Rice database (ftp://ftp.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_7.0/, downloaded on September 1, 2014) using blastp (PMID: 9254694). Although not as comprehensive as the NCBI database, the TIGR Rice database provides a large number of protein annotations

about the sequences it contains. For each identified protein sequence, the annotation of its best match from the TIGR Rice database was reported.

Data analysis

The software PatternLab²⁰ was used to identify unique proteins that were statistically differentially expressed in rice leaves under the control and mite-infested conditions. Spectral counting (as used by PatternLab) was used to estimate relative protein abundance²¹. Proteins that were not detected in at least two out of the three replicates per condition were not considered. Statistical significance of differential expression was calculated using a *t*-test. The resulting *p*-values were adjusted for multiple hypothesis testing and transformed to *q*-values using the Benjamini-Hochberg (BH) correction. Proteins were deemed significantly differentially expressed when associated to a BH *q*-value < 0.05 (5% FDR) and an absolute fold change greater than two. Low abundance proteins (low spectral count values) with low *q*-values and a significant fold change were discarded using the L-stringency of 0.4. PatternLab's Approximate Area Proportional Venn Diagram (AAPVD) module was used for pinpointing unique proteins that were identified in a given condition.

The Blast2GO tool (<http://www.blast2go.org>)²² was used to identify proteins with known Gene Ontology annotations²³. Detected proteins were also analyzed using the B2G Kegg maps.

Validation of proteomic data through enzymatic assays

For the protease assay, 10 μ L of sample was incubated in 20 mM Tris-HCl buffer, pH 7.4. The reactions were initiated by adding DL-BAPNA (benzoyl-DL-arginine- ρ NA) at 0.2 mM (final concentration)²⁴. Kinetic assays were monitored at 37 °C for 30 min in a microplate reader SpectraMax (Molecular Devices, USA). One protease unit (U) was defined as the amount of enzyme that produces one μ mol of ρ -nitroaniline per minute under the assay conditions described.

Phospholipase activity assay was evaluated using ρ -nitrophenylphosphorylcholine (ρ NPPC), as described previously²⁵. The reaction contained ρ NPPC 20 mM in 50 mM Tris-HCl pH 8.0 and 60% sorbitol. Samples (10 μ L) were mixed with 90 μ L of substrate solution and incubated at 37 °C for 1 h. Absorbance was read at 410 nm using a microplate reader SpectraMax (Molecular Devices, USA). One unit of PLC was defined as the amount of enzyme that releases 1 μ mol ρ -nitrophenol per min.

Phosphatase activity was measured by the rate of ρ -nitrophenol (ρ -NP) production as previously described²⁶. Samples were incubated for 60 min at room temperature in 0.2 mL of reaction mixture containing 116 mM NaCl, 5.4 mM KCl, 30 mM Hepes-Tris buffer pH 7.0, and 5 mM ρ -nitrophenylphosphate (ρ -NPP) as substrate. The reaction was stopped by the addition of 0.2 mL of 20% trichloroacetic acid. Subsequently, the reaction mixture was centrifuged at 1,500 g for 15 min at 25 °C. The absorbance was measured spectrophotometrically at 405 nm using a microplate reader SpectraMax (Molecular Devices, USA). The concentration of released ρ -nitrophenolate in the reaction was determined using a standard curve of ρ -nitrophenolate for comparison.

Glutathione S-transferase (GST) activity was measured as described previously²⁷, using 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma, USA) as substrate. Ninety microliters of the reaction mixture, consisting of 50 mM CDNB in methanol, 5 mM glutathione in 100 mM Tris-HCl (pH 7.5), and 10 μ L of purified enzyme in 100 mM Tris-HCl (pH 7.5). The absorbance (A_{340 nm}) was measured during 5 min at 25 °C in a microplate reader SpectraMax (Molecular Devices, USA). The concentration of the product formed was calculated using the extinction coefficient of 9.6 mM cm⁻¹.

The lactate dehydrogenase activity (LDH) was measured based on its ability to reduce pyruvate using NADH as a cofactor, generating lactate and NAD⁺. Briefly, samples were incubated at 37 °C with an assay buffer containing 0.2 M Tris-HCl, pH 7.5 and 6 mM pyruvate. After 2–3 min, 20 mM NADH was added and the kinetics of NAD⁺ formation was monitored by the decrease of absorbance at 340 nm by 10 min. LDH activity was calculated based on a standard curve made with known concentrations of NADH and purified LDH. All the measurements were done using the lactate dehydrogenase activity assay kit (MAK066) from Sigma-Aldrich (Saint Louis, MO, USA).

The aminotransferase activity was measured based on the transfer of amino groups of aspartate to α -ketoglutarate, leading to formation of glutamate and oxaloacetate. Samples were incubated in phosphate buffer containing L-aspartic acid and α -ketoglutarate in pH 7.4 followed by the addition of dinitrophenylhydrazine in alkaline medium. The intensity of the hydrazone formed was detected at 505 nm and is directly proportional to the amount of oxaloacetate generated by aminotransferase. All the measurements were done using the aminotransferase activity assay kit from Bioclin-Quibasa (Belo Horizonte, MG, Brazil).

All enzymatic assays were performed in triplicates, with results obtained from at least two separate experiments. RT-qPCR analyses were also used to validate the proteomic data at the RNA level, as described below.

RNA extraction and cDNA synthesis

Total RNA was extracted from rice leaves using NucleoSpin RNA Plant (Macherey-Nagel, Düren, Germany). cDNA was prepared using the SMART PCR cDNA Synthesis Kit by Clontech Laboratories (Mountain View, CA, USA), according to the manufacturer's instructions. First-strand cDNA synthesis was performed with reverse transcriptase (M-MLV, Invitrogen, Carlsbad, CA, USA) using 1 μ g of total RNA.

Quantitative RT-PCR and data analysis

RT-qPCRs were carried out in a StepOne Real-Time Cycler (Applied Biosystems). All primers (listed in Table S-1) were designed to amplify 100–150 bp of the 3'-UTR of the genes and to have similar T_m values (60 °C $\pm$ 2). Reaction settings were composed of an initial denaturation step of 5 min at 94 °C, followed by 40 cycles of 10 s at 94 °C, 15 s at 60 °C, 15 s at 72 °C and 35 s at 60 °C (fluorescence data collection). Samples were held for 2 min at 40 °C for annealing of the amplified products and then heated from 55 to 99 °C with a ramp of 0.1 °C/s to produce the denaturing curve of the amplified products. RT-qPCRs were carried out in 20 μ L final volume composed of 10 μ L of each reverse transcription sample diluted 100 times, 2 μ L of 10X PCR buffer, 1.2 μ L of 50 mM MgCl₂, 0.1 μ L of 5 mM dNTPs,

0.4 µl of 10 µM primer pairs, 4.25 µl of water, 2.0 µl of SYBR green (1:10,000, Molecular Probe), and 0.05 µl of Platinum Taq DNA polymerase (5 U/µl, Invitrogen, Carlsbad, CA, USA). Gene expression was evaluated using a modified 2^{-CT} method²⁸, which takes into account the PCR efficiencies of each primer pair (Relative expression $\frac{\text{TESTED GENE}}{\text{CONTROL GENE}} = \frac{(\text{PCR}_{\text{eff}} \text{ CG})^{\text{Ct CG}}}{(\text{PCR}_{\text{eff}} \text{ TG})^{\text{Ct TG}}}$). *OsUBQ5* gene expression was used as an internal control to normalize the relative expression of the tested genes²⁹. Each data point corresponds to three biological and four technical replicate samples.

Determination of protein carbonylation

The concentration of protein bound carbonyls was determined following the method of Levine *et al.*³⁰. Leaf samples (0.5 g) were homogenized in 1 mL of 1M Tris-HCl buffer (pH 8.0) containing 50 mM EDTA, 100 mM phenylmethane sulphonyl fluoride (PMSF), and 100 mM Benzamidine. The homogenate was centrifuged at 11,000 g for 15 min at 4 °C. Supernatants (500 µL) were mixed with 10% (w/v) streptomycin sulphate for 15 min to remove the nucleic acids. After centrifugation at 11,000 g for 10 min at 4 °C, supernatants were mixed with 500 µL of 20% TCA for additional 15 min. Supernatants were discarded and precipitates were dissolved in 500 µL of 10 mM 2,4-dinitrophenyl hydrazine (DNPH) (freshly prepared) in 2 M HCl. After 1 h incubation at room temperature, proteins were precipitated with pre-cooled 20% (w/v) trichloroacetic acid (TCA) and the pellets were washed three times with 1 mL of ethanol:ethylacetate (1:1). The pellets were then dissolved in 6 M Urea and the absorbance was measured at 370 nm using UV-Vis spectrophotometer. Carbonyl concentration was calculated using a molar absorption coefficient for aliphatic hydrazones as 22,000 M⁻¹ cm⁻¹ and expressed in terms of nmol carbonyl/mg protein.

Chlorophyll a fluorescence transients

The chlorophyll fluorescence transient was measured on the third upper leaves of ten plants, using a portable fluorometer (OS30p, Optosciences, UK). Before the measurements, plants were dark adapted for 20 minutes and the fluorescence intensity was measured by applying a saturating pulse of 3,000 µmol photons m⁻² s⁻¹ and the resulting fluorescence of the chlorophyll a measured from 0 to 1 s, OJIP curve³¹. This data was used to calculate parameters of the JIP Test^{31,32}. It is important to highlight that measures were performed in visually not damaged parts of the mite-infested leaves.

Statistical analysis

Data generated from enzymatic activities and RT-qPCR were analyzed statistically using the Student's *t*-test (*p*-value 0.05, 0.01 and 0.001) using SPSS Base 21.0 for Windows (SPSS Inc., USA).

Results and discussion

Overview of the proteomic analysis

Using the MudPIT approach, a total of 1,592 proteins were identified in control leaves and 2,446 in early-infested (EI) leaves (Figure 2). The majority of these proteins (1,574 or 63.9%) were detected in both conditions, while only 18 (0.7%) were uniquely identified in

control and 872 (35.4%) in EI samples. The list of all unique or differentially expressed proteins identified in this work is presented in Table S-2. Only 32 of the 1,574 proteins identified in both conditions (control and EI) (2.0%) were considered differentially expressed proteins, and all of them were more expressed in control leaves than in EI leaves.

In order to increase the stringency and, consequently, improve the significance of our results, a minimum of ten spectral counts was further used as cut-off to specifically discuss the unique and differentially expressed proteins statistically identified in PatternLab's analysis. Applying this additional threshold limit, we were able to detect 109 proteins. The corresponding sequence of each identified protein was submitted to NCBI protein BLAST in order to identify specific domains, molecular functions and protein annotations. Afterwards, proteins were arbitrarily categorized in functional categories, according to its putative molecular function. Among these proteins, 5 and 72 were detected uniquely in control and EI leaves, respectively, and 32 had higher expression level in control than in EI leaves (Table 1 and Table 2). Highly expressed or unique proteins identified in control samples include the following functional categories: photosynthesis, carbohydrate metabolism and energy production, oxidative stress, protease inhibitor and lipid metabolism. Proteins uniquely found in EI leaves were classified in the following functional categories: protein modification/degradation, amino acid metabolism, carbohydrate metabolism and energy production, lipid metabolism oxidative stress, translation, transport, jasmonic acid synthesis, phosphatase, general metabolic processes, stress response, purine and pyrimidine metabolism, RNA-related stress, detoxification, hormone and calcium signaling, chitin-related, and nitrogen metabolism.

Enrichment of GO terms comparing control and early-infested leaves and KEGG pathway analysis

Gene Ontology (GO) analyses provided an overview of rice molecular response to *S. oryzae* infestation. The GO annotations of all 922 differentially expressed and unique proteins identified are shown in Figure 3. Response to stimulus, primary metabolism processes and cellular metabolic processes were the most regulated biological processes. A smaller number of proteins involved with signaling, developmental processes, growth, immune system process (defense-related), localization, response to stress and nitrogen metabolic process, were also modified in mite-infested leaves. The molecular functions of catalytic activity and binding were the most regulated. The molecular functions of transmembrane transport, structural molecule activity, antioxidant system, hydrolase activity and electron transport (photosynthesis-related) were also represented in our dataset.

To identify specific pathways affected by *S. oryzae* infestation in rice, KEGG pathways were also analyzed. Ninety-five different pathways were associated with proteins identified as up- or down-regulated (data not shown). The following KEGG pathways involved more than 10 proteins identified in our dataset: biosynthesis of antibiotics (43), starch and sucrose metabolism (23), purine metabolism (16), amino sugar and nucleotide sugar metabolism (16), aminoacyl-tRNA biosynthesis (11) and galactose metabolism (10). Some pathways were represented only by proteins uniquely found in mite-infested leaves: phenylpropanoid biosynthesis, α -linolenic acid metabolism and fatty acid degradation. Phenylpropanoid

compounds play a role in plant defense, ranging from preformed or inducible physical and chemical barriers against infection to signal molecules involved in local and systemic signaling³³. In response to arthropod feeding, linolenic acid is released from membrane lipids and then converted enzymatically into jasmonic acid, which causes the transcriptional activation of genes resulting in increased production of defense-related compounds^{3,34}. On the other hand, fatty acid biosynthesis pathway is clearly down-regulated in EI leaves. The pathway related to glutathione metabolism shows that glutathione can be accumulated in mite-infested rice leaves, and probably used for rice defense against oxidative stress. We observe that pathways related to carbon assimilation and energy production are down-regulated, while gene expression at the translational level pathway is up-regulated in rice leaves infested by *S. oryzae* (data not shown).

Photosynthesis- and energy production-related proteins

The functional category of photosynthesis presented the highest number of differentially expressed proteins. We found 15 proteins related to photosynthesis with higher expression in control leaves than EI leaves (including Oxygen evolving enhancer protein 3 domain and Chlorophyll A-B binding protein, 18.9 and 12.6 fold more expressed in control than in EI leaves, respectively), along with two proteins uniquely found in control leaves (Photosystem II reaction center protein and Cytochrome b6-f complex subunit 4) (Table 1). Of note, no protein related to photosynthesis process was detected as more expressed in EI than in the control leaves, suggesting that the rice photosynthetic apparatus is damaged during *S. oryzae* leaf infestation. This process may affect the ability of plants to produce energy.

It was described that rice plants modulate the expression of several genes (including photosynthesis-related ones) under biotic stress conditions³⁵. This is probably due to the excessive loss of plant assimilates, decreased leaf area and wilting^{36,37}. Yuan *et al.*³⁸ showed that genes involved in photosynthesis were down-regulated following Brown Planthopper (*Nilaparvata lugens*) infestation in rice leaves. Later, Wei *et al.*³⁹ and Sangha *et al.*⁴⁰ confirmed these results using proteomic approaches, including the down-regulation of an Oxygen evolving enhancer protein, also found in our work. Ferry *et al.*⁴¹ detected a decrease in the expression of a subunit of cytochrome b6-f complex (also detected in our experiment) in wheat leaves following aphid (*Sitobion avenae*) infestation. Five proteins related to Calvin Cycle were detected by Kim *et al.*⁴² with reduced abundance in leaves infested by the necrotrophic fungal pathogen *Cochliobolus miyabeanus*, which causes brown spot disease in rice leaves.

To investigate the impact of *S. oryzae* infestation in the chlorophyll *a* fluorescence of rice leaves, we analyzed control and EI leaves using some parameters of the JIP-Test³². To the best of our knowledge, this is the first work that uses this type of photosynthetic analysis in plants infested by phytophagous mite. Three out (PI_{ABS} , Sm and N) of the 16 analyzed parameters were reduced by *S. oryzae* infestation (Figure 4). The photosynthetic performance index in relation to absorption (PI_{ABS}), which measures energy conservation from photons absorbed by PSII antenna, to the reduction of plastoquinone B, is one of the main parameters used to analyze the plant responses to stress conditions⁴³, being used to estimate plant vitality³¹. Zivcak *et al.*⁴⁴ used this parameter as a sensitive indicator of water

stress in *Triticum aestivum*. Similarly, Jafarinia and Shariati⁴⁵ showed that Pi_{ABS} is sensitive enough to salt stress to be used as a parameter to screen the activity of the photosynthetic apparatus in canola plants. In the same work, the N parameter (related to the acceptor side of electrons in PSII, which measures the number of plastoquinone A redox turnovers until the maximal fluorescence intensity is reached) was also reduced with an increase in salt concentration. The third affected parameter (Sm) is also related to the acceptor side of electrons in PSII, or more specifically, to the number of electron carriers per electron transport chain⁴³. Both parameters (Sm and N) were significantly reduced when *Wolffia arrhiza* plants were exposed to high salinity levels⁴⁶. Based on our results, we suggest that the electron transport chain/acceptor side of PSII is affected by the *S. oryzae* infestation. This probably constitutes the first major target of biotic stress in the photosynthetic apparatus of rice plants. These data are also reinforced by the down-regulation of PSII-related proteins^{47,48} (PSII protein H, Plastocyanin, PsbQ and PsbH - Table 1 and Table S-2), indicating that the activity of the donor and acceptor sides of PSII are reduced in EI leaves.

The carbohydrate metabolism and energy production category was also affected by the *S. oryzae* infestation. Some proteins were only detected in the infested condition and others were more expressed in the control condition. UDP-glucose 6-dehydrogenase is involved in diverting UDP-Glc to the cell wall biosynthesis, which is important to maintain the cell integrity under stress conditions. Short exposure to cold⁴⁹ and heat⁵⁰ conditions can increase the UDP-glucose 6-dehydrogenase protein abundance. However, its role in plant tolerance/resistance to stress conditions is unclear. Starch synthesis does not seem to be affected by *S. oryzae* infestation in rice, since we detected two similar starch synthase proteins, each one uniquely detected in control or in EI leaves (Table 1 and Table 2). Citrate synthase, which was only found in EI leaves, is involved in combination of oxaloacetate and acetyl-CoA to produce citrate. Citrate plays an important role in Krebs cycle, -oxidation of fatty acids and photorespiratory glycolate pathway⁵¹. This suggests that EI leaves present high levels of Acetyl-CoA, possibly as a consequence of enhanced -oxidation of fatty acids. Also, it seems that EI leaves with reduced photosynthetic capacity make use of photorespiration process of energy dissipation to avoid photo-inhibition of photosynthetic apparatus. The biological functions of glycosyl hydrolases include degradation of structural polysaccharides, which is important for cell wall remodeling under stress conditions and during plant growth⁵². One β -galactosidase protein (which belong to glycosyl hydrolases family 35) was only detected in EI leaves, and three glycosyl hydrolase family 17 proteins, which hydrolyze 1,3- β -glucan polysaccharides found in the cell wall matrix of plants⁵³, were more expressed in control than EI leaves (Table 1). Glycosyl hydrolase family 17 proteins form a universal mechanism of growth in plants⁵⁴, suggesting that rice leaves infested by *S. oryzae* could present reduced growth rate, if the infestation occurred in an early vegetative stage. Another protein which could also be related to cell wall structure⁵⁵ (NAD dependent epimerase/dehydratase) was only detected in EI leaves. Although a NAD dependent epimerase/dehydratase was also detected as more expressed in control than EI leaves, these data indicate that cell wall remodeling is important for the rice response to mite infestation.

Lactate/malate dehydrogenases play a crucial role in many important metabolic pathways including the Krebs cycle, the glyoxylate bypass, gluconeogenesis, and the facilitation of metabolites exchange between cytoplasm and subcellular organelles⁵⁶. Lower expression of

two lactate/malate dehydrogenases in EI leaves (Table 1), which was also confirmed with our enzymatic assay (Table 3), indicates that several metabolic responses and activities should be negatively affected by *S. oryzae* infestation. Two ATP synthases (gamma and epsilon chain) were more expressed in control leaves. This is also indicative of a decreased capability of carbon assimilation and energy production of EI leaves. A similar response has been observed in rice under heat stress⁵⁷, drought⁵⁸, and brown planthopper infestation⁴⁰.

Oxidative stress and jasmonic acid-related proteins

Rapid production of reactive oxygen species (ROS) is a well-documented early plant response to biotic stress⁴¹. To prevent oxidative damage of ROS to different macromolecules, plants fortify their resistance mechanisms, such as their anti-oxidative system. Three proteins related to oxidative stress were down-regulated in EI leaves: Thioredoxin, Cu/Zn superoxide dismutase (SOD) and 2-Cys peroxiredoxin (Prx) (Table 1). During photosynthesis process, electron transfer to O₂ generates O₂⁻, which is dismutated by SOD to produce H₂O₂. H₂O₂ is decomposed by ascorbate peroxidases (APX) to yield H₂O. In the overall reaction, electrons abstracted from H₂O through the oxygen-evolving complex of photosystem II are transferred to O₂ again and a water-water cycle is established. Frequently, Prx proteins can replace APX to establish an alternative pathway for this water-water cycle. In this way, 2-Cys Prx (also known as chloroplast Prx), has specific roles in photosynthesis process, acting as an alternative water-water cycle for detoxification of photochemically produced H₂O₂⁵⁹. After each catalytic cycle, oxidized Prx needs a reductive regeneration, and thioredoxin and glutathione function as regenerators⁶⁰. Due to the reduced photosynthetic capacity of *S. oryzae* infested plants, a reduction in these three protein abundances is not surprising. It suggests that the chloroplast ROS detoxification network is potentially affected by mite infestation.

According to our results, the expression of classical ROS-related protein such as Catalase, SOD, and APX was not induced by *S. oryzae* infestation, as previously observed by Mahmood *et al.*⁶¹ after methyl jasmonate (MeJA) application on rice leaves. On the other hand, four antioxidant proteins were only detected in EI leaves: Oxidoreductase, Leucoanthocyanidin reductase and two Glutathione S-transferase (GST) (Table 2). Oxidoreductase proteins have been implicated in several abiotic stress responses⁶². It is however the first time that such proteins are detected in response to mite infestation. Leucoanthocyanidin reductase (LAR) is a key enzyme of the proanthocyanidins (PAs) biosynthesis, which plays important roles in the protection plants against herbivores and pathogens⁶³. Transcripts of PA biosynthetic genes rapidly accumulate in response to infection by the *Marssonina brunnea* f.sp. *multigermmtubi* fungus. This results in an accumulation of PA in poplar leaves⁶⁴. Based on the results of Yuan *et al.*⁶⁵, in which *PtLAR3*, a gene encoding leucoanthocyanidin reductase from *Populus trichocarpa* that enhances fungal resistance in transgenic plants, was over-expressed, we can hypothesize that over-expression of the gene encoding LAR could be an effective way to generate *S. oryzae*-resistant rice plants. Enhancement of GST expression and activity has been found to be a marker for stress response in plants. GST activity in EI rice leaves compared to non-infested control leaves is in accordance to our proteomic results (Tables 1 and 2). GSTs play a role reducing oxidative damage and are responsible for scavenging free radicals in the wake of

the oxidative burst produced in response to aphid attack in *Arabidopsis*⁶⁶ and wheat^{41,67}. Recently, Chronopoulou *et al.*⁶⁸ showed that the expression of a GST protein from *Phaseolus vulgaris* is induced upon biotic stress treatment (*Uromyces appendiculatus* infection). The authors suggested that GSTs play a pivotal role in biotic stress response. Also, MeJA application on *Arabidopsis* leaves enhances GST expression⁶⁹.

We detected two JA biosynthesis-related proteins only in EI leaves: Lipoygenase (LOX) (Table 2) and Jacalin-like lectin (Table S-2). LOXs are known to be involved in biotic stress responses in plants. According to Hildebrand *et al.*⁷⁰, the activity of the LOX enzyme increases proportionally to the size of the *Tetranychus urticae* mite population in soybean leaves. Diminished expression of a LOX gene caused reduced JA levels and improved the performance of striped stem borer (*Chilo suppressalis*) larvae in rice plants⁶⁴. Lectins are described as anti-insect proteins commonly induced upon herbivore attacks which negatively affect development or population growth^{3,71}. According to Vandenborre *et al.*⁷², cell-content-feeding spider mites can induce lectin expression in *Nicotiana tabacum*. The over-expression of *Ta-JA1*, a jacalin-like lectin from wheat, confers resistance to *Pseudomonas syringe* infection⁷³. A similar improved resistance against the rice herbivore fall armyworm (*Spodoptera frugiperda*) has been observed in rice plants over-expressing a jacalin-like lectin protein⁷⁴. Even though JA pathway plays a central role in plant defense responses against insects, it is important to highlight that the presence of an active JA-synthesis pathway *per se* is not indicative of a plant resistance to a biotic stress. Tong *et al.*⁷⁵ noted that JA plays a positive role in the rice resistance to the striped stem borer chewing herbivore (*Chilo suppressalis*), but a negative role in the resistance to the phloem feeder brown planthopper (*Nilaparvata lugens*). Also, Zhou *et al.*⁷⁶ showed that silencing a chloroplast-localized LOX gene of rice (*OsHI-LOX*) makes rice more susceptible to the chewing of herbivores, but enhances their resistance to a phloem feeder. The observation that suppression of JA activity results in an increased resistance to an insect indicates that a better understanding of plant defense models in monocotyledons is required to develop novel strategies to protect rice against herbivores.

Protein modification and degradation

Several proteins related to protein modification and degradation were detected only in EI leaves, including proteolysis and ubiquitin-related proteins (Table 2). Corroborating our proteomic data, protease activity was also higher in EI rice leaves compared to control (Table 2). Proteolysis-related proteins has been shown to be induced by MeJA application on rice leaves⁷⁷ and by brown planthopper (*Nilaparvata lugens*) infestation in leaf sheaths⁴⁰. Proteolysis processes may be associated with oxidative stress, and cells exhibit increased rates of proteolysis following exposure to oxidative stress inducing agents⁷⁸. Intracellular proteins damaged by oxidative stress could be selectively recognized and preferentially degraded by intracellular proteolytic enzymes⁷⁹. This suggests that proteolysis-related proteins may have a role in the oxidative burst in rice plants in response to mite infestation. Enhanced accumulation of proteins related to ubiquitin/proteasome degradation pathway also hints to a high protein turnover stimulated by *S. oryzae* stress injuries. It is already known that ubiquitin/proteasome pathways are involved in JA signaling and plant defense mechanisms against biotic stresses, including herbivore attacks⁸⁰. For instance, COP9

signalosome interacts physically with SCF^{COI1} E3 ubiquitin ligase and modulates JA responses⁸¹. Taken together, our data suggest that the *S. oryzae* infestation in rice leaves increases the rate of degradation and recycling of damaged or modified intracellular proteins.

On the other hand, the lower expression of two protease inhibitors in EI than in control leaves (Table 1) shows that the rice defense may not be effective against *S. oryzae*. Indeed, the inhibition of the mite's protease activity is a defense strategy used by plants to impair many physiological activities of mites, such as their nutrition, reproduction, and development³. Such inhibition of the proteolysis content of vegetal cells and tissues, when effective, decreases the accessibility to essential amino acids and causes higher mite mortality⁸².

Protein modifications due to overproduced ROS may occur as a result of direct oxidation of an amino acid side chain leading to formation of carbonyl groups⁸³. Tissues injured by oxidative stress generally contain an increased concentration of carbonylated proteins, which are widely used as markers of protein oxidation⁸⁴. We detected higher levels of protein carbonylation in EI rice leaves than in the control leaves (Figure 5a). Enhanced carbonyl modification of proteins has been previously reported in rice plants under various stresses conditions^{85–87}. However, this is the first time that an increase in protein carbonylation is reported in rice plants under a biotic stress. The increased carbonyl concentration combined with a higher proteolysis suggests that the *S. oryzae* infestation in rice leaves increases the production of ROS, consequently causing enhanced oxidative damage to proteins and creating an increased susceptibility to proteolysis for rice proteins. It has been shown that carbonylated proteins, upon their accumulation in the cells caused by the unfolding of protein target domains, become more susceptible to proteolysis⁸⁸.

Amino acid and lipid metabolism

Proteins related to amino acid synthesis were also only identified in EI leaves (Table 2). These include 3-isopropylmalate dehydratase (3-IPD) that catalyzes the second step of the biosynthesis of leucine and is involved in the methionine chain elongation cycle for glucosinolate formation⁸⁹. Glucosinolate is a toxic metabolite that can reduce plant digestibility. This is a common chemical defense mechanism that was described for a wide range of potential plant consumers^{3,90}. Also, 3-IPD was identified as a JA-responsive gene and it is involved in oxidative response⁹¹ and plant defense⁹². Intriguingly, rice plants do not produce glucosinolates. Therefore, the relation between high expression of 3-IPD and rice defense against mite infestation still need to be elucidated. Several amino acid metabolism-related proteins (including aspartate aminotransferase, which was detected in our proteomic analysis and our enzymatic assays) were identified in the rice-*Cochliobolus miyabeanus* fungal interaction⁴².

As commented above, β -oxidation of fatty acids appears to be enhanced in EI rice leaves. Such hypothesis is reinforced by the identification of two enzymes (acyl-CoA oxidase and 3-ketoacyl-CoA thiolase) related to this process uniquely expressed in EI leaves. β -oxidation process is essential to JA biosynthesis, and is probably required for anti-herbivore resistance in plants⁹³. The lower expression of enoyl-acyl-carrier-protein reductase (related to fatty

acid biosynthesis) in EI leaves compared to control provides another indication that *S. oryzae* infestation promotes reduced fatty acid biosynthesis. We also identified two lipid metabolism-related proteins that were only detected in EI leaves: phosphatidylinositol-specific phospholipase and 3,4-dihydroxy-2-butanone kinase. Phosphatidylinositol-specific phospholipase C is a signaling enzyme that was also detected in our enzymatic assays to be present with higher levels of activity in EI rice (Table 2). This enzyme cleaves phosphatidylinositol (4,5) biphosphate into two initial secondary messengers, myo-inositol-1,4,5-trisphosphate and diacylglycerol (DAG). DAG may be used as a precursor for phosphatidic acid (PA) biosynthesis, an important signaling molecule involved in biotic stress tolerance⁹⁴. Protein 3,4-dihydroxy-2-butanone kinase is involved in flavin biosynthesis. According to Asai *et al.*⁹⁵, reduced levels of flavins compromise NO and ROS production and hypersensitive cell death response. This increases the susceptibility of *Nicotiana benthamiana* plants to oomycete *Phytophthora infestans* and ascomycete *Colletotrichum orbiculare* infections. Hence, *S. oryzae* infestation in rice leaves may cause a cellular reprogramming, which includes amino acid and lipid metabolism changes.

Proteins only detected in *S. oryzae* infested leaves that are associated to other functional annotations

We also identified proteins that were only detected in EI leaves that are associated to several other functional categories (Table 2). These proteins are related to translation, transport, general metabolic processes, stress-response, hormone signaling, and nitrogen metabolism. This hints that mite infestation may induce a metabolic shift in rice leaves. The identification of three translation-related proteins is consistent with previous findings showing that JA increases gene expression at the translational level in Arabidopsis and rice plants^{69,77}. In line with these findings, we detected a higher expression of amino acid synthesis-related genes (Table 2) and a higher abundance of total soluble proteins in rice leaves infested with *S. oryzae*, when compared to control leaves (Figure 5b). This constitutes the likely explanation as to why a higher number of proteins were identified in EI leaves than in the control ones (Figure 2).

Transport-related proteins expressed only in EI leaves suggest that protein partitioning to different organelles is highly active during mite infestation. Hermsmeier *et al.*⁹⁶ also found high expression of an importin protein that mediates the import of cytosolic proteins into the nucleus in leaf cells of *Nicotiana attenuata* interacting with the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae).

High levels of aldehyde dehydrogenase (ALDH) detected in EI leaves are in accordance with previous reports showing that plant ALDHs play crucial roles in stress responses. When overexpressed in Arabidopsis plants, ALDH induces abiotic stress tolerance and protection against lipid peroxidation and oxidative stress⁹⁷. According to Kumari *et al.*⁹⁸, reduction of ROS accumulation also seems to be a mechanism by which cyclophilin proteins (also identified in our experiments, only in EI leaves) alleviate stress conditions. This was demonstrated in *Pseudomonas syringae* infection of tobacco plants⁹⁹.

The high expression of gibberellin (GA) receptor (GID1L2) in EI leaves is not surprising, since recent studies have revealed that an intensive crosstalk between GA and JA signaling is

involved in the plant defense system against biotic stresses¹⁰⁰. RGL3, a repressor of GA-responsive growth, positively regulates the JA-mediated resistance to the necrotroph *Botrytis cinerea* and the susceptibility to the hemibiotroph *Pseudomonas syringae* in Arabidopsis¹⁰¹. As GA antagonizes JA-mediated defense, the high expression of a GA receptor is indicative of a decrease in GA levels and in plant growth. This is due to the fact that JA prioritizes defense mechanisms over growth by interfering with the GA signaling cascade¹⁰². In line with these findings, we detected lower expression of three GA biosynthetic pathway-related genes (*OsGA2ox1*, *OsGA2ox3* and *OsGA20ox1*) in rice leaves infested with *S. oryzae*, when compared to control leaves (Figure 6a–c). The expression of *OsAOS* (*allene oxide synthase*), which catalyzes the committed step in JA biosynthesis, was only detected in rice leaves infested with *S. oryzae* (Figure 6d). Another protein related to hormone signaling that was identified in EI leaves is nitrilase, which is involved in ethylene and defense responses¹⁰³. The higher expression of 1-aminocyclopropane-1-carboxylate (ACC) deaminase (which catalyzes the hydrolytic cleavage of ACC, the immediate precursor of ethylene, and is therefore an inhibitor of ethylene biosynthesis) in EI than in control leaves (Table S-2) is another indication that *S. oryzae* infestation interfere with ethylene biosynthesis. Several transgenic plants have been engineered to express the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase in order to decrease plant ethylene levels and enhance tolerance to biotic and abiotic stresses¹⁰⁴. Probably as a consequence of ACC deaminase activity (which results in low ACC levels for ethylene biosynthesis), we detected higher expression of three ACC oxidase genes (*OsACO3*, 5 and 7) in rice leaves infested with *S. oryzae*, when compared to control leaves (Figure 6e–g).

NifU proteins are highly conserved and serve as the scaffold for assembly of FeS clusters. A NifU-like gene was up-regulated when wild rice (*Oryza minuta*) was exposed to *Magnaporthe grisea* infection¹⁰⁵. Villins are actin modulating proteins¹⁰⁶. Together with the down-regulation of actin proteins in EI leaves, we suggest that *S. oryzae* can cause an actin cytoskeleton remodeling in rice leaf cells. Protein disulfide isomerases (PDIs) are normally referred as molecular chaperones. Previous studies have identified high PDI expression under biotic stresses, such as in the context of *Cochliobolus miyabeanus* fungal infection in rice plants⁴². It is proposed that increased expression of molecular chaperones under stress conditions might provide enhanced plant defense against pathogen attacks⁷⁷.

Validation of proteomic data

To validate the proteomic analysis, we performed enzymatic assays and RT-qPCR analysis. All enzymatic assays results reflected those of our proteomic analysis. The enzymatic activities presented in Table 3 corresponding to glutathione S-transferase, phosphatase, aspartate aminotransferase, protease, and phospholipase, all included proteins that were only detected in EI rice leaves in proteomic results and consequently resulted in higher levels of activity. Of note, LDH activity was found to be higher in non-infested rice leaves than in EI. This result was also reflected in our proteomic analysis. The identification and validation of known enzymes in the context of the interaction of pathogen/parasite with its specific host supports the use of the experimental design presented here and elsewhere¹⁰⁷.

The mRNA expression of seven genes (*Chlorophyll A-B binding protein*, *Photosystem II reaction center protein*, *Cytochrome b6-f complex subunit 4*, *Ubiquitin carboxyl-terminal hydrolase*, *Ubiquitin-conjugating enzyme*, *3-isopropylmalate dehydratase large Aconitase*, and *Glutathione S-transferase C-terminal*, all listed in Table S-1) corresponding to seven rice proteins that were identified as differentially expressed during *S. oryzae* infestation, was further evaluated in control and early-infested (EI) leaves by quantitative RT-PCR (Figure 7). The differences in expression highlighted by the MudPIT methodology were confirmed for the seven tested genes, even though the fold change detected at the mRNA (Figure 7) and protein levels (Table 1 and Table 2) were different, probably due to regulation at the post-transcriptional level. In a wider view, the relation of the proteomic data with mRNA expression, and also enzymatic activity, in the case of glutathione S-transferase, reinforce the involvement of these specific proteins/genes in rice responses to mite infestation.

Conclusions

This is the first report evaluating the rice response to phytophagous mite *Schizotetranychus oryzae* infestation using a high-throughput proteomic approach. Among the 2,464 total proteins detected, we found 872 proteins that were unique of EI leaves, 18 unique of control leaves, and 32 more expressed in control than in EI leaves. Even though we have focused on proteins identified with at least 10 spectral counts, we provide a large and high-quality dataset of differentially expressed proteins in rice leaves following infestation of *S. oryzae* phytophagous mite (Table S-2). A schematic model of the main molecular processes induced or repressed in rice leaves by *S. oryzae* infestation is shown in Figure 8. Altogether, these processes and the identification of differentially expressed proteins are helpful to reveal the molecular mechanisms involved in the response of rice to biotic stress and yield a set of interesting potential targets for future studies aiming to promote mite resistance in rice leaves.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

GO	gene ontology
JA	jasmonic acid
PSII	photosystem II

SA salicylic acid

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Figure 1.

Visual characteristics of leaves from control and early-infested (EI) leaves (a). Detailed view of these leaves under stereomicroscope (b and c). Bars indicate 1 cm in (a), 0.5 cm in (b) and 0.25 cm in (c).

Figure 2. Distribution and overlap of rice proteins identified in the control (non-infested) and mite-infested leaves. The data presented in the Venn diagram was produced with PatternLab's AAPV module, using 0.01 probability. Green circle: control condition; yellow circle: early-infested condition.

Figure 3. Gene Ontology annotation. Biological Processes and Molecular Functions are represented at multilevel (2 and 3) of hierarchical categorization for differentially expressed and unique proteins obtained from control and early-infested rice leaves.

Figure 4.

JIP-test parameters calculated from the chlorophyll a fluorescence transient in control and early-infested (EI) rice leaves: (a) Pi_{ABS} , (b) Sm , and (c) N . Represented values are the averages of ten samples $\pm$ SE. Mean values with one asterisk are different by Student's *t* test (*p*-value < 0.05).

Figure 5. Carbonyl concentration (a) and total soluble protein concentration (b) in control and early-infested (EI) rice leaves. Represented values are the average of ten (a) and three (b) samples $\pm$ SE. Mean values with one asterisk are different by Student's *t* test (*p*-value ≤ 0.05). DW = dry weight.

Figure 6.

Relative expression levels (RT-qPCR, relative to *OsUBQ5* expression) of genes related to the biosynthesis of gibberellin (a) *OsGA2ox1*, (b) *OsGA2ox3*, and (c) *OsGA20ox1*; jasmonic acid (d) *OsAOS*; and ethylene (e) *OsACO3*, (f) *OsACO5*, and (g) *OsACO7*, in control and early-infested (EI) rice leaves. Represented values are the averages of three samples $\pm$ SE. Mean values with one or two asterisks are different by Student's *t* test (*p*-value $\leq$ 0.05 or 0.01, respectively). ND = not detected.

Figure 7.

Relative expression levels (RT-qPCR, relative to *OsUBQ5* expression) of seven selected genes (a) *Chlorophyll A-B binding protein*, (b) *Photosystem II reaction center protein*, (c) *Cytochrome b6-f complex subunit 4*, (d) *Ubiquitin carboxyl-terminal hydrolase*, (e) *Ubiquitin-conjugating enzyme*, (f) *3-isopropylmalate dehydratase large Aconitase*, and (g) *Glutathione S-transferase C-terminal*, for which the encoded proteins were identified by MudPIT as differentially expressed between the control and early-infested (EI) rice leaves. Represented values are the averages of three samples $\pm$ SE. Mean values with one or two asterisks are different by Student's *t* test (*p*-value ≤ 0.05 or 0.01 , respectively). ND = not detected.

Figure 8.

Schematic model of the rice processes stimulated and inhibited (red and green color, respectively) by *S. oryzae* infestation based on the proteomic and physiological results presented in this study. JA: jasmonic acid; PS: photosystem II; ROS: reactive oxygen species. Plant cell model modified from Raven et al. Biology 9th Edition (with kind permission from McGraw-Hill Education).

Table 1

List of unique or differentially expressed proteins identified by MudPIT in rice leaves under control condition, in relation to early-infested condition. Only proteins with at least 10 spectral counts are shown in this table.

PROTEINS UNIQUE OR MORE EXPRESSED IN CONTROL LEAVES					
Functional categories	Description	Locus	E-value	Spectral counts Control/Infested	Fold change
Photosynthesis	Oxygen evolving enhancer protein 3 domain	LOC_Os07g36080	7e-152	795/42	18.9
	Chlorophyll A-B binding	LOC_Os04g38410	4e-180	428/34	12.6
	Chlorophyll A-B binding	LOC_Os11g13890	0	502/42	12.0
	Chlorophyll A-B binding	LOC_Os07g37550	0	557/48	11.6
	Chlorophyll A-B binding	LOC_Os06g21590	9e-180	259/23	11.3
	Ribulose biphosphate carboxylase large subunit	LOC_Os10g21268	8e-137	6018/549	11.0
	<u>Chlorophyll A-B binding</u>	LOC_Os09g17740	0	703/68	10.3
	Cytochrome b6-f complex iron-sulfur	LOC_Os07g37030	2e-166	443/45	9.8
	Chlorophyll A-B binding	LOC_Os01g41710	0	551/57	9.7
	Chlorophyll A-B binding	LOC_Os01g52240	0	351/39	9.0
	Ribulose biphosphate carboxylase large subunit	LOC_Os01g58020	4e-104	1560/176	8.9
	Chlorophyll A-B binding	LOC_Os07g38960	0	257/34	7.6
	Ribulose biphosphate carboxylase large subunit	LOC_Os10g21268	2e-125	4332/573	7.6
	Plastocyanin, chloroplast precursor	LOC_Os06g01210	2e-107	1038/140	7.4
	Chlorophyll A-B binding	LOC_Os08g33820	0	192/33	5.8
Carbohydrate metabolism and energy production	<u>Photosystem II reaction center protein</u>	LOC_Os08g15296	3e-50	37	-
	<u>Cytochrome b6-f complex subunit 4</u>	LOC_Os10g21326	8e-95	29	-
	Lactate/malate dehydrogenase	LOC_Os01g61380	0	96/12	7.5
	Lactate/malate dehydrogenase	LOC_Os03g56280	0	533/71	7.5
	Glycosyl hydrolases family 17	LOC_Os01g71820	0	58/9	6.4
	Glycosyl hydrolases family 17	LOC_Os01g71830	0	44/4	6.4
	ATP synthase gamma chain	LOC_Os07g32880	0	537/96	5.6
	NAD dependent epimerase/dehydratase	LOC_Os05g01970	0	106/23	4.6
	ATP synthase epsilon chain	LOC_Os01g58000	2e-94	173/42	4.1
	Glycosyl hydrolases family 17	LOC_Os01g71860	0	10	-

PROTEINS UNIQUE OR MORE EXPRESSED IN CONTROL LEAVES					
Functional categories	Description	Locus	E-value	Spectral counts Control/Infested	Fold change
	Starch synthase	LOC_Os06g04200	0	19	-
Oxidative stress-related	Thioredoxin	LOC_Os12g08730	1e-124	214/23	9.3
	Cu/Zn superoxide dismutase	LOC_Os03g22810	7e-105	58/8	7.3
	2-Cys peroxiredoxin BAS1, chloroplast	LOC_Os04g33970	2e-52	135/23	5.9
Protease inhibitors	LTPL113 - Protease inhibitor/seed storage/LTP	LOC_Os02g44320	4e-86	289/19	15.2
	LTPL122 - Protease inhibitor/seed storage/LTP	LOC_Os04g46830	7e-91	141/11	12.8
Lipid metabolism	Enoyl-acyl-carrier-protein reductase	LOC_Os08g23810	0	65/17	3.8
Others	Actin	LOC_Os12g44350	0	94/8	11.8
	Cupin	LOC_Os08g35760	7e-152	318/53	6.0
	DEAD-box ATP-dependent RNA helicase	LOC_Os06g48750	0	123/38	4.4
	Ubiquitin	LOC_Os05g38310	2e-57	24	-
	Expressed protein	LOC_Os05g48630	2e-97	289/17	17.0

Obs. 1: "E-value" is related to the protein identification by sequence similarity; "... signal means "uniquely found in control condition"; "Spectral counts" represent the sum from three replicates.

Obs. 2: The entire list of unique proteins obtained from the MudPIT experiments in control leaves is shown in Table S-2. Bold and underlined sequences were confirmed by RT-qPCR.

Table 2

List of unique proteins identified by MudPIT in rice leaves under early-infested condition. Only proteins with at least 10 spectral counts are shown in this table.

PROTEINS UNIQUE IN EARLY-INFESTED LEAVES				
Functional categories	Description	Locus	E-value	Spectral counts
Protein modification and degradation	Aspartyl aminopeptidase	LOC_Os12g13390	0	19
	OsPOP12 - Putative Prolyl Oligopeptidase	LOC_Os06g11180	0	18
	Puromycin-sensitive aminopeptidase	LOC_Os08g30810	0	16
	Zinc peptidase	LOC_Os02g46520	0	15
	Ubiquitin carboxyl-terminal hydrolase	LOC_Os01g08200	0	14
	Aspartic proteinase oryzain-1 precursor	LOC_Os05g49200	0	13
	Peptidase, M24	LOC_Os05g28280	0	12
	Ubiquitin-conjugating enzyme	LOC_Os09g12570	1e-109	12
	COP9 signalosome complex subunit 5b	LOC_Os04g56070	0	11
	Proteasome/cyclosome repeat containing	LOC_Os04g51910	0	10
Amino acid metabolism	3-isopropylmalate dehydratase large Aconitase	LOC_Os02g03260	0	15
	Aminotransferase, classes I and II	LOC_Os01g08270	0	14
	Cysteine desulfurase 1, mitochondrial	LOC_Os09g16910	0	13
	D-aminoacylases	LOC_Os05g08930	0	13
	Peptidyl-prolyl isomerase	LOC_Os08g41390	0	11
	Argininosuccinate lyase	LOC_Os03g19280	0	10
	Aspartate aminotransferase	LOC_Os09g28050	0	10
	Amine oxidase, flavin-containing	LOC_Os03g08570	0	10
Carbohydrate metabolism and energy production	UDP-glucose 6-dehydrogenase	LOC_Os03g55070	0	12
	UDP-glucose 6-dehydrogenase	LOC_Os12g25690	0	12
	Soluble starch synthase 3, chloroplast	LOC_Os04g53310	0	11
	Beta-galactosidase	LOC_Os01g72340	0	10
	Citrate synthase	LOC_Os02g13840	0	10
	NAD dependent epimerase/dehydratase	LOC_Os05g32140	0	10
	Sucrase-related	LOC_Os02g49320	0	10
Lipid metabolism	Acyl-coenzyme A oxidase 1.2, peroxisomal	LOC_Os06g01390	0	14
	2-oxo acid dehydrogenases acyltransferase	LOC_Os12g08170	0	13
	3-ketoacyl-CoA thiolase, peroxisomal	LOC_Os10g31950	0	11
	3,4-dihydroxy-2-butanone kinase	LOC_Os03g51000	0	11
	Phosphatidylinositol-specific phospholipase	LOC_Os09g36520	0	11
Oxidative stress-related	Oxidoreductase, aldo/keto reductase	LOC_Os07g04990	0	21
	Glutathione S-transferase, C-terminal	LOC_Os08g43680	0	18
	Leucoanthocyanidin reductase	LOC_Os04g53810	0	17
	Glutathione S-transferase	LOC_Os10g38780	2e-168	11

PROTEINS UNIQUE IN EARLY-INFESTED LEAVES				
Functional categories	Description	Locus	E-value	Spectral counts
Translation-related	Tyrosyl-tRNA synthetase	LOC_Os01g31610	0	20
	Methionyl-tRNA synthetase	LOC_Os06g31210	0	18
	Cysteiny-tRNA synthetase	LOC_Os09g38420	0	10
Transport-related	Mitochondrial import receptor subunit	LOC_Os01g69250	2e-151	12
	Importin subunit beta	LOC_Os12g38110	0	10
	Cysteine-rich repeat secretory	LOC_Os05g02200	0	10
Jasmonic acid biosynthesis	Lipoxygenase 2.1, chloroplast precursor	LOC_Os12g37260	0	39
	Lipoxygenase	LOC_Os12g37350	0	14
General metabolic processes	Aldehyde dehydrogenase	LOC_Os02g07760	1e-130	14
	Aldehyde dehydrogenase	LOC_Os01g40860	0	11
	Ser/Thr protein phosphatase	LOC_Os01g49690	0	11
	Ser/Thr protein phosphatase	LOC_Os02g57450	0	10
Stress response	Cyclophilin	LOC_Os09g39780	1e-131	13
	WD domain, G-beta repeat domain containing	LOC_Os06g19660	0	10
Purine and pirimydin metabolism	5-formyltetrahydrofolate cyclo-ligase	LOC_Os07g39070	0	12
	Adenylosuccinate synthetase, chloroplast	LOC_Os03g49220	0	11
RNA related-stress	TUDOR protein with multiple Snc domains	LOC_Os02g32350	0	19
Detoxification	Metallo-beta-lactamase	LOC_Os01g47690	0	15
Hormone signaling	Gibberellin receptor GID1L2	LOC_Os07g06830	0	15
	Nitrilase	LOC_Os02g42330	0	14
Chitin-related	Pro-resilin	LOC_Os06g12580	0	12
Nitrogen metabolism	NifU	LOC_Os05g06330	0	12
Calcium signaling	Peflin	LOC_Os11g04480	0	10
Signal transduction	Ras-related protein	LOC_Os06g35814	4e-159	10
Others	Carboxyvinyl-carboxyphosphonate phosphorylmutase	LOC_Os12g08760	0	15
	Transposon protein, putative, CACTA	LOC_Os11g06570	1e-103	14
	Haloacid dehalogenase-like hydrolase	LOC_Os10g32730	0	13
	Villin	LOC_Os03g24220	0	12
	O-methyltransferase	LOC_Os08g06100	0	11
	Lissencephaly type-1-like homology motif	LOC_Os03g14980	0	11
	HEAT	LOC_Os07g38760	0	11
	MA3 domain-containing protein	LOC_Os08g02690	0	11
	Protein disulfide isomerase (PDI)	LOC_Os03g29190	0	11
	DnaK	LOC_Os02g48110	0	10

PROTEINS UNIQUE IN EARLY-INFESTED LEAVES				
Functional categories	Description	Locus	E-value	Spectral counts
	CGMC_MAPKCMGC_2_ERK.12	LOC_Os06g06090	0	10
Unknown	expressed protein	LOC_Os03g58372	5e-08	15
	expressed protein	LOC_Os01g70400	9e-126	12
	expressed protein	LOC_Os11g21990	0	11

Obs. 1: "E-value" is related to the protein identification by sequence similarity; "Spectral counts" represent the sum from three replicates.

Obs. 2: The entire list of unique proteins obtained from the MudPIT experiments in early-infested leaves is shown in Table S-2. Bold and underlined sequences were confirmed by RT-qPCR.

Table 3

Validation of proteomic results using enzymatic assays.

Enzyme	Sample		Specific activity ^a
	Control	Early infested	
Glutathione S-transferase	0.2 ± 0.1	0.4 ± 0.05 **	μmol/mL/min/mg
Phosphatase	710.4 ± 6.99	1098.5 ± 6.59 ***	mOD/min/mg
Lactate dehydrogenase	325.6 ± 0.02	288.7 ± 0.005 *	mOD/min/mg
Aspartate aminotransferase	13.1 ± 3.7	537.3 ± 13.88 ***	U/mL/mg
Protease	1.8 ± 0.07	2.8 ± 0.04 *	pmol/mL/min/mg
Phospholipase	26.7 ± 0.7	33.5 ± 0.4 ***	μmol/mL/min/mg

^aUnits as described in methods section for each specific assay.*
p-value 0.05**
p-value 0.01***
p-value 0.001