Genetics and Molecular Research

Proteomic analysis of mycelial proteins from *Magnaporthe oryzae* under nitrogen starvation

X.-G. Zhou[1,2], P. Yu[2], C. Dong[2], C.-X. Yao[2], Y.-M. Ding[2], N. Tao[2] and Z.-W. Zhao[1]

[1]State Key Laboratory for Conservation and Utilization of Bio-Resources in Yunnan, Yunnan University, Kunming, China
[2]Key Laboratory of Southwestern Crop Gene Resources and Germplasm Innovation, Ministry of Agriculture and Yunnan Provincial Key Laboratory of Agricultural Biotechnology, Biotechnology and Germplasm Resources Institute, Yunnan Academy of Agricultural Sciences, Kunming, China

Corresponding author: Z.W. Zhao
E-mail: zhaozhw@ynu.edu.cn

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ABSTRACT. *Magnaporthe oryzae* is an important model system in studies of plant pathogenic fungi, and nitrogen is a key nutrient source affecting microbial growth and development. In order to understand how nitrogen stress causes changes in mycelial proteins, we analyzed differentially expressed mycelial proteins from the *M. oryzae* virulent strain CH-63 using two-dimensional electrophoresis and mass spectrometry in complete medium or under nitrogen starvation conditions. A total of 975 ± 70 and 1169 ± 90 protein spots were detected in complete medium and under nitrogen starvation conditions, respectively. Forty-nine protein spots exhibited at least 2-fold up-regulation or down-regulation at the protein level according to PDQuest7.4. Moreover, 43 protein spots were successfully identified by matrix-assisted laser desorption/ionization-time-of-flight/time-of-flight mass spectrometry. Among these spots, 6 proteins were functionally unknown and 37 proteins were categorized into 5 groups according to

their functions, including development, metabolism, biosynthesis, and biological process. These 37 proteins were further analyzed for their enriched metabolic pathways by KOBAS2.0, and 14 proteins were found to be involved in glycolysis, tricarboxylic acid cycle, and nitrogen metabolism. Taken together, the regulation of *M. oryzae* growth under the nitrogen starvation conditions appears to be complex because of the various proteins and enzymes involved.

Key words: Mass spectrometry; Mycelium; Nitrogen stress; Proteome; Rice blast fungus

INTRODUCTION

Rice (*Oryza sativa*) is a major crop that feeds millions of people worldwide, particularly in Asia and Africa (Talbot, 2003). Cultivated rice is exposed to numerous abiotic and biotic stresses (Mahajan and Tuteja, 2005). Rice blast disease caused by *Magnaporthe oryzae* is the most devastating disease in rice-growing regions worldwide (Liu et al., 2010). The control of rice blast depends on breeding resistant cultivars and applying chemical fungicides. However, it is important to understand the mechanisms of pathogenesis and host resistance to control fungal infection. Therefore, the molecular characterization of *M. oryzae* may reveal effective strategies for preventing various plant diseases. To evaluate the interactions between rice plants and *M. oryzae* on the molecular, biochemical, and physiological levels, high-throughput technologies such as transcriptomics, proteomics, and metabolomics have been used (Agrawal and Rakwal, 2011; Mathioni et al., 2011; Jones et al., 2011).

Nitrogen is an important nutrient source for microbial growth and development. Stress caused by a lack of nitrogen source is one of the major environmental stresses for *M. oryzae* in rice. Nitrogen deficiency can induce *M. oryzae* to secret pathogenic proteins (Wang et al., 2011) and protoplast-disrupting proteins (Rathour et al., 2003).

Proteomics technology can be used to rapidly analyze proteins from different individuals, organizations, and growth periods. In particular, two-dimensional electrophoresis (2-DE) has become an important research tool. Recently, a variety of plant pathogenic fungi has been investigated through proteomics (Garg et al., 2002). The pathogenesis of most of these fungi has been explored based on secreted proteins (Fernández-Acero et al., 2010), mycelia (Bregar et al., 2012), and conidia (Noir et al., 2009).

Proteomics studies for *M. oryzae* have been conducted on secreted proteins (Wang et al., 2011) and conidia (Bhadauria et al., 2010), but not on mycelium. However, secreted proteins, all from hyphae, are the main factors causing rice blast. Therefore, it is important to characterize *M. oryzae* mycelium using proteomics in order to understand the pathogenic mechanism.

MATERIAL AND METHODS

Materials

Strains and media

Magnaporthe oryzae CH-63 was kindly provided by Chengyun Li from Yunnan

Agricultural University, China. The following culture media were used in this study:

1) Solid medium: 15.0 g glucose, 5.0 g yeast extract, and 12.0 g agar in 1.0 L H_2O.

2) Trace elements: 2.20 g $ZnSO_4 \cdot 7H_2O$, 10 g H_3BO_3, 0.50 g $MnCl_2 \cdot 4H_2O$, 0.50 g $FeSO_4 \cdot 7H_2O$, 0.16 g $CoCl_2 \cdot 6H_2O$, 0.16 g $CuSO_4 \cdot 5H_2O$, 0.11 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 5 g Na_2-EDTA in 1.0 L H_2O.

3) Liquid complete medium (CM): 6 g $NaNO_3$, 0.52 g KCl, 0.52 g $MgSO_4 \cdot 7H_2O$, 1.52 g KH_2PO_4, 10 g glucose, 2 mL trace elements, 2 g peptone, 1 g yeast extract, and 1 g casein in 1.0 L H_2O.

4) N starvation complete medium (CM-N): 0.52 g KCl, 0.52 g $Mg_2SO_4 \cdot 7H_2O$, 1.52 g KH_2PO_4, 10 g glucose, and 2 mL trace elements in 1.0 L H_2O.

Strain culture and mycelium preparation

Strains of *M. oryzae* were grown on solid medium at 28°C for 7 days in the dark. Three 0.5 x 0.5-cm plugs of mycelia were added to 50 mL CM and incubated at 28°C and 150 rpm for 3 days. Cultures were filtered and rinsed with sterile water. A part of the mycelia was moved to 50 mL CM, while another part was moved to 50 mL CM-N. These samples were incubated at 28°C and 150 rpm for 2 days. Finally, the mycelia were filtered and rinsed.

Methods

Mycelial protein extraction

Briefly, 1 g of mycelia was ground to a fine powder in liquid nitrogen and suspended in 30 mL acetone containing 10% trichloroacetic acid, 0.1% dithiothreitol (DTT) and 1% phenylmethanesulfonyl fluoride. The mycelium suspension was incubated at -20°C overnight and centrifuged at 10,000 *g* for 10 min. The precipitate was washed with 30 mL 80% acetone 3 times, incubated at -20°C for 2 h, centrifuged at 10,000 *g* for 10 min, and then vacuum-dried. Crude mycelial protein was dissolved in rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT, 0.2% IPG buffer, 0.001% bromophenol blue) at a ratio of 1:15 (mg:μL). Protein solution was incubated at room temperature for 1 h and then centrifuged at 10,000 *g* for 10 min. The obtained supernatant was precipitated by the addition of 4 volumes of ice-cold acetone containing 1% DTT at -20°C for 2 h. The pellet was dissolved in rehydration buffer. Protein concentration was determined using the Bradford method (Bradford, 1976).

2-DE and image analysis

Within a pH range of 4-7, 24-cm immobilized pH gradient (IPG) strips (GE Healthcare, Little Chalfont, UK) were hydrated according to the manufacturer instructions. Briefly, 600 mg total proteins were dissolved in 500 μL rehydration buffer. Isoelectric focusing was conducted using an IPGphor II (GE Healthcare) according to the manufacturer instructions under the following conditions: 250 V for 30 min, 500 V for 30 min, 1000 V for 30 min, 8000 V for 4 h, and 8000 V for 75,000 Vh. The strips were then sequentially placed in equilibration buffer-1 (6 M urea, 2% sodium dodecyl sulfate, 50 mM Tris-HCl pH 8.8, 20% glycerol, 2% DTT) and equilibration buffer-2 (6 M urea, 2% sodium dodecyl sulfate, 50 mM Tris-HCl, 20% glycerol, 2.5% iodoacetamide) for 15 min, respectively. The equilibrated strips were run in a Ettan DAL Tsix (GE Healthcare) using 12% sodium dodecyl sulfate-polyacrylamide

gel electrophoresis at 10 mA/gel for 30 min, and then 30 mA/gel until the bromophenol blue reached the bottom of the gel. The gels were stained by Coomassie Brilliant Blue G-250, scanned using a UMAX Power Look scanner at a resolution of 300 dpi in gray scale mode, and then analyzed using PDQuest 7.4 (Bio-Rad, Hercules, CA, USA). A total of six maps, including three replicate maps, were analyzed. Spots were detected, matched, and normalized based on total density in the gel image according to the software guide. Isoelectric point and molecular weight (Mr) values were calculated according to the IEF strips range and protein standard markers, respectively. To create a statistical analysis set, the Student *t*-test was performed at a significance level of 95%.

Mass spectrometry and database searching

The intensity of each spot was measured in three biological replicates. Protein spots with an expression fold difference of >2 were selected for further identiðcation. Digging and mass spectrometry were performed on these protein spots. Trypsin digestion and 4800 Proteomics Analyzer MALDI-TOF/TOF of ABI (Applied Biosystems, Foster City, CA, USA) were performed as described previously (Yu et al., 2015). Protein sequences meeting the requirements were functionally classified through metabolic pathway analysis based on Gene Ontology (http://www.geneontology.org/) and KOBAS2.0 (http://kobas.cbi.pku.edu.cn/home.do).

RESULTS

2-DE imaging for mycelial protein

Figure 1 shows the 2-DE image of mycelial proteins from *M. oryzae* CH-63 cultured in CM and CM-N. The image shows a light background, good dyeing effect, clear separation of protein points, and no significant horizontal or vertical tails. Protein spots were distributed throughout the 24-cm IPG strips and a 12% gel. Experiments were performed in triplicate, and the 2-DE maps were analyzed using PDQuest 7.4 (Bio-Rad). A total of 975 ± 70 and 1169 ± 90 protein spots were detected in CM and CM-N, respectively. Our results revealed that nitrogen starvation induced the expression of additional proteins. Figure 2 shows enlarged pictures of the 43 proteins.

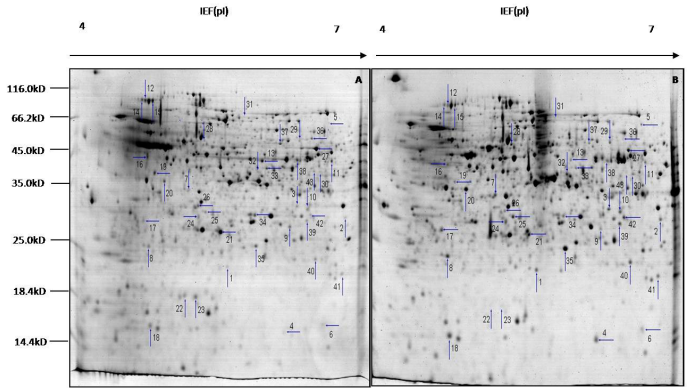

Figure 1. 2-DE maps of mycelial proteins cultivated in CM and CM-N from *Magnaporthe oryzae*. **A.** *M. oryzae* cultivated in CM. **B.** *M. oryzae* cultivated in CM-N.

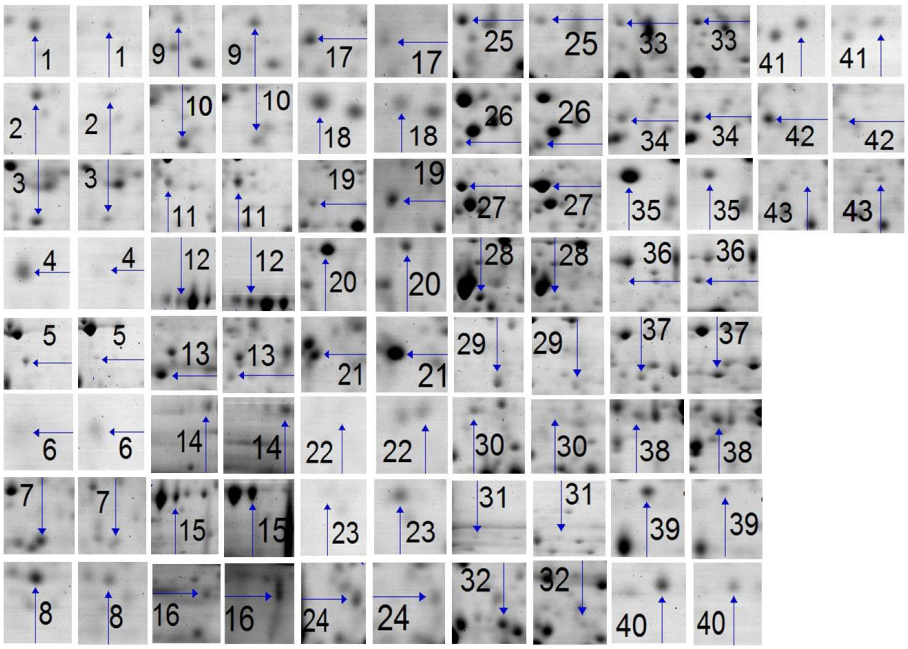

Figure 2. Enlarged pictures of differently expressed proteins.

Protein expression and mass spectrometry analysis

Compared with samples cultured under CM conditions, 49 protein spots showed a >2-fold change at the protein level. These differentially expressed proteins were analyzed by matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry (MS)/MS. Forty-three proteins were successfully identified, and the remaining 6 proteins were considered as failures. Table 1 shows the preliminary identification of 43 protein spots, including 23 up-regulated and 20 down-regulated proteins. Identified proteins were functionally classified into 5 known categories and 1 unknown group: development (23.3%), metabolism (41.9%), translation (2.3%), transport (2.3%), cellular component (16.3%), and unknown function (14%).

KEGG analysis

Among the 43 proteins, spots 29 spots 33 were the same proteins elongation factor 1-gamma. Spots 7, spots 14, spots 15 were the same proteins trehalase precursor. Thus, 40 proteins were analyzed by KEGG. However, only 24 proteins were categorized into metabolic pathways by KEGG (Table 2). We found that 8 proteins were involved in carbon metabolism and 12 proteins in glycometabolism (Figure 3A), amino acid metabolism (Figure 3B), and nitrogen metabolism (Figure 3C). In addition, spots 10, 19, and 28 were involved in oxidative phosphorylation; spot 16 was involved in protein processing in the endoplasmic reticulum; spot 21 was involved in cell cycling; spot 41 was involved in the proteasome; spot 12 was involved in the phagosome; spots 3 and 30 were involved in butanoate metabolism; and spot 36 was involved in tyrosine metabolism. Furthermore, 3 up-regulated proteins (trehalase,

glucose-6-phosphate isomerase, phosphoglycerate kinase) and 3 down-regulated proteins (transaldolase, aldose reductase, enolase) were involved in glycometabolism. Thus, *M. oryzae* CH-63 responded to nitrogen starvation by regulating carbon metabolism.

Table 1. Differentially expressed mycelial proteins in *Magnaporthe oryzae* cultivated with CM and CM-N.

Spot	Loc.No.	Protein	Change	Th Mr	Th pI	Ex Mr	Ex pI	Scores	MP
Development									
6	gi|15216342	Putative vacuolar subtilisin-like serine protease	+	57.48	6.21	7.78	6.66	265	8
8	gi|58257441	Transaldolase-like protein	+	35.81	5.38	33.66	5.14	69	11
13	gi|59803071	Oxalate decarboxylase-like protein /OXDC	-	55.03	5.62	45.96	5.98	719	18
17	gi|145616440	Enolase	+	47.19	5.19	24.03	4.63	172	6
19	gi|145608354	ATP synthase beta chain, mitochondrial	-	55.08	5.42	40.41	4.76	385	15
29	gi|39978103	Elongation factor 1-gamma	+	47.36	5.78	57.02	6.40	235	12
33	gi|39978103	Elongation factor 1-gamma	-	47.36	5.78	42.43	6.03	472	24
34	gi|145603053	Cytochrome c peroxidase	-	40.06	8.47	25.76	5.90	699	19
36	gi|39974845	Homogentisate 1,2-dioxygenase	-	55.26	5.94	58.37	6.54	441	14
37	gi|39944658	Trichothecene 3-O-acetyltransferase	-	54.63	5.74	34.21	6.12	442	19
Metabolism									
1	gi|351649361	Nitroreductase	+	32.96	9.30	27.45	7.94	663	9
2	gi|58257473	Formate dehydrogenase-like protein, FDH	+	40.00	6.03	25.13	6.93	469	16
3	gi|31321885	Aldose reductase	+	34.52	6.01	28.12	6.37	826	18
4	gi|145607549	Catalase-peroxidase 1	+	82.87	5.84	76.33	5.52	326	13
5	gi|59802854	Phosphoglycerate kinase-like protein	-	44.60	6.16	21.47	5.51	42	8
7	gi|23507179	Trehalase precursor	-	78.81	4.97	87.84	4.65	419	15
9	gi|5759121	Vacuolar-atpase	+	20.20	6.96	18.43	4.69	307	7
11	gi|59803007	Malate dehydrogenase-like protein	+	35.40	8.26	27.15	6.49	113	11
14	gi|23507179	Trehalase precursor	-	78.81	4.97	88.39	4.62	185	12
15	gi|23507179	Trehalase precursor	-	78.81	4.97	87.33	4.74	300	15
22	gi|145608638	Glucose-6-phosphate isomerase	-	61.22	5.87	11.13	5.09	269	16
25	gi|145603214	Spermidine synthase	+	38.23	6.23	26.54	5.35	723	16
27	gi|39952039	4-aminobutyrate aminotransferase	-	55.82	7.16	51.97	6.59	1090	22
28	gi|39942328	Vacuolar ATP synthase subunit B	+	56.85	5.33	57.51	5.32	992	27
30	gi|145616020	Acetyl-coa acetyltransferase	+	41.91	6.56	41.13	6.64	469	14
39	gi|351645211	S-formylglutathione hydrolase	+	34.52	6.06	24.12	6.49	577	13
41	gi|351648385	Proteasome component PRE3	+	24.76	5.66	33.87	4.96	626	12
42	gi|39973277	Dihydrodipicolinate synthase	+	33.10	5.92	26.06	6.53	668	13
Translation									
43	gi|351647828	Mitochondrial ribosomal small subunit component	-	43.90	7.66	41.15	6.57	620	24
Transport									
10	gi|58257421	NADH-ubiquinone oxidoreductase-like protein	-	33.35	8.54	22.75	6.29	780	16
Cellular component									
12	gi|15021730	Actin	-	3.97	9.30	45.85	6.77	44	3
16	gi|39951351	Deubiquitination-protection protein dph1	-	42.82	4.89	48.06	4.68	143	6
18	gi|39977911	Alkaline foam protein B	+	16.58	5.70	7.74	4.71	104	4
21	gi|39953425	Transcriptional repressor rco 1	+	66.12	6.39	35.95	5.20	690	26
23	gi|145609229	3-hydroxybutyryl-coa dehydrogenase	-	34.15	8.71	10.75	5.22	614	15
32	gi|39963452	Diphosphomevalonate decarboxylase	+	40.98	5.79	40.79	5.93	660	16
38	gi|39975311	Bacterial hemoglobin	-	48.68	5.84	46.46	6.38	683	20
Unknown									
20	gi|145605595	Conserved hypothetical protein	+	31.38	4.83	37.00	4.87	579	10
24	gi|351644770	Conserved hypothetical protein	+	27.51	5.56	25.67	5.26	261	6
26	gi|145613347	Conserved hypothetical protein	-	35.66	5.91	28.44	5.25	748	14
31	gi|145614628	Conserved hypothetical protein	-	89.23	6.50	72.84	5.83	868	39
35 gi|145613040		Conserved hypothetical protein	+	25.18	5.73	18.50	5.91	659	17
40	gi|145616036	Conserved hypothetical protein	+	23.59	6.3	16.17	6.58	269	8

Loc. No., locus number; MP, number of matched peptides; Ex Mr, experimental molecular weight; Th Mr, theoretical molecular weight; Ex pI, experimental isoelectric point; Th pI, theoretical isoelectric point; +, upregulated proteins; -, downregulated proteins.

DISCUSSION

As the key enzyme in the catabolic process of trehalose, trehalase directly affects trehalose content. Trehalose stores carbohydrates not only to cope with carbon starvation, but also in response to abiotic stress, such as salt and high temperature (Garg et al., 2002; Cortina and Culiáñez-Macià, 2005; Salmerón-Santiago et al., 2011). Our data showed that in response to nitrogen starvation, *M. oryzae* reduced the expression levels of trehalase in order to increase trehalose accumulation to enhance resistance. Fructose-6P synthesis is blocked from glucose-6P by glucose-6-phosphate isomerase, while it is promoted by other pathways involving transaldolase and aldose reductase. In addition, transaldolase plays a key role in a variety of life activities, development, and metabolism. Gene expression levels increase during the early stage of *M. oryzae* infestation. Aldose reductase, in the presence of NADH,

Table 2. Metabolic pathways of mycelial proteins by KEGG analysis in *Magnaporthe oryzae* cultivated with CM and CM-N.

Term	ID	Input number	Background number	P value	Input
Carbon metabolism	mgr01200	8	99	0.002239875	11|39|17|22|30|2|5|8
Butanoate metabolism	mgr00650	3	22	0.016811924	30|27|23
Glyoxylate and dicarboxylate metabolism	mgr00630	3	23	0.018706966	11|2|30
Methane metabolism	mgr00680	3	25	0.022838937	39|2|17
Glycolysis / Gluconeogenesis	mgr00010	3	39	0.064298189	5|17|22
Propanoate metabolism	mgr00640	2	17	0.064561199	27|30
Terpenoid backbone biosynthesis	mgr00900	2	18	0.070732466	32|30
beta-Alanine metabolism	mgr00410	2	18	0.070732466	25|27
Phenylalanine metabolism	mgr00360	2	20	0.08361611	4|23
Pentose phosphate pathway	mgr00030	2	23	0.104136343	8|22
Synthesis and degradation of ketone bodies	mgr00072	1	5	0.125358225	30
Tryptophan metabolism	mgr00380	2	26	0.125857062	30|4
Valine, leucine and isoleucine degradation	mgr00280	2	29	0.148540925	27|30
Pyruvate metabolism	mgr00620	2	33	0.17992082	11|30
Phagosome	mgr04145	2	36	0.204065975	12|28
Cysteine and methionine metabolism	mgr00270	2	38	0.220361583	11|25
Oxidative phosphorylation	mgr00190	3	73	0.234802078	19|10|28
Starch and sucrose metabolism	mgr00500	2	40	0.236765163	7|22
Fatty acid degradation	mgr00071	1	18	0.346125281	30
Lysine degradation	mgr00310	1	18	0.346125281	30
Metabolic pathways	mgr01100	18	734	0.391343851	11|25|27|17|22|23|19|32|30|28|36|3|2|5|4|7|8|10
Glutathione metabolism	mgr00480	1	22	0.402223541	25
Fatty acid metabolism	mgr01212	1	24	0.42846056	30
Citrate cycle (TCA cycle)	mgr00020	1	26	0.453558385	
Glycerolipid metabolism	mgr00561	1	27	0.465695471	3
Tyrosine metabolism	mgr00350	1	27	0.465695471	36
Alanine, aspartate and glutamate metabolism	mgr00250	1	27	0.465695471	27
Biosynthesis of amino acids	mgr01230	3	116	0.491958538	8|5|17
Biosynthesis of secondary metabolites	mgr01110	7	295	0.504013509	11|22|17|32|30|5|8
Proteasome	mgr03050	1	34	0.543505597	41
Arginine and proline metabolism	mgr00330	1	36	0.563601056	25
Amino sugar and nucleotide sugar metabolism	mgr00520	1	41	0.610093064	22
RNA degradation	mgr03018	1	47	0.659457103	17
Protein processing in endoplasmic reticulum	mgr04141	1	74	0.815282306	16
Cell cycle - yeast	mgr04111	1	76	0.823495045	21
Ribosome	mgr03010	1	107	0.91304204	20

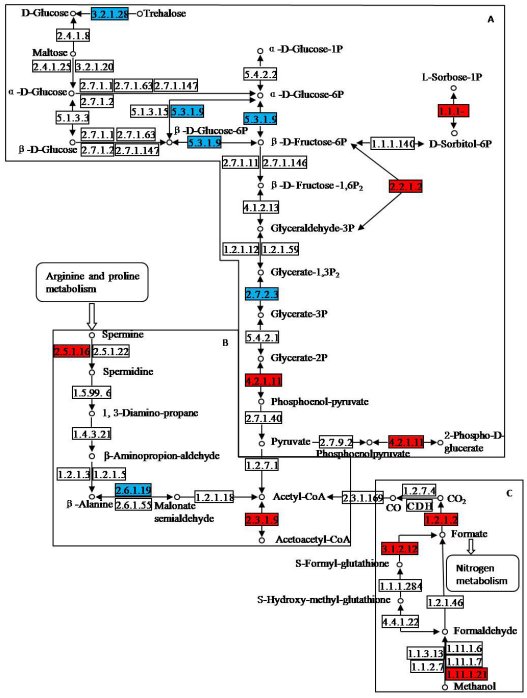

Figure 3. Images of differentially expressed proteins in KEGG pathway from *Magnaporthe oryzae*. Red boxes: up-regulated enzymes, blue boxes: down-regulated enzymes.

deoxygenates glyceraldehyde to glycerol. Glycerol accumulation induces mycelium infection. Moreover, aldose reductase also plays a key role in inducing β-galactosidase and cellulose by lactose. However, cellulose, as a hydrolase of polysaccharides in the plant cell wall, plays an important role in pathogenic formation. Transaldolase and aldose reductase are up-regulated in the mycelium of *M. oryzae* under nitrogen starvation stress conditions, suggesting that *M. oryzae* manipulates nutrient metabolism and pathogenicity by regulating specific reactions of glucose metabolism during nitrogen starvation. As a multi-functional protein, enolase plays an important role in infection with various pathogens (Marcos et al., 2012). This enzyme was up-regulated under nitrogen starvation conditions, which may be related to pathogenicity. Formate dehydrogenase is widely used in NADH recycling. This enzyme oxidizes formic acid to CO_2, while it reduces the coenzyme NAD^+ to NADH. Production of this enzyme marks the start adaptive metabolic processes after *M. oryzae* mycelia are placed into rice internal structures. *S*-Formyl glutathione hydrolase is regulated by carbon and nitrogen source and is involved in the glutathione-dependent formaldehyde oxidation pathway. Its products can be used as carbon sources for growth (Yurimoto et al., 2003). Our results showed that the expression of *S*-formyl glutathione hydrolase in the mycelia was down-regulated under low nitrogen stress, suggesting that *M. oryzae* enhanced nitrogen metabolism in the mycelia and improved the synthetic ability of the desired carbon and nitrogen in order to ameliorate the adaptability of strains to adverse environmental conditions of nitrogen deficiency.

As a host barrier in the infection process, serine proteases function to degrade proteins and are pathogenic determinants. Subtilisin-like serine protease, a pathogenesis-related protein of *M. oryzae*, can regulate *M. oryzae* mycelial growth within the host cell. Dean et al. (2005) showed that the gene for serine protease in *M. oryzae* mycelia was more highly expressed under nitrogen stress conditions, indicating that this gene is associated with pathogenicity. Donofrio et al. (2006) suggested that the subtilase family of *M. oryzae* plays a key role in pathogenesis. Saitoh et al. (2009) showed that subtilisin-like serine protease, encoding gene *SPM1* of *M. oryzae*, is closely related to the growth rate of mycelia, the ascus size of conidia, spore germination, and spore production. It is also a virulence-related gene that plays an important role in the autophagy process of *M. oryzae* infection. In the present study, we found that subtilisin-like serine protease was up-regulated, suggesting that nitrogen affects the expression of subtilisin-like serine protease in the mycelia to enhance disease resistance.

A variety of fungi can secrete oxalic acid during the pathogenic process, leading to hundreds of plant diseases and seriously impair crop production worldwide (Williams et al., 2011). Oxalic acid becomes calcium oxalates by chelating calcium in the cell wall to break the cell wall structure for pathogenic fungal invasion (Heller et al., 2013). Oxalate decarboxylase is an enzyme that can degrade oxalic acid to formic acid and CO_2. Moreover, it can further alleviate fungal diseases to some extent. In this study, oxalate decarboxylase in the mycelia was down-regulated under nitrogen stress conditions, suggesting that *M. oryzae* regulates oxalate decarboxylase content to increase oxalic acid formation.

In the present study, we analyzed total mycelial proteins of *M. oryzae* under nitrogen stress conditions using 2-DE. A total of 23 up-regulated proteins and 20 down-regulated proteins were identified by MS. These proteins were primarily involved in development and metabolic processes of the pathogen. *M. oryzae* altered the process of growth and development of various physiological processes by regulating protein expression to adapt to nitrogen starvation stress and to improve resistance. In future studies, the morphology and pathogenicity of mycelia under nitrogen starvation stress should be investigated. Additionally, the expression of related

proteins identified by proteomics should be verified at the mRNA level using real-time fluorescence quantitative polymerase chain reaction. Specific genes should be also screened to improve the understanding of their functions and underlying molecular pathways.

Conflicts of interest

The authors declared no conflict of interest.

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