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Research Paper

High infestation levels of *Schizotetranychus oryzae* severely affects rice metabolism

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ABSTRACT

High levels of *Schizotetranychus oryzae* phytophagous mite infestation on rice leaves can severely affect productivity. Physiological characterization showed that *S. oryzae* promotes a decrease in chlorophyll concentration and the establishment of a senescence process in rice leaves. Late-infested leaves also present high levels of superoxide radical and hydrogen peroxide accumulation, along with high levels of membrane integrity loss, which is indicative of cell death. To better understand the rice molecular responses to high levels of mite infestation, we employed the Multidimensional Protein Identification Technology (MudPIT) approach to identify differentially expressed proteins. We identified 83 and 88 proteins uniquely present in control and late-infested leaves, respectively, along with 11 and one proteins more abundant in control and late-infested leaves, respectively. *S. oryzae* infestation induces a decreased abundance of proteins related to translation, protease inhibition, and photosynthesis. On the other hand, infestation caused increased abundance of proteins involved in protein modification and degradation. Our results also suggest that *S. oryzae* infestation interferes with intracellular transport, DNA structure maintenance, and amino acid and lipid metabolism in rice leaves. Proteomic data were positively correlated with enzymatic assays and RT-qPCR analysis. Our findings describe the protein expression patterns of late-infested rice leaves and suggest several targets which could be tested in future biotechnological approaches aiming to avoid the population increase of phytophagous mite in rice plants.

1. Introduction

Rice is the staple food for over half of the world's population. Approximately 715 million metric tons of paddy rice, which generates about 480 million metric tons of milled rice, are produced annually in over a hundred countries (Muthayya et al., 2014). Rice is the primary source of calorie intake for an estimated 3.5 billion people worldwide. This is especially the case in the developing world, where it accounts for approximately 50% of the dietary caloric supply and a substantial part of the protein intake, being therefore critical for food security (Muthayya et al., 2014). For this reason, rice production faces the challenge to be enhanced by 50% by year 2030 to meet the growth of the population in rice-eating countries (Ahmadi et al., 2014). This is definitely not an easy task, since the oscillations observed in annual production of this culture derive mainly from abiotic and biotic stresses, which limit plant germination, development, and productivity (Lim et al., 2013; Dametto et al., 2015). The estimates for the potential losses caused by animal pests are around 25% worldwide (Oerke, 2006). One of the main reasons for losses in rice productivity is phytophagous mite infestation (Blasi et al., 2015; Buffon et al., 2016) that can damage rice plants during its entire development, depending on the mite species and infestation level.

Abbreviations: GO, gene ontology; PSII, photosystem II

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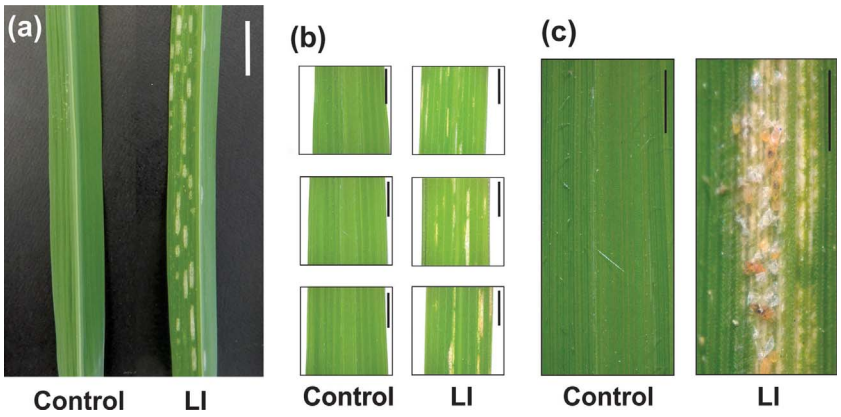

Fig. 1. Visual characteristics of leaves from control and late-infested (LI) leaves (a). Detailed view of these leaves under stereomicroscope (b and c). Bars indicate 1 cm in (a), 0.5 cm in (b) and 0.25 cm in (c).

Phytophagous mites of the Tarsonemidae (*Steneotarsonemus furcatus* De Leon and *Steneotarsonemus spinki* Smiley) and Tetranychidae (*Schizotetranychus oryzae* Rossi de Simons and *Oligonychus oryzae* Hirst) families are frequently found infesting rice culture as reviewed by Blasi et al. (2015). The spider mite *Schizotetranychus oryzae* (Acari: Tetranychidae) was first found in Argentina, and has been reported in rice plantations in Brazil and other South American countries (Ferla et al., 2013). Rice leaves infested with *S. oryzae* may present yellowish white elongated areas on the upper side of the leaves that corresponds to mite colonies in different stages of development. These colonies are located on the lower surface of the leaves (Buffon et al., 2016). In order to defend themselves from herbivore attacks, plants employ a wide range of induced defenses, including mostly jasmonic acid- and salicylic acid-dependent (Agut et al., 2015). These defenses result in morphological changes and synthesis of secondary metabolites that cause a decrease in herbivore performance (Alba et al., 2011; de Oliveira et al., 2016). The induction of these plant defenses depends on the ability of the plant to identify and recognize its attackers, and varies with the herbivore species and infestation level or the amount of time since the attack (Kant et al., 2004; Wu and Baldwin, 2009; de Oliveira et al., 2016). For example, in early attack stage plant responses are regulated through phytohormones, whereas late stage responses typically involve the synthesis of phytoalexins and secondary defensive metabolites (Agut et al., 2015). Rice responses to early *S. oryzae* infestation include down-regulation of photosynthetic performance, carbon assimilation and energy production, fatty acid biosynthesis and actin cytoskeleton remodeling. On the other hand, gene expression at the translational level, protein partitioning to different organelles, protein modification and degradation, β-oxidation of fatty acids (essential to jasmonic acid biosynthesis) and cell wall remodeling are up-regulated in response to early infestation (Buffon et al., 2016). It is important to highlight that a small/limited number of mites on rice plants can induce all of these molecular effects. In late stages, where there is a substantial increase in herbivore activity promoted by a larger number of mites infesting rice plants, the responses are still completely unknown.

Even though mite infestations in rice plantations can cause drastic yield reductions of up to 90% (Hummel et al., 2009), most of the plant-mite interaction studies have been limited to the visual effects of the infestation (population growth rate and injury symptoms) or physiological changes (Evaristo et al., 2013; Jaimez-Ruiz et al., 2015). Thus, the molecular mechanisms specifically elicited by mite infestation in rice plants remain virtually unexplored. In recent years, rice proteomics has achieved tremendous progress in development of highly efficient sample preparation methods and physiological and biochemical pathway data analysis under different developmental stages, in various tissues, organs, and organelles, and against biotic and abiotic stresses (Kim et al., 2014). Previously, we detected differentially expressed proteins in rice leaves after *S. oryzae* phytophagous mite early infestation (Buffon et al., 2016). The understanding of rice response to an intense parasitic activity promoted by *S. oryzae* in late infestation on rice leaves is important since the main damages directly affecting rice productivity occurs in this stage. In this work, we focused on the physiological and molecular alterations in rice leaves exposed to high levels of *S. oryzae* infestation, hypothesizing that we could use proteomic approach to identify potential targets to avoid the population increase of phytophagous mite in rice plants. Our findings will be helpful for future biotechnological and molecular breeding efforts that aim to promote mite resistance in rice leaves.

2. Materials and methods

2.1. Plant growth and mite infestation conditions

Plant growth and mite infestation conditions were performed according to Buffon et al. (2016). In all the experiments, we analyzed healthy (control) and late-infested leaves (LI, 60 days after infestation, containing about 177.8 ± 22.4 mites per leaf) (Fig. 1). It is important to highlight that IRGA 424 cultivar is not affected by the means of plant height and tiller number, but some leaves present high infestation levels after only 30 days, and the grain yield is reduced significantly (data not shown).

2.2. Total chlorophyll concentration

Samples containing 100 mg of leaves from rice plants submitted to control or infested conditions were ground in liquid nitrogen and chlorophyll extracted in 85% (v/v) acetone. Chlorophyll *a* and *b* were quantified by measuring absorbance at 663 and 645 nm and the concentrations calculated according to Ross (1974).

2.3. In situ histochemical localization of O_2^- and H_2O_2

In situ accumulation of O_2^- and H_2O_2 was detected by histochemical staining with nitro blue tetrazolium (NBT) and diaminobenzidine (DAB), according to Shi et al. (2010) with minor modifications. For O_2^- detection, leaves of control and late-infested plants were excised and immersed in a 1 mg ml^{-1} solution of NBT in 10 mM phosphate buffer (pH 7.8) at room temperature. Immersed leaves were illuminated for 2 h until appearance of dark spots, characteristic of blue formazan precipitates. For localization of H_2O_2, another set of leaves was sampled and immersed in DAB solution (1 mg ml^{-1}, pH 3.8) in 10 mM phosphate buffer (pH 7.8), and incubated at room temperature for 8 h in the light until brown spots were visible, which are derived from the reaction of DAB with H_2O_2. For both staining methods, leaves were bleached in boiling concentrated ethanol to visualize the blue and brown spots, which were kept in 70% ethanol for taking pictures with a digital camera coupled to a stereomicroscope.

2.4. Detection of cell death by loss of plasma membrane integrity

To determine changes in cell viability by *S. oryzae* late infestation, excised leaves were immersed for 5 h in a 0.25% (w/v) aqueous solution of Evans Blue (Romero-Puertas et al., 2004). Leaves were discolored in boiling concentrated ethanol to develop the blue precipitates, which correspond to the localization of dead cells, followed by photo documentation with a digital camera coupled to a stereomicroscope.

2.5. Protein extraction and Rubisco depletion

Protein extraction and determination of protein concentration were performed according to Buffon et al. (2016), using Plant Total Protein Extraction Kit (Sigma-Aldrich) and BCA assay, respectively. Krishnan and Natarajan (2009) method was used for depletion of Rubisco proteins.

2.6. Sample preparation for mass spectrometry

Sample preparation for mass spectrometry was performed according to Buffon et al. (2016), using approximately 100 µg of Rubisco depleted protein extracts as starting material.

2.7. MudPIT and mass spectrometry conditions

MudPIT and mass spectrometry conditions were the same used by Buffon et al. (2016). Three biological replicates were analyzed for both control and late-infested samples using an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA).

2.8. Data analysis

Data analysis was performed according to Buffon et al. (2016). In brief, PatternLab software (Carvalho et al., 2012) was used to identify unique and differentially expressed proteins (through the analysis of spectral counts) in both tested conditions. To be considered differentially expressed, proteins should have q-value < 0.05 (5% FDR) and fold change > 2.0. Approximate Area Proportional Venn diagram (A-APVD) module of PatternLab was used to highlight the number of unique proteins in each sample. We also analyzed the proteins using Blast2GO tool (http://www.blast2go.org – Conesa et al., 2005) and B2G Kegg maps.

2.9. Validation of proteomic data through enzymatic assays

Glutathione S-trasferase (GST) activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma, USA) as substrate. Phosphatase activity was measured by the rate of ρ-nitrophenol (ρ-NP) production. Lactate dehydrogenase activity (LDH) was measured based on its ability to reduce pyruvate using NADH as a cofactor, generating lactate and NAD^+. Aminotransferase activity was measured based on the transfer of amino groups of aspartate to α-ketoglutarate, leading to formation of glutamate and oxaloacetate. Protease activity was measured based on the production of ρ-nitroaniline. Reaction conditions were the same as used previously (Buffon et al., 2016).

The peroxidase activity was determined by measuring the absorbance at 470 nm of tetraguaiacol produced by the reaction between 0.05 M guaiacol solution and 10.3 mM hydrogen peroxide solution in 0.1 M phosphate buffer pH 7.0 at 25 °C. The increase in absorbance at 470 nm was monitored for 5 min. One activity unit was defined as the amount of enzyme that causes an increase of 0.001 in absorbance per hour.

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

2.10. 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 µg of total RNA.

2.11. Quantitative RT-PCR and data analysis

RT-qPCRs were carried out in a StepOne Real-Time Cycler (Applied Biosystems). All primers (listed in Supplementary Table 1) were designed to amplify 100–150 bp of the 3′-UTR of the genes and to have similar Tm values (60 °C ± 2). RT-qPCR reactions and gene expression evaluations were performed according to Buffon et al. (2016). *OsUBQ5* gene expression was used as an internal control to normalize the relative expression of the tested genes (Jain et al., 2006). Each data point corresponds to three biological and four technical replicate samples.

The expression of a senescence marker gene (*Staygreen* gene, *OsSGR*, a chloroplast protein which regulates chlorophyll degradation by inducing LHCII disassembly through direct interaction; Park et al., 2007) was also analyzed in control and LI leaves.

2.12. Statistical analysis

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

3. Results and discussion

3.1. Physiological characterization of late-infested rice leaves

Rice leaves highly infested with *Schizotetranychus oryzae* showed a decrease in total chlorophyll concentration (Fig. 2a), which is indicative of a well-established senescence process. Such hypothesis was confirmed by the higher expression of the senescence marker *OsSGR* gene (Park et al., 2007) in late-infested leaves when compared to control (Fig. 2b). It is important to highlight that *OsSGR* expression on early-infested leaves was at the same level of control leaves (data not shown), suggesting that a high *S. oryzae* population is needed to trigger the endogenously degenerative senescence process that ultimately leads to plant death (Yoshida, 2003). A reduction in total chlorophyll

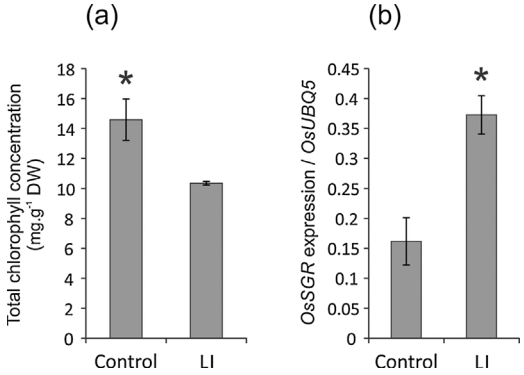

Fig. 2. Total chlorophyll concentration (a) and *OsSGR* expression (b) in rice leaves of control and late-infested (LI) plants. Averages of three samples ± SE are presented. Mean values with one asterisk are significantly different as determined by a Student's t test (p-value ≤ 0.05). DW = dry weight.

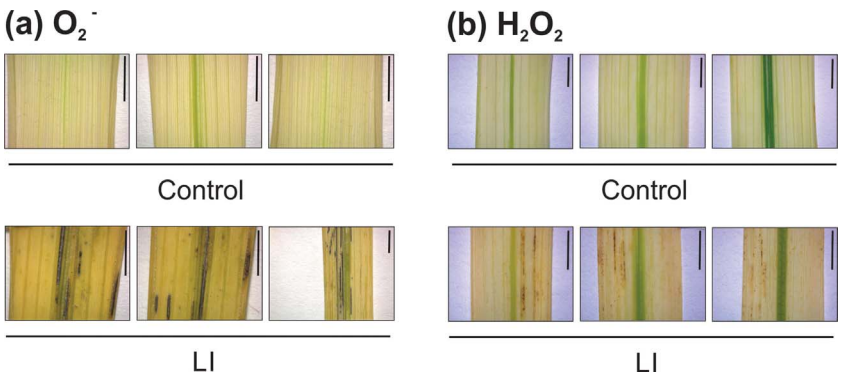

Fig. 3. Histochemical staining assay of O_2^- (a) and H_2O_2 (b) by nitro blue tetrazolium (NBT) and diaminobenzidine (DAB), respectively, in rice leaves of control and late-infested (LI) plants. The positive staining (detected in higher levels on infested leaves) in the photomicrographs shows as bright images (blue-color for NBT and brown-color for DAB). Bars in figures indicate 0.5 cm (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

concentration was reported in cucumber and rose after *Tetranychus urticae* mite infestation (Park and Lee, 2002; Landeros et al., 2004). In addition, the establishment of a senescence process in tomato plants was detected after *T. urticae* infestation (Alba et al., 2015). However, this is the first time that such events are reported after *S. oryzae* infestation in rice plants.

An imbalance between production of ROS and sufficient enzymatic and non-enzymatic protection has been implicated in senescence processes (Afify et al., 2011). To check if the senescence process detected in late-infested leaves is followed by an accumulation of superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2), we used NBT and DAB for *in situ* histochemical localization. As seen in Fig. 3a and b, high levels of both ROS can be detected only on LI rice leaves. The accumulation is exactly on the infestation site, and is not spreading over the entire leaf blade. Although bean and Arabidopsis plants infested by *T. urticae* also accumulate H_2O_2 in their leaf blades (Farouk and Osman, 2012; Santamaria et al., 2012), this is the first report of ROS accumulation in rice leaves after *S. oryzae* infestation. The high accumulation of ROS on the infestation site can indicate that defense strategies in rice are not effective against *S. oryzae* infestation, since this broad range of oxidative responses may initiate destructive oxidative processes, such as chlorophyll bleaching, lipid peroxidation, protein oxidation, and damage to nucleic acids, ultimately leading to cell death (Farouk and Osman, 2012). As seen in Fig. 4, Evans Blue staining indicated for the first time that *S. oryzae* late-infested rice leaves exhibit high levels of plasma membrane integrity loss, indicating cell death promoted by high levels of mite infestation.

3.2. Overview of the proteomic analysis results

Using MudPIT, a total of 2731 different proteins were identified in both conditions (Supplementary Table 2). After statistical analysis (PatternLab program), only 183 proteins were considered significantly differentially expressed, being 95 in control leaves and 100 in late-infested (LI) leaves (Fig. 5). Only 12 of these proteins (6.5%) were detected in both conditions (11 were considered significantly over-expressed in control leaves and one in LI), while 83 (45.3%) were uniquely identified in control and 88 (48.1%) in LI samples. The lists of these proteins, unique or significantly differentially expressed, are presented in Tables 1 and 2.

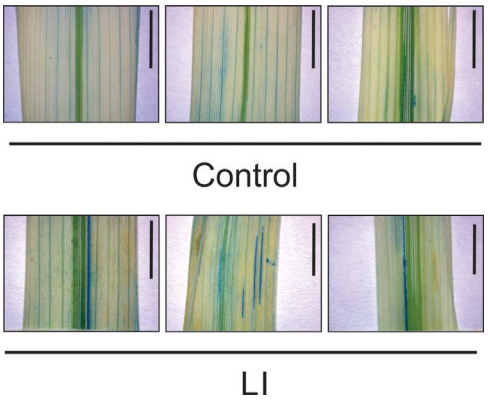

Fig. 4. Loss of plasma membrane integrity in rice leaves of control and late-infested (LI) plants, detected by histochemical staining using Evans Blue reagent. The positive staining (detected in higher levels on infested leaves) in the photomicrographs is shown as bright images (blue-color). Bars in figures indicate 0.5 cm (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

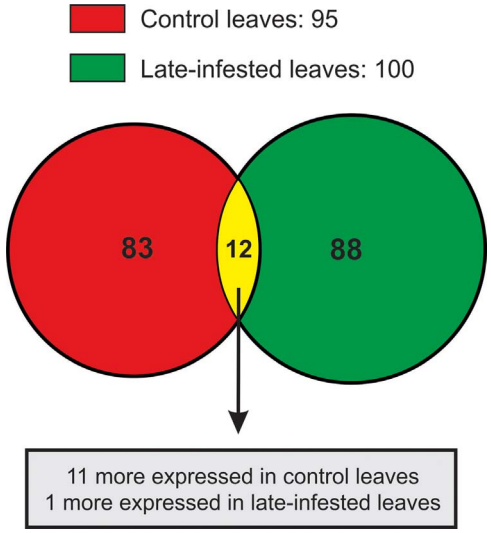

Fig. 5. Overlap of significantly differentially expressed rice proteins identified in the control (non-infested) and late-infested leaves. The data presented in the Venn diagram was produced with PatternLab's AAPV module, using a 0.01 probability threshold. Red circle: unique in control condition; green circle: unique in late-infested condition; yellow intersection: found in both conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The corresponding sequence of each identified protein was submitted to NCBI blastp in order to identify and annotate the protein. Specific domains and molecular functions were manually checked. Afterwards, proteins were arbitrarily classified in functional categories, according to its putative molecular function. Proteins exclusively found or over-expressed in control leaves include the following major functional categories: translation, carbohydrate metabolism and energy production, protease inhibitors, photosynthesis, stress response, general metabolic processes, oxidative stress, transport, amino acid metabolism, protein modification or degradation, and DNA structure maintenance (Table 1). Proteins exclusively found or over-expressed in late-infested leaves include the following major functional categories: general metabolic processes, protein modification or degradation, carbohydrate metabolism and energy production, translation, amino acid metabolism, oxidative stress, DNA structure maintenance, transport, hormone signaling, lipid metabolism, photosynthesis, and protease

Table 1
List of unique or differentially expressed proteins identified by MudPIT in rice leaves under control condition, in relation to late-infested condition.

Proteins EXCLUSIVELY FOUND or more expressed in Control leaves					
Functional categories	Description	Locus	E-value	Spectral counts Control/ Infested	Fold change
Translation-related	ribosome recycling factor	LOC_Os07g38300	0	131/12	10.92
	DEAD-box ATP-dependent RNA helicase	LOC_Os06g48750	0	123/36	3.42
	tRNA synthetase class II domain containing	**LOC_Os02g46130**	**0**	**12**	–
	60S ribosomal protein L18a	LOC_Os05g49030	1e–132	11	–
	elongation factor	LOC_Os01g52470	0	11	–
	eukaryotic translation initiation factor	LOC_Os07g02210	2e–118	11	–
	eukaryotic translation initiation factor	LOC_Os05g49150	0	8	–
	60S ribosomal protein L15	LOC_Os05g19370	4e–147	7	–
	initiation factor 2 subunit family domain	LOC_Os11g11050	0	5	–
	50S ribosomal protein L15, chloroplast	LOC_Os03g12020	0	3	–
	60S ribosomal protein L27a-3	LOC_Os02g07890	9e–101	3	–
	L1P family of ribosomal proteins domain	LOC_Os02g21660	6e–159	3	–
	40S ribosomal protein S4	LOC_Os01g25610	0	2	–
	elongation factor Tu	LOC_Os02g25870	0	2	–
	DNA directed RNA polymerase, 7 kDa subunit	LOC_Os01g34614	3e–32	2	–
	ribosomal protein L37	LOC_Os02g02130	4e–63	2	–
	tRNA synthetases class II domain containing	LOC_Os07g30200	0	2	–
Carbohydrate metabolism and energy production	**phosphoglycerate kinase**	**LOC_Os06g45710**	**0**	**113/13**	**8.69**
	ATP synthase gamma chain	**LOC_Os07g32880**	**0**	**537/120**	**4.48**
	ATP synthase	LOC_Os06g45120	0	112/50	2.24
	NAD dependent epimerase/dehydratase	LOC_Os07g40974	0	12	–
	NAD dependent epimerase/dehydratase	LOC_Os03g16980	0	11	–
	glycosyl hydrolases family 17	LOC_Os01g71860	0	10	–
	sucrose-phosphatase	LOC_Os02g05030	0	10	–
	FGGY family of carbohydrate kinases	LOC_Os07g44660	0	5	–
	UDP-glucoronosyl and UDP-glucosyl transferase	LOC_Os07g32010	0	5	–
	electron transfer flavoprotein subunit	LOC_Os04g10400	0	3	–
Protease inhibitors	LTPL122 – Protease inhibitor/seed storage	LOC_Os04g46830	7e–91	141/6	23.50
	LTPL113 – Protease inhibitor/seed storage	LOC_Os02g44320	4e–86	289/20	14.45
	LTPL11 – Protease inhibitor/seed storage	**LOC_Os12g02310**	**1e–70**	**29**	**-**
	cysteine proteinase inhibitor precursor	LOC_Os05g41460	2e–75	10	–
	LTPL152 – Protease inhibitor/seed storage	LOC_Os05g47700	1e–59	8	–
	LTPL17 – Protease inhibitor/seed storage	LOC_Os05g40010	6e–83	8	–
	LTPL160 – Protease inhibitor/seed storage	LOC_Os10g36170	7e–58	6	–
	serpin domain containing protein	LOC_Os03g41419	2e–168	2	–
Photosynthesis	pre-protein translocase subunit secA	LOC_Os01g21820	4e–33	7	–
	cytochrome c	LOC_Os05g34770	7e–80	3	–
	divinyl reductase (DVR)	LOC_Os03g22780	0	3	–
	apocytochrome f precursor	**LOC_Os08g15280**	**9e–71**	**102/4**	**25.50**
	photosystem II 5 kDa protein, chloroplast	**LOC_Os02g37060**	**6e–77**	**432/135**	**12.34**
	photosystem I iron-sulfur center	LOC_Os10g21406	1e–54	439/37	11.86
	photosystem II D2	LOC_Os10g41689	0	98/26	3.77
Stress response	peptidyl-prolyl *cis*-trans isomerase	LOC_Os07g09040	9e–123	11	–
	RALFL23 – Rapid Alkalinization Factor	LOC_Os11g26190	2e–71	8	–
	polyprenyl synthetase	LOC_Os01g50760	0	5	–
	stress responsive protein	LOC_Os03g21040	0	5	–
	WD domain, G-beta repeat domain containing	LOC_Os05g47890	0	4	–
	ranBP1	LOC_Os03g18180	4e–148	2	–
General metabolic processes	**pyruvate kinase**	**LOC_Os12g05110**	**0**	**38**	–
	aldehyde dehydrogenase	LOC_Os02g49720	0	9	–
	dehydrogenase	LOC_Os08g01760	0	8	–
	dehydrogenase	LOC_Os03g01190	0	6	–
	aldehyde dehydrogenase	LOC_Os11g08300	0	2	–
	iron-sulfur cluster assembly enzyme	LOC_Os01g47340	2e–122	2	–
Oxidative stress-related	ferredoxin-thioredoxin reductase	LOC_Os02g42570	2e–104	33	–
	oxidoreductase, aldo/keto reductase	LOC_Os10g02480	0	6	–
	thioredoxin	LOC_Os01g07376	2e–94	6	–
	thioredoxin	LOC_Os05g07690	4e–95	5	–
	thioredoxin	LOC_Os04g53740	2e–93	3	–
Transport-related	transporter-related	LOC_Os02g49260	1e–32	7	–
	mitochondrial import inner membrane	LOC_Os03g61019	4e–57	4	–
	vesicle transport protein GOT1B	LOC_Os07g40320	9e–39	4	–
	mitochondrial import inner membrane	LOC_Os03g19290	5e–126	2	–
Amino acid metabolism	2-isopropylmalate synthase B	LOC_Os11g04670	1e–44	9	–
	fumarylacetoacetase	LOC_Os03g61330	3e–163	5	–
	methionine sulfoxide reductase	LOC_Os03g24600	4e–98	2	–
	heat shock protein	LOC_Os08g39140	9e–118	8	–

(*continued on next page*)

Table 1 (*continued*)

Proteins EXCLUSIVELY FOUND or more expressed in Control leaves					
Functional categories	Description	Locus	E-value	Spectral counts Control/ Infested	Fold change
Protein modification/degradation	OsClp9 – Putative Clp protease homologue	LOC_Os06g04530	0	6	–
	ubiquitin-like protein 5	LOC_Os12g04924	7e–48	5	–
	chaperone protein dnaJ	LOC_Os03g44620	0	2	–
DNA structure maintenance	histone H1	LOC_Os04g18090	1e–119	4	–
	regulator of chromosome condensation	LOC_Os02g34860	0	3	–
Nitrogen metabolism	nitrogen regulatory protein P-II	LOC_Os05g04220	4e–152	4	–
Development-related	homeobox protein knotted-1	LOC_Os02g08544	8e–158	2	–
Signal transduction	ras-related	LOC_Os07g31370	5e–152	2	–
Lipid metabolism	acyl-coenzyme A dehydrogenase, mitochondrial	LOC_Os05g03480	0	2	–
Others	regulator of ribonuclease	LOC_Os02g52450	1e–118	11	–
	plastid developmental protein DAG	LOC_Os04g51280	9e–62	7	–
	thiamine biosynthesis protein thiC	LOC_Os03g47610	1e–88	6	–
	heavy metal-associated domain containing	LOC_Os02g32814	0	4	–
	RNA recognition motif	LOC_Os06g02240	0	4	–
	T-complex protein	LOC_Os06g36700	0	4	–
	HAD-superfamily hydrolase	LOC_Os03g19760	3e–48	3	–
	haloacid dehalogenase-like hydrolase	LOC_Os06g45440	0	3	–
	RNA recognition motif	LOC_Os04g50110	0	3	–
	RNA recognition motif	LOC_Os10g17454	0	2	–
Unknown	expressed protein	LOC_Os07g05290	5e–60	8	–
	expressed protein	LOC_Os02g57030	0	7	–
	expressed protein	LOC_Os01g50450	0	5	–
	B12D	LOC_Os06g13680	1e–47	4	–
	expressed protein	LOC_Os04g54320	2e–113	4	–
	expressed protein	LOC_Os01g52170	0	4	–
	expressed protein	LOC_Os04g35530	7e–153	3	–
	hairpin-induced protein 1 domain containing	LOC_Os02g33550	1e–162	3	–

Obs. 1: "E-value" represents the expected value of the match between the identified sequence and the sequence blastp found an alignment for in the TIGR Rice database; "-" signal means "uniquely found in control condition"; "Spectral counts" represent the sum from three replicates.
Obs. 2: Bold and underlined sequences were confirmed by RT-qPCR.

inhibitors (Table 2). It is well known that biotic and abiotic stress can affect several molecular functions in rice (Narsai et al., 2013). The same result could be noticed in our Gene Ontology (GO) analyses. The GO annotations of all 183 differentially expressed and unique proteins identified are shown in Fig. 6. Metabolic processes, cellular processes and response to stimulus were the most regulated biological processes (Fig. 6a). The molecular functions of catalytic activity and binding were the most regulated (Fig. 6b).

According to KEGG analysis, fifty-two different pathways were associated with proteins identified as up- or down-regulated (data not shown). The following KEGG pathways involved more than three proteins identified in our dataset: biosynthesis of antibiotics (10), glycolysis/gluconeogenesis (7), amino/nucleotide sugar metabolism (6), aminobenzoate degradation (4), pyruvate metabolism (4), aminoacyl-tRNA biosynthesis (4), purine metabolism (4), starch and sucrose metabolism (4), cysteine/methionine metabolism (4), terpenoid backbone biosynthesis (4). Terpenoids have an extensive range of important roles in plants, including plant-pathogen interactions (Fall and Solomon, 2011), and the presence of a large number of terpenoid compounds in rice has been positively correlated with reduced pathogen infection (Peters, 2006). The aminobenzoate degradation pathway, which has functions in xenobiotics biodegradation and metabolism (Li et al., 2016), was represented only by proteins uniquely found in LI leaves. We hypothesize that the well-established senescence process in LI leaves is partially responsible for degradation of cell compounds and generation of metabolic byproducts. In addition, *S. oryzae* infestation probably generates toxic compounds in rice cells that can be treated as xenobiotics. In our analysis, we identified a glutathione S-transferase protein only in LI leaves (Table 2). According to Noctor et al. (2012), these proteins can function in the detoxification of heavy metals and xenobiotics. We observed that the pyruvate metabolism pathway is down-regulated, while amino/nucleotide sugar and cysteine/methionine metabolism are up-regulated (data not shown). This suggests a severe modification in primary metabolic pathways.

3.3. Differentially expressed proteins in rice leaves infested with S. oryzae

Several proteins of essential processes as translation, carbohydrate metabolism/energy production, defense-related protease inhibitors, photosynthesis, and stress response were found as unique or more abundant in control leaves compared to LI. It indicates that high infestation of *S. oryzae* can cause serious and irreversible damage in rice leaves, since all these processes are seriously affected and less active in LI leaves, according to protein abundance (Table 1). In general, *S. oryzae* high infestation stress induces profound alterations in protein network including those participating in general metabolic processes and protein modification/degradation (Table 2).

The "translation-related" functional category presented the highest number of differentially expressed proteins (17), with higher expression in control leaves than LI leaves (Table 1). Interestingly, Buffon et al. (2016) showed that rice leaves infested by a small population of *S. oryzae* increases the expression of translation-related proteins and, consequently, increase protein level. Therefore, it seems that the progress of infestation and its intensity leads to a negative effect on protein synthesis in rice leaves. According to Narsai et al. (2013), this is a common response of rice cultivars susceptible to bacterial and fungal infection, while resistant cultivars display up-regulation of translation during parasite infection. Another plausible explanation for such a decrease in expression of translation-related proteins in LI leaves is that translation is finely repressed under stress conditions to prevent

Table 2
List of unique or differentially expressed proteins identified by MudPIT in rice leaves under late-infested condition, in relation to control condition.

Proteins EXCLUSIVELY FOUND or more expressed in LATE-INFESTED leaves					
Functional categories	Description	Locus	E-value	Spectral counts Control/ Infested	Fold change
General metabolic processes	NADH dehydrogenase 1 alpha sub-complex	LOC_Os02g57180	0	5	–
	inositol-1-monophosphatase	LOC_Os07g09330	0	5	–
	amine oxidase, flavin-containing domain	LOC_Os03g08570	0	4	–
	polyprenyl synthetase	LOC_Os12g17320	0	4	–
	cysteine-rich repeat secretory protein	LOC_Os05g02200	0	3	–
	hydrolase	LOC_Os01g37960	0	3	–
	methyltransferase domain containing	LOC_Os01g51530	0	3	–
	dehydrogenase, putative, expressed	LOC_Os09g32640	0	2	–
	kinase	LOC_Os06g12590	0	2	–
	nucleotide pyrophosphatase/phosphodiesterase	LOC_Os01g58640	0	2	–
	thiamine pyrophosphate enzyme, C-terminal	LOC_Os01g32080	0	2	–
	methylthioribose kinase	LOC_Os04g57400	0	2	–
	methyltransferase domain containing	LOC_Os06g43800	0	2	–
Protein modification or degradation	oryzain gamma chain precursor	LOC_Os09g27030	0	7/64	−9.14
	aspartic proteinase oryzasin-1 precursor	LOC_Os05g49200	0	6	–
	ubiquitin-conjugating enzyme	LOC_Os01g46926	6e–108	4	–
	Ser/Thr protein phosphatase	LOC_Os01g49690	0	3	–
	ubiquitin family	LOC_Os07g31540	0	3	–
	OsDegp15 – Putative Deg protease homologue	LOC_Os12g42210	1e–109	3	–
	OsSub30 – Putative Subtilisin homologue	LOC_Os03g40830	0	2	–
	OsSub53 – Putative Subtilisin homologue	LOC_Os07g39020	0	2	–
	Ser/Thr protein phosphatase	LOC_Os06g43640	0	2	–
	Ser/Thr protein phosphatase	LOC_Os12g44020	0	2	–
	ubiquitin family domain containing protein	LOC_Os01g68940	3e–70	2	–
Carbohydrate metabolism and energy production	ATP synthase protein 9, mitochondrial	LOC_Os12g34108	4e–28	93	–
	NAD dependent epimerase/dehydratase	LOC_Os02g35039	0	17	–
	amidase	LOC_Os04g02754	0	14	–
	maltose excess protein 1-like, chloroplast	LOC_Os04g51330	0	6	–
	aldose 1-epimerase	LOC_Os04g38530	0	4	–
	mannose-1-phosphate guanyl transferase	LOC_Os01g62840	0	4	–
	lactate/malate dehydrogenase	LOC_Os02g01510	0	3	–
	glycosyl hydrolase	LOC_Os01g47070	0	2	–
	glycosyl hydrolases family 17	LOC_Os01g71380	0	2	–
Translation-related	methionyl-tRNA synthetase	LOC_Os03g11120	0	7	–
	threonyl-tRNA synthetase, mitochondrial	LOC_Os08g19850	0	3	–
	polyadenylate-binding protein	LOC_Os08g22354	0	3	–
	ribosomal protein	LOC_Os03g55930	3e–162	2	–
	40S ribosomal protein S26	LOC_Os01g60790	6e–92	2	–
	60S ribosomal protein L8	LOC_Os12g38000	2e–24	2	–
	40S ribosomal protein S28	LOC_Os10g27174	5e–39	2	–
Amino acid metabolism	cysteine desulfurase 1, mitochondrial	LOC_Os09g16910	0	8	–
	3-isopropylmalate dehydratase	LOC_Os02g03260	0	6	–
	argininosuccinate lyase	LOC_Os03g19280	0	5	–
	OsSCP3 – Putative Serine Carboxypeptidase	LOC_Os01g22980	0	3	–
	aspartate aminotransferase	LOC_Os09g28050	0	3	–
Oxidative stress-related	glutathione S-transferase	LOC_Os10g38780	2e–168	7	–
	peroxidase	LOC_Os07g02440	0	3	–
	glutathione peroxidase	LOC_Os11g18170	4e–88	2	–
	peroxidase	LOC_Os08g02110	0	2	–
DNA structure maintenance	**core histone H2A/H2B/H3/H4**	**LOC_Os05g02300**	**1e–104**	**49**	–
	histone H3	**LOC_Os06g04030**	**1e–94**	**18**	–
	Core histone H2A/H2B/H3/H4	LOC_Os10g28230	4e–94	6	–
	HMG1/2	LOC_Os04g47690	3e–87	4	–
Transport-related	importin subunit beta	LOC_Os12g38110	0	10	–
	TOC159	LOC_Os05g05950	0	3	–
	mitochondrial import inner membrane	LOC_Os05g02060	8e–105	3	–
	adaptin protein	LOC_Os03g23950	0	3	–
Hormone signaling	gibberellin receptor GID1L2	LOC_Os07g06830	0	5	–
	tetratricopeptide repeat containing	LOC_Os05g31770	1e–117	3	–
Lipid metabolism	3-deoxy-manno-octulosonate cytidylyltransferase	LOC_Os05g48750	0	12	–
	acyl CoA binding protein	LOC_Os08g06550	3e–61	8	–
Photosynthesis	chloroplast post-illumination chlorophyll	LOC_Os03g38950	0	4	–
	PAP fibrillin family domain containing	LOC_Os07g28790	0	2	–

(*continued on next page*)

Table 2 (*continued*)

Proteins EXCLUSIVELY FOUND or more expressed in LATE-INFESTED leaves					
Functional categories	Description	Locus	E-value	Spectral counts Control/ Infested	Fold change
Protease inhibitors	LTPL129 – Protease inhibitor/seed storage	LOC_Os06g43600	1e–166	15	–
	LTPL114 – Protease inhibitor/seed storage	LOC_Os03g01300	1e–89	5	–
Transferase	phosphoribosyl transferase	LOC_Os02g40010	4e–151	5	–
Nucleotide metabolism	adenylosuccinate synthetase, chloroplast	LOC_Os03g49220	0	4	–
Secondary metabolism	lycopene beta cyclase, chloroplast precursor	LOC_Os02g09750	0	4	–
Cell wall-related	alpha-N-arabinofuranosidase A	LOC_Os03g20420	0	3	–
Stress response	hevamine-a-like	LOC_Os01g64110	0	7	–
	CHIT1 – Chitinase family protein precursor	LOC_Os02g39330	0	2	–
Stress-response/allergens	pathogenesis-related Bet v I family	LOC_Os12g36850	3e–113	2	–
Others	ki1	LOC_Os09g36520	0	5	–
	HEAT repeat family protein	LOC_Os02g07120	0	3	–
	bifunctional protein folD	LOC_Os02g02850	0	3	–
	pantothenate kinase 4	LOC_Os06g21980	0	2	–
	methylcrotonoyl-CoA carboxylase beta	LOC_Os08g32850	0	2	–
	haloacid dehalogenase-like hydrolase	LOC_Os09g24230	3e–136	2	–
	actin-depolymerizing factor	LOC_Os03g60580	5e–107	2	–
Unknown	expressed protein	LOC_Os07g29240	0	12	–
	expressed protein	LOC_Os11g21990	0	12	–
	expressed protein	LOC_Os10g39150	2e–120	9	–
	expressed protein	LOC_Os01g66980	2e–166	7	–
	expressed protein	LOC_Os02g30410	8e–135	4	–
	expressed protein	LOC_Os01g26039	0	3	–
	expressed protein	LOC_Os03g03470	0	3	–
	expressed protein	LOC_Os10g22460	0	3	–
	expressed protein	LOC_Os12g06335	0	3	–
	uncharacterized conserved membrane protein	LOC_Os05g33290	4e–58	2	–

Obs. 1: "E-value" represents the expected value of the match between the identified sequence and the sequence blastp found an alignment for in the TIGR Rice database; "-" signal means "uniquely found in late-infested condition"; "Spectral counts" represent the sum from three replicates.
Obs. 2: Bold and underlined sequences were confirmed by RT-qPCR.

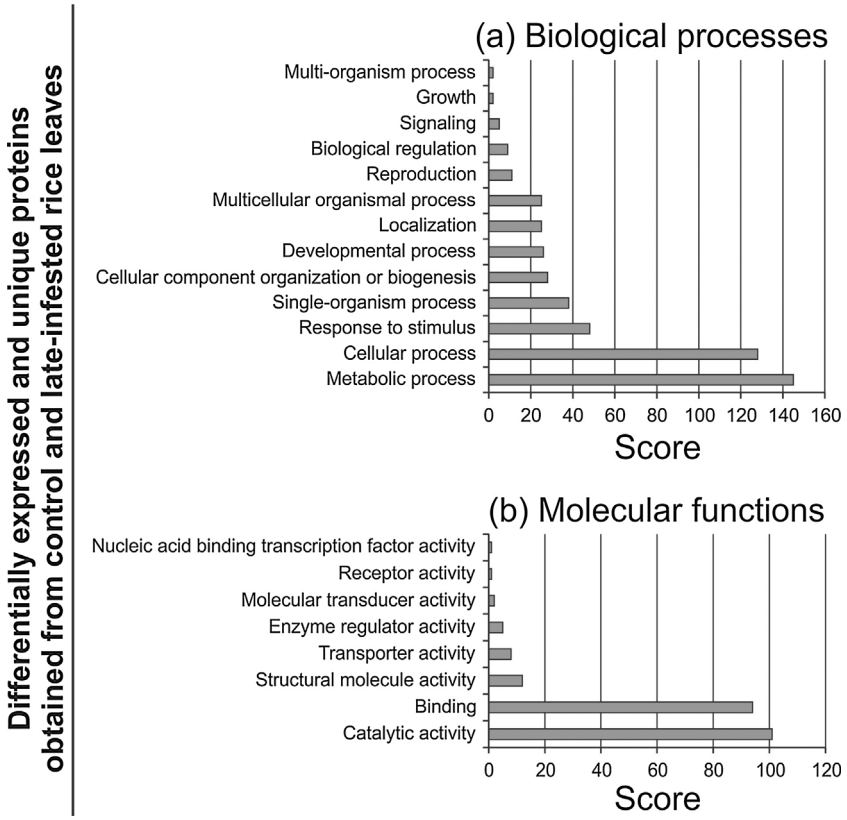

Fig. 6. Gene Ontology annotation. Biological processes (a) and Molecular functions (b) for differentially expressed and unique proteins obtained from control and late-infested rice leaves.

production of peptides from aberrant transcripts (Cooper et al., 2003). Anyway, it would be interesting to test if the maintenance of translation-related proteins expression could effectively avoid the increase of *S. oryzae* population number in rice leaves.

Even though the functional category "carbohydrate metabolism/energy production" was found up- and down-regulated by *S. oryzae* late infestation in rice leaves (Tables 1 and 2), we believe that infested leaves present a markedly reduction in energy production. This hypothesis is reinforced by 1) two ATP synthases more abundant in control than in LI leaves; 2) KEGG analysis showed that pyruvate metabolism pathway is down-regulated in LI leaves; 3) seven proteins related to photosynthesis are also down-regulated in LI leaves, which probably impact NADPH production and, consequently, glucose and ATP synthesis; and 4) decrease in carbon assimilation and energy production was also detected in rice under brown planthopper infestation (Sangha et al., 2013). Previously, we also found down-regulation of photosynthesis- and carbohydrate metabolism/energy production-related proteins in rice leaves early infested by *S. oryzae* (Buffon et al., 2016); however, the identities of the proteins are mostly different in early and LI leaves. It is interesting to highlight that the reduction in the expression of proteins related to photosynthesis was more apparent in early responses to *S. oryzae* infestation (Buffon et al., 2016), even though we were only able to detect a reduction in chlorophyll concentration and the establishment of a senescence process in highly infested leaves (Fig. 2).

According to our results, even under intense infestation by *S. oryzae,* proteins commonly used in plant defense strategy to prevent herbivory attack were down regulated. Several protease inhibitors were found more abundant or uniquely identified in control leaves (Table 1). Plant protease inhibitors are important natural plant defenses targeting herbivorous proteases (War et al., 2012). Down-regulation of protease inhibitors indicates that the defense mechanism based on inhibition of mite proteases was not effective and shut down, contributing to the fast increase in mite population. Strategies to exploit plant protease inhibitors to protect crops against herbivorous are not novel. Several bioassays show that pests exposed to protease inhibitors have a slower growth rate, retarded development, and increased mortality (Mosolov and Valueva, 2008; Gatehouse, 2011). It would be interesting to test if the increased expression of protease inhibitors could protect rice plants against *S. oryzae* infestation, as previously suggested by Gatehouse (2011) and Kunert et al. (2015).

Several proteins related to protein modification/degradation were found up-regulated in LI leaves (Table 2). It is known that senescence process is accompanied by an increase in protein degradation level, and such a process can be stimulated by biotic stress (Diaz-Mendoza et al., 2016). LI leaves showed an early senescence process (Fig. 2), it is therefore not surprising that several proteins related to proteolysis are highly expressed in these leaves. We detected three proteins related to ubiquitination process expressed only in LI leaves (Table 2). Ubiquitination process is important in protein degradation/signaling and regulation of several mechanisms related to stress responses (Dametto et al., 2015), including defense against biotic threats (Dreher and Callis, 2007). According to Jeon et al. (2012), ectopic expression of ubiquitin-conjugating enzyme gene from wild rice *Oryza grandiglumis* (*OgUBC1*) confers resistance against *Botrytis infection* in Arabidopsis. We also detected proteins related to protein modification only in LI leaves (Table 2), including proteases and phosphatases. Wang et al. (2015) showed that aspartic proteases might be associated with virus-induced cell death in rice leaves, and in certain plant-pathogen interactions, the cell death seems to be mediated by specific proteases (Fagundes et al., 2015). Interestingly, in our study LI leaves presented higher levels of cell death than the control leaves (Fig. 4). Ser/Thr-phosphatases normally act as negative regulators of plant defense responses against pathogens, suggesting the existence of active signaling pathways in the absence of stress that require continuous Ser/Thr phosphatase activity to remain inhibited (País et al., 2009a). In agreement with our results, transcripts of the catalytic subunits of Ser/Thr phosphatase PP2A subfamily I are up-regulated by fungal elicitors in potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*) (País et al., 2009b). As activation of defense-related protein phosphorylation cascades leads to oxidative burst and localized cell death (as seen in Figs. 3 and 4), the up-regulation of potential negative modulators by pathogens may be a protective mechanism in order to prevent extensive damage to host tissues (País et al., 2009a).

Six proteins belonging to the "stress response" functional category were found only in control leaves, while two proteins associated with this same category were uniquely identified in LI leaves (Tables 1 and 2). Peptidyl-prolyl *cis-trans* isomerase was recently described as responsive to *Puccinia striiformis* f. sp. *tritici* infection in wheat plants, regulating the immune response (Yang et al., 2016). Rapid Alkalinization Factors (RAF's) are small peptides affecting acidification and cell expansion during growth and development (Murphy and De Smet, 2014). The absence of these proteins in LI leaves suggests that *S. oryzae* causes a deregulation of the immune response (resulting in a mite-susceptible phenotype) and a restriction in cell expansion. It would be interesting to test if both proteins expressed in rice leaves during the entire development period could enhance rice resistance to *S. oryzae*. On the other hand, the presence of both hevamine-a-like and chitinase solely in LI leaves suggest that these leaves are still trying to combat *S. oryzae* infestation, as previously reported by Maserti et al. (2011) in citrus leaves infested by *Tetranychus urticae*.

The "oxidative stress-related" functional category comprehend five proteins exclusively identified in control leaves (Table 1) and four proteins expressed only in LI leaves (Table 2). Chloroplast thioredoxins are reduced by ferredoxin-thioredoxin reductases (Serrato et al., 2004) that are required for proper chloroplast development and is involved in the regulation of plastid gene expression in Arabidopsis (Wang et al., 2014). According to Lim et al. (2010), silencing a ferredoxin-thioredoxin reductase induces pathogenesis-related genes and pathogen resistance in tomato plants. Since we found a down regulation of this same protein in our results, it could be an evolutionary conserved and complementary defense strategy used by rice leaves infested with *S. oryzae*. On the other hand, three peroxidases expressed only in LI leaves suggest a hazardous H_2O_2 accumulation in these leaves, as confirmed by histochemical analysis (Fig. 3b). Also, the presence of one glutathione S-transferase (the same expressed in early-infested leaves – Buffon et al., 2016) only in LI leaves suggest a long exposure of the host tissues to oxidative burst caused by herbivorous attack (Ferry et al., 2011).

For several functional categories, we were not able to detect a consistent response to *S. oryzae* late-infestation, solely based on the differentially expressed proteins. Even though, we can certainly claim that high levels of *S. oryzae* mites on rice leaves affect intracellular transport, amino acid metabolism, DNA structure maintenance, and lipid metabolism (Tables 1 and 2). More studies are needed to elucidate if these proteins can be used in biotechnological approaches to generate rice plants more resistant to *S. oryzae* infestation.

3.4. Validation of proteomic data

To validate the proteomic analysis, we performed enzymatic assays and RT-qPCR analysis. The enzymatic assay results reflected those of our proteomic analysis, except for lactate dehydrogenase. The enzymatic activities presented in Fig. 7 corresponding to glutathione S-transferase, phosphatase, aspartate aminotransferase, protease, and peroxidase (Fig. 7a, b, d, e, f), all included proteins that were only detected in LI rice leaves in proteomic results and consequently resulted in higher levels of activity. Lactate dehydrogenase activity (Fig. 7c) was found to be higher in control than in LI leaves, which is not reflected in our proteomic analysis, evidencing some kind of post-translational control mechanism. The identification and validation of known enzymes in the context of the interaction of pathogen/parasite with its

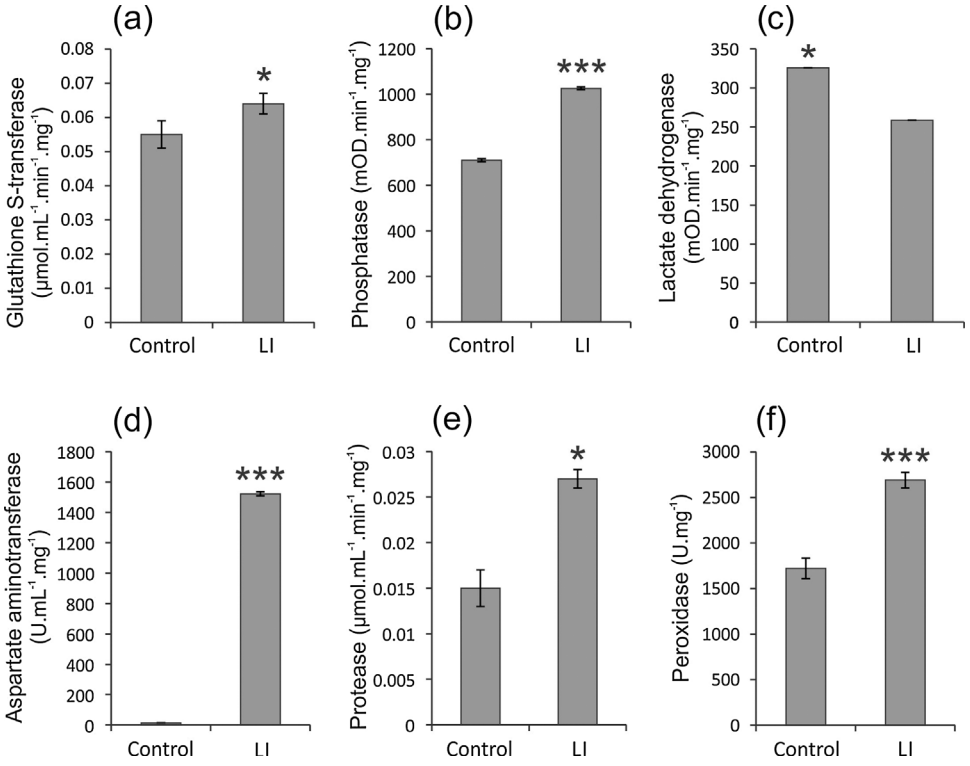

Fig. 7. Validation of proteomic results using enzymatic assays. Enzymatic activities of Glutathione S-transferase (a), Phosphatase (b), Lactate dehydrogenase (c), Aspartate aminotransferase (d), Protease (e), and Peroxidase (f) in rice leaves of control and late-infested (LI) plants. Represented values are the averages of three samples ± SE. Mean values with one, two or three asterisks are different by Student's *t* test (*p*-value ≤ 0.05, 0.01, and 0.001, respectively).

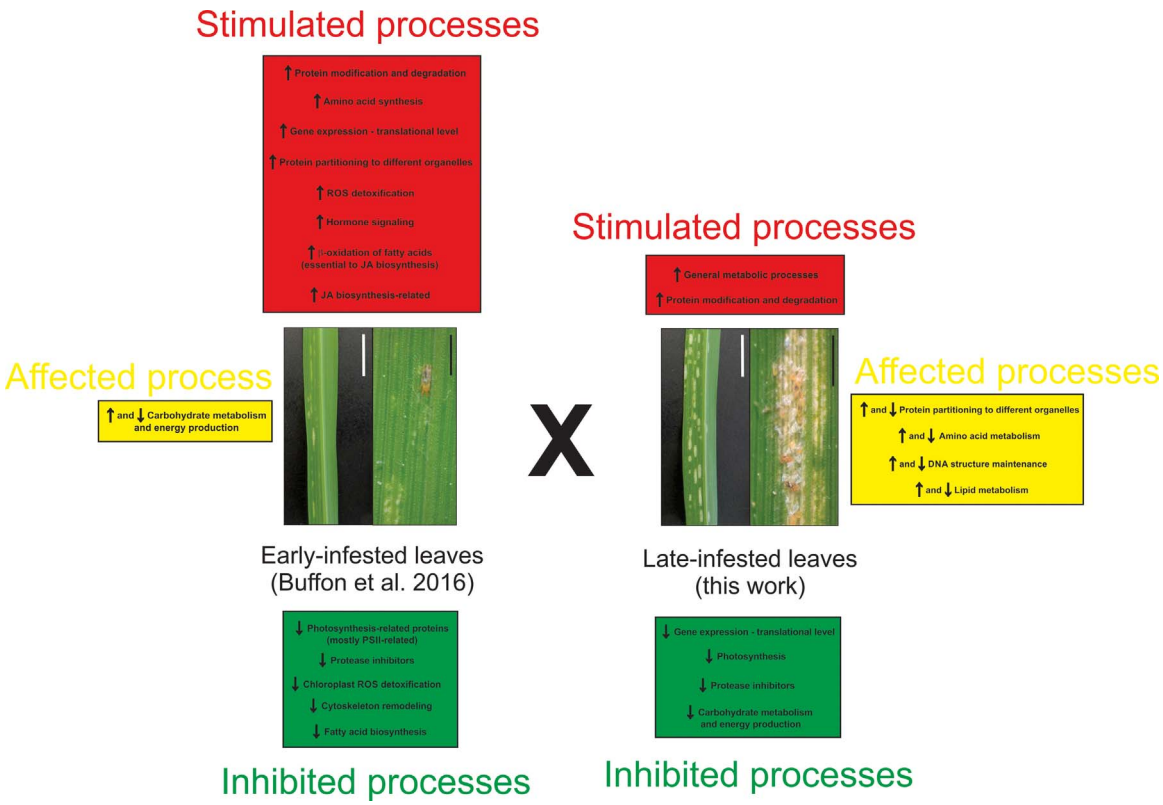

Fig. 8. Schematic model of the rice processes stimulated (red color), inhibited (green color), and affected (yellow color) by *S. oryzae* early (Buffon et al., 2016) and late infestation (this work), based on the proteomic and physiological results presented in both studies. JA: jasmonic acid; PS: photosystem II; ROS: reactive oxygen species. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

specific host supports the use of the experimental design presented here.

The mRNA expression of nine genes (all listed in Supplementary Table 1) corresponding to proteins identified as differentially expressed during *S. oryzae* infestation was further evaluated in control and LI leaves by quantitative RT-PCR (Supplementary Fig. 1). The differences in expression highlighted by the MudPIT methodology were confirmed for the nine tested genes, even though the fold change detected at the

mRNA (Supplementary Fig. 1) and protein levels (Tables 1 and 2) were different. This was probably due to a regulation at the post-transcriptional level. In a wider view, the correlation of the proteomic data with mRNA expression, and also enzymatic activity, reinforces the involvement of these specific proteins in rice responses to mite LI.

4. Conclusions

This is the first report evaluating the rice response to phytophagous mite *S. oryzae* late infestation using a high-throughput proteomic approach. The schematic model in Fig. 8 shows how specific these findings are in relation to that found in "early-infested" rice leaves (Buffon et al., 2016). The main molecular processes repressed in rice leaves by *S. oryzae* late infestation are photosynthesis, translation, carbohydrate metabolism/energy production, and production of protease inhibitors. On the other hand, high infestation levels induce protein modification/degradation pathways. Altogether, these processes and the identification of differentially expressed proteins are helpful to reveal the molecular mechanisms involved in the response of rice to high levels of mite infestation, and yield a set of interesting potential targets for future studies aiming to promote phytophagous mite resistance in rice plants, or, at least, to prevent the mite population increase.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2017.10.005.

References

Afify, A.E.M.M.R., El-Beltagi, H.S., Fayed, S.A., Shalaby, E.A., 2011. Acaricidal activity of different extracts from *Syzygium cumini* L. Skeels (Pomposia) against *Tetranychus urticae* Koch. Asian Pac. J. Trop. Biomed. 1, 359–364.

Agut, B., Gamir, J., Jaques, J.A., Flors, V., 2015. *Tetranychus urticae*-triggered responses promote genotype dependent conspecific repellence or attractiveness in citrus. New Phytol. 207, 790–804.

Ahmadi, N., Audebert, A., Bennett, M.J., Bishopp, A., de Oliveira, A.C., et al., 2014. The roots of future rice harvests. Rice 7, 29.

Alba, J.M., Glas, J.J., Schimmel, B.C.J., Kant, M.R., 2011. Avoidance and suppression of plant defenses by herbivores and pathogens. J. Plant Interact. 6, 1–7.

Alba, J.M., Schimmel, B.C.J., Glas, J.J., Ataide, L.M.S., Pappas, M.L., et al., 2015. Spider mites suppress tomato defenses downstream of jasmonate and salicylate independently of hormonal crosstalk. New Phytol. 205, 828–840.

Blasi, E.A.R., Buffon, G., da Silva, R.Z., Stein, C., Dametto, A., et al., 2015. Alterations in rice, corn and wheat plants infested by phytophagous mite. Int. J. Acarol. 41, 10–18.

Buffon, G., Blasi, E.A.R., Adamski, J.M., Ferla, N.J., Berger, M., et al., 2016. Physiological and molecular alterations promoted by Schizotetranychus oryzae mite infestation in rice leaves. J. Proteome Res. 15, 431–446.

Carvalho, P.C., Fischer, J.S., Xu, T., Yates, J.R., Barbosa, V.C., 2012. PatternLab: from mass spectra to label-free differential shotgun proteomics. Curr. Protoc. Bioinf. 13, 13–19.

Conesa, A., Götz, S., García-Gómez, J.M., Terol, J., Talón, M., et al., 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21, 3674–3676.

Cooper, B., Clarke, J.D., Budworth, P., Kreps, J., Hutchison, D., et al., 2003. A network of rice genes associated with stress response and seed development. Proc. Natl. Acad. Sci. U. S. A. 100, 4945–4950.

Dametto, A., Buffon, G., Blasi, E.A.R., Sperotto, R.A., 2015. Ubiquitination pathway as a target to develop abiotic stress tolerance in rice. Plant Signal. Behav. 10, e1057369.

de Oliveira, E.F., Pallini, A., Janssen, A., 2016. Herbivores with similar feeding modes interact through the induction of different plant responses. Oecologia 180, 1–10.

Diaz-Mendoza, M., Velasco-Arroyo, B., Santamaria, M.E., González-Melendi, P., Martinez, M., et al., 2016. Plant senescence and proteolysis: two processes with one destiny. Genet. Mol. Biol. 39, 329–338.

Dreher, K., Callis, J., 2007. Ubiquitin, hormones and biotic stress in plants. Ann. Bot. 99, 787–822.

Evaristo, A.B., Venzon, M., Matos, F.S., de Freitas, R.G., Kuki, K.N., et al., 2013. Susceptibility and physiological responses of Jatropha curcas accessions to broad mite infestation. Exp. Appl. Acarol. 60, 485–496.

Fagundes, D., Bohn, B., Cabreira, C., Leipelt, F., Dias, N., et al., 2015. Caspases in plants: metacaspase gene family in plant stress responses. Funct. Integr. Genomics 15, 639–649.

Fall, L.A.D., Solomon, P.S., 2011. Role of cereal secondary metabolites involved in mediating the outcome of plant-pathogen interactions. Metabolites 1, 64–78.

Farouk, S., Osman, M.A., 2012. Alleviation of oxidative stress induced by spider mite invasion through application of elicitors in bean plants. Egypt J. Biol. 14, 1–13.

Ferla, N.J., Rocha, M.S., Freitas, T.F.S., 2013. Fluctuation of mite fauna associated to rice culture (Oryza sativa L.: poales, Poaceae) in two regions in the State of Rio Grande do Sul, Brazil. J. Agric. Sci. Technol. 3, 525–533.

Ferry, N., Stavroulakis, S., Guan, W., Davison, G.M., Bell, H.A., et al., 2011. Molecular interactions between wheat and cereal aphid (Sitobion avenae): analysis of changes to the wheat proteome. Proteomics 11, 1985–2002.

Gatehouse, J.A., 2011. Prospects for using proteinase inhibitors to protect transgenic plants against attack by herbivorous insects. Curr. Prot. Pept. Sci. 12, 409–416.

Hummel, N.A., Castro, B.A., McDonald, E.M., Pellerano, M.A., Ochoa, R., 2009. The panicle rice mite, Steneotarsonemus spinki Smiley, a rediscovered pest of rice in the United States. Crop Prot. 28, 547–560.

Jaimez-Ruiz, I.A., Otero-Colina, G., Valdovinos-Ponce, G., Villanueva-Jiménez, J.A., Vera-Graziano, J., 2015. Population growth and characterization of plant injuries of Steneotarsonemus spinki Smiley (Acari: Tarsonemidae) on rice. Neotrop. Entomol. 44, 294–300.

Jain, M., Nijhawan, A., Tyagi, A.K., Khurana, J.P., 2006. Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. Biochem. Biophys. Res. Commun. 345, 646–651.

Jeon, E.H., Pak, J.H., Kim, M.J., Kim, H.J., Shin, S.H., et al., 2012. Ectopic expression of ubiquitin-conjugating enzyme gene from wild rice, OgUBC1, confers resistance against UV-B radiation and Botrytis infection in Arabidopsis thaliana. Biochem. Biophys. Res. Commun. 427, 309–314.

Kant, M.R., Ament, K., Sabelis, M.W., Haring, M.A., Schuurink, R.C., 2004. Differential timing of spider mite-induced direct and indirect defenses in tomato plants. Plant Physiol. 135, 483–495.

Kim, S.T., Kim, S.G., Agrawal, G.K., Kikuchi, S., Rakwal, R., 2014. Rice proteomics: a model system for crop improvement and food security. Proteomics 14, 593–610.

Krishnan, H.B., Natarajan, S.S., 2009. A rapid method for depletion of Rubisco from soybean (*Glycine max*) leaf for proteomic analysis of lower abundance proteins. Phytochemistry 70, 1958–1964.

Kunert, K.J., van Wyk, S.G., Cullis, C.A., Vorster, B.J., Foyer, C.H., 2015. Potential use of phytocystatins in crop improvement, with a particular focus on legumes. J. Exp. Bot. 66, 3559–3570.

Landeros, J., Guevara, L.P., Badii, M.H., Flores, A.E., Pámanes, A., 2004. Effect of different densities of the twospotted spider mite *Tetranychus urticae* on CO_2 assimilation, transpiration, and stomatal behaviour in rose leaves. Exp. Appl. Acarol. 32, 187–198.

Li, S.W., Shi, R.F., Leng, Y., Zhou, Y., 2016. Transcriptomic analysis reveals the gene expression profile that specifically responds to IBA during adventitious rooting in mung bean seedlings. BMC Genomics 17, 43.

Lim, C.J., Kim, W.B., Lee, B.S., Lee, H.Y., Kwon, T.H., et al., 2010. Silencing of SlFTR-c, the catalytic subunit of ferredoxin:thioredoxin reductase, induces pathogenesis-related genes and pathogen resistance in tomato plants. Biochem. Biophys. Res. Commun. 399, 750–754.

Lim, S.D., Cho, H.Y., Park, Y.C., Ham, D.J., Lee, J.K., et al., 2013. The rice RING finger E3 ligase, OsHCI1, drives nuclear export of multiple substrate proteins and its heterogeneous overexpression enhances acquired thermotolerance. J. Exp. Bot. 60, 2899–2914.

Maserti, B.E., Carratore, R.D., Croce, C.M.D., Podda, A., Migheli, Q., et al., 2011. Comparative analysis of proteome changes induced by the twospotted spider mite *Tetranychus urticae* and methyl jasmonate in citrus leaves. J. Plant Physiol. 168, 392–402.

Mosolov, V.V., Valueva, T.A., 2008. Proteinase inhibitors in plant biotechnology: a review. Appl. Biochem. Microbiol. 44, 233–240.

Murphy, E., De Smet, I., 2014. Understanding the RALF family: a tale of many species. Trends Plant Sci. 19, 664–671.

Muthayya, S., Sugimoto, J.D., Montgomery, S., Maberly, G.F., 2014. An overview of global rice production, supply, trade, and consumption. Ann. N.Y. Acad. Sci. 1324, 7–14.

Narsai, R., Wang, C., Chen, J., Wu, J., Shou, H., et al., 2013. Antagonistic, overlapping and distinct responses to biotic stress in rice (*Oryza sativa*) and interactions with abiotic stress. BMC Genomics 14, 93.

Noctor, G., Mhamdi, A., Chaouch, S., Han, Y., Neukermans, J., et al., 2012. Glutathione in plants: an integrated overview. Plant Cell Environ. 35, 454–484.

Oerke, E.C., 2006. Crop losses to pests. J. Agric. Sci. 144, 31–43.

País, S.M., Téllez-Iñón, M.T., Capiati, D.A., 2009a. Serine/threonine protein phosphatases type 2A and their roles in stress signaling. Plant Signal. Behav. 4, 1013–1015.

País, S.M., González, M.A., Téllez-Iñón, M.T., Capiati, D.A., 2009b. Characterization of potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*) protein phosphatases type 2A catalytic subunits and their involvement in stress responses. Planta 230, 13–25.

Park, Y.L., Lee, J.H., 2002. Leaf cell and tissue damage of cucumber caused by Twospotted Spider Mite (Acari: Tetranychidae). J. Econ. Entomol. 95, 952–957.

Park, S.Y., Yu, J.W., Park, J.S., Li, J., Yoo, S.C., et al., 2007. The senescence-induced staygreen protein regulates chlorophyll degradation. Plant Cell 19, 1649–1664.

Peters, R.J., 2006. Uncovering the complex metabolic network underlying diterpenoid

phytoalexin biosynthesis in rice and other cereal crop plants. Phytochemistry 67, 2307–2317.
Romero-Puertas, M.C., Rodríguez-Serrano, M., Corpas, F.J., Gómez, M., Del Río, L.A., et al., 2004. Cadmium-induced subcellular accumulation of O_2^- and H_2O_2 in pea leaves. Plant Cell Environ. 27, 1122–1134.
Ross, C.W., 1974. Plant Physiology Laboratory Manual. Wadsworth Publishing Company, Inc., Belmont, CA, USA.
Sangha, J.S., Chen, Y.H., Kaur, J., Khan, W., Abduljaleel, Z., et al., 2013. Proteome analysis of rice (*Oryza sativa* L.) mutants reveals differentially induced proteins during Brown Planthopper (*Nilaparvata lugens*) infestation. Int. J. Mol. Sci. 14, 3921–3945.
Santamaria, M.E., Cambra, I., Martinez, M., Pozancos, C., González-Melendi, P., et al., 2012. Gene pyramiding of peptidase inhibitors enhances plant resistance to the Spider Mite *Tetranychus urticae*. PLoS One 7, e43011.
Serrato, A.J., Pérez-Ruiz, J.M., Spínola, M.C., Cejudo, F.J., 2004. A novel NADPH thioredoxin reductase, localized in the chloroplast, which deficiency causes hypersensitivity to abiotic stress in *Arabidopsis thaliana*. J. Biol. Chem. 279, 43821–43827.
Shi, J., Fu, X.Z., Peng, T., Huang, X.S., Fan, Q.J., et al., 2010. Spermine pretreatment confers dehydration tolerance of citrus in vitro plants via modulation of antioxidative capacity and stomatal response. Tree Physiol. 30, 914–922.
Wang, P., Liu, J., Liu, B., Da, Q., Feng, D., et al., 2014. Ferredoxin:thioredoxin reductase is required for proper chloroplast development and is involved in the regulation of plastid gene expression in *Arabidopsis thaliana*. Mol. Plant 7, 1586–1590.
Wang, B., Hajano, J.U.D., Ren, Y., Lu, C., Wang, X., 2015. iTRAQ-based quantitative proteomics analysis of rice leaves infected by Rice stripe virus reveals several proteins involved in symptom formation. Virol. J. 12, 99.
War, A.R., Paulraj, M.G., Ahmad, T., Buhroo, A.A., Hussain, B., et al., 2012. Mechanisms of plant defense against insect herbivores. Plant Signal. Behav. 7, 1306–1320.
Wu, J.Q., Baldwin, I.T., 2009. Herbivory-induced signalling in plants: perception and action. Plant Cell Environ. 32, 1161–1174.
Yang, Y., Yu, Y., Bi, C., Kang, Z., 2016. Quantitative proteomics reveals the defense response of wheat against *Puccinia striiformis* f. sp. *tritici*. Sci. Rep. 6, 34261.
Yoshida, S., 2003. Molecular regulation of leaf senescence. Curr. Opin. Plant Biol. 6, 79–84.