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Extraction and characterisation of proteins from a Malaysian Isolate of *Magnaporthe grisea*

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ABSTRACT

Aims: Rice blast, a disease caused by the fungus *Magnaporthe grisea* is one of the serious diseases of rice in the world. The main objective of this study is to isolate and characterise the proteins extracted from the rice blast fungus, *M. grisea* 7.6.
Methodology and results: Through comparative 2-D analyses of the crude protein extracts obtained from this fungus, we were able to identify 88 protein spots through MALDI-TOF. These proteins were then classified into 8 functional groups through the Pfam and KEGG databases into hypothetical, transferases, energy and carbon metabolism, oxidoreductases, molecular chaperone, hydrolases, structural organisation and kinases. The individual protein's functions were then identified and their possible role in pathogenesis, virulence and proliferation of *M. grisea* 7.6 were predicted.
Conclusion, significance and impact of study: Through the assays conducted, we were able to identify some proteins and pathways that could be targeted in developing fungicides and used in future mutagenesis studies.

Keywords: Magnaporthe grisea, protein, elicitor, proliferation, stress

INTRODUCTION

Rice blast disease is the number one disease that causes massive reduction in rice yield worldwide. The causative agent of this disease is a phytopathogenic ascomycete fungus, *Magnaporthe grisea.* The fungus is easily transmitted from one plant to another through spores that are airborne. Hence, once the cultivation area is infected, it may lead to large scale devastation in rice yield. The infection process starts with the attachment of the spores to the host surface, followed by the sensory reaction of the hyphal tip which will determine if the host is appropriate for appressorium formation, penetration and growth (Hamer *et al*., 1988). Appressorium is a specialised structure which assists in the process of penetration into host cells. The appressoria of *M. grisea* are melanin-pigmented and dome-shaped. The functionality of this structure is supported by biosynthesis and availability of various components such as melanin which causes cell wall thickening, and the presentation of turgor pressure by the accumulation of lipid and glycerol within the structure (Howard *et al*., 1991, Kim *et al*., 2004).

Recent studies have been directed towards the molecular mechanisms underlying the infection process of *M. grisea* into the host. Through these molecular studies, infection-related fungal genes have been identified. Functional characterisation of these genes was conducted by researchers through isolation and cloning of genes to determine their contribution to the appressorium formation process (Balhadère *et al*., 2001; Kim *et al*., 2004). Some of the genes that have been isolated and characterised are hydrophobin gene (*MPG1*) (Soanes *et al*., 2008), mitogen-activated protein kinase gene (*PMK1*) (Bruno *et al.*, 2004), a subunit of trimeric G-protein, and adenylate cyclase (Ramanujam *et al*., 2013). These genes collectively have been shown to play a role in the formation of the appressorium. Genes involved in melanin biosynthesis and enzymes associated with peroxisomal fatty acid β-oxidation were also demonstrated to be important in appressorium formation (Wang *et al*., 2007). The characterisation of genes like subtilisin protease (*SPM1*) and a NAD specific glutamate dehydrogenase (*Mgd1*) revealed the importance of protein degradation and amino acid metabolism in the process of infection (Oh *et al.*, 2008). High-throughput approaches have also been used recently to isolate the genes involved in *M. grisea* appressorium formation and pathogenicity. These include the generation of expressed sequence tags, microarrays (Wang *et al*., 2017) and the use of the SAGE technique (Irie *et al*., 2003).

In the era of post genomic studies, proteomic analysis contributes towards the understanding of the organism's functionality. *M. grisea* contains vast amounts of secreted

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and unsecreted proteins, and therefore the study of its proteome will enable us to draw some correlation between the proteins and their function. Proteomic studies on rust fungi identified proteins that show stage specific localisation in infected host cells (Kemen *et al.*, 2005). Likewise, studies have also been conducted on *M. grisea* and *M. oryzae* where the protein constituents have been studied in these isolates. Through the studies done by Dean *et al.* (2005), a large number of these proteins have been identified in *M. grisea* 70-15 with specialised functions in pathogenicity and virulence. Here we have subjected our own field isolate to protein isolation and characterisation for the identification of proteins that may play a critical role in fungal pathogenicity and virulence. However, among all the proteins isolated, only a few were known and characterised to be involved in appressorium formation through annotation against the published genome sequence of *M. grisea* (Kim *et al*., 2004; Zhou *et al*., 2016).

In this article, we report the extraction and characterisation of crude proteins from *M. grisea* 7.6. The findings of our research facilitate the identification of a plethora of proteins in this local rice blast isolate and further characterises the function of these proteins into the survival and infectivity of the pathogen. It is hoped that through the understanding of these proteins, novel strategies for controlling plant disease (Dixon, 2001) may be developed. The findings of this study have lead to further analysis of key pathways that are important in lipid and glycerol metabolism and biosynthesis.

MATERIALS AND METHODS

Source and growth of fungi

The *M. grisea* 7.6 isolate was obtained from the MARDI station at Bumbung Lima, Seberang Perai, Malaysia. The isolate was grown on potato dextrose agar (PDA) at 28 °C for seven days and the resulting mycelia was harvested for its spores and inoculated aseptically into yeast peptone glucose (YPG) medium for seven days with agitation at 120 rpm, at 28 °C until mycelia balls were formed.

Extraction of total protein

The mycelium obtained from the liquid media above is then washed thoroughly with sterile distilled water and strained. The resulting mat is then ground in a sterile mortar using liquid nitrogen. The ground tissue is then subjected to total protein extraction from 1 g of fungal mycelia using the method employed by Bhadauria *et al*. (2010).

Determination of protein concentration in sample

Purified elicitor and glycoprotein concentration were determined via the 2D-Quan Kit (GE Healthcare, Uppsala, Sweden). A Standard Curve was prepared using the bovine serum albumin provided in the kit (2 mg/mL).

Preparation of protein extract

The EttanIPGphor III system (GE Healthcare, Uppsala, Sweden) was used. The IPG Strip (pH 4-7 Linear, 18 cm) was hydrated overnight with 340 µL hydration buffer containing 200 µg protein. Focusing was carried out at 300 V for one hour, 500 V for two hours, six hours at 1,000 V (gradient / gradient mode), three hours at 8, 000 V (gradient mode / gradient) and finally 8,000 V for 70 minutes. After isoelectric focusing (IEF), the strips were equilibrated twice in the SDS buffer with gentle agitation. The first equilibration was carried out in a solution containing 50 mM Tris-HCl pH 8.8), 6 M urea, 30% v/v glycerol, 2% w/v SDS, 0.002% w/v bromophenol blue, and 1% w/v DTT. In the second step, DTT was replaced with 2.5% w/v iodoacetamide. Separation of the second dimension was with the Ettan Daltsix Large Vertical System (GE Healthcare, Uppsala, Sweden). The second dimension SDS-PAGE run was at 15 °C with 1 W / gel for 1 hour and is followed by 13 W / gel. SDS-PAGE was conducted with 12% gels using 120 V for 0.5 h, followed by 240 V, until the bromophenol blue dye front left the gel. Protein spots in gels were visualised by silver staining. Each experiment was repeated three times at a biological level.

Imaging and analysis of proteins

The protein spots were visualised with Silver staining. Gels were scanned at 300 dots per inch (dots per inch, dpi) resolution using ImageScanner III LabScanTM, Version 6.0 (GE Healthcare, Uppsala, Sweden) and analysis of protein spots in 2-DE gel runs were conducted with Image MasterTM 2- D Platinum, Version 6.0 (GE Healthcare, USA). Cut-off spots were placed inside the ZipPlate (wells) for washing and hydration with 100 µL Buffer 1 [25 mM ammonium bicarbonate (ABC) / 5% acetonitrile] for 30 minutes. The wash is repeated twice and finally 200 µL of 100% acetonitrile was added into the ZipPlate well, incubated, and vacuum dried. This was followed by trypsin (11 ng / uL) digestion in ABC 25 mM buffer with overnight incubation at 30 °C. Extraction was performed by inserting 8 µL acetonitrile into the resin in the well using the pipette. This was followed by two washes using 100 µL extraction / washing solution (0.2% TFA) that was added to each well and incubated at room temperature for 30 min. First wash was removed through partial vacuum while second through full vacuum. Twenty microlitre elution solution (0.1% TFA / 50% acetonitrile) was and vacuumed to exclude peptides from microtiter plates. Peptide extract is evaporated through flushing at room temperature. The extract was dissolved in 1 µL matrix solution [5 mg/mL a-siano-4-hydroxyisinamic acid (CHCA) in 0.1% TFA, 50% ACN in MilliQ water].

Identification of proteins MALDI TOF

The protein from SDS-PAGE is cut and floated in distilled water in microfib tubes and then sent to Protein and Proteomics Center at National University of Singapore

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(NUS) for MALDI-TOF/ TOF analysis. The protein pack identifier is achieved using MALDI mass spectrum data and MASCOT search engine (Matrix Science, London, United Kingdom) against NCBI_100907 database (11613246 sequence; 3967887859 residue). The digestive enzyme used is trypsin (Promega, USA). A fixed modification of cysteine residues is carboxymethyl. The peptide identifier is performed on the basis of the presence of a tryptic peptide with no more than one splice site omitted. Peptide mass tolerance is ± 100 ppm and fragment mass for monochromatic peptide is ± 0.2 D (according to MALDI tool sensitivity).

RESULTS AND DISCUSSION

Identification of total protein from *M. grisea* 7.6

Total protein of *M. grisea* 7.6 was extracted and identified using 2D-PAGE and MALDI-TOF. Mascot search was conducted using the NCBI nr 100907 database (11613246 sequence; 3967887859 residue) (ftp://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/nr.gz) and repeated with sequential data that is contained in the Genome Initiative / Broad Institute of MIT and Harvard (www.broadinstitute.org). Out of the 328 spots produced on 2D-PAGE gel, only 88 protein spots have been identified (Figure 1) and the remaining 240 of spots were not identifiable (no hits - data not shown). The protein spots in the SDS PAGE gel ranged from 5 kDa to 118 kDa while the 88 identifiable spots ranged from 10 - 110 kDa. This result resembles the findings made by Bhadauria *et al*. (2010) who reported the formation of proteins in the range of 14.4 - 97 kDa using the same approach to *M. oryzae* P131 fungus. The result of the molecular weight distribution of protein molecules from this study shows that 61% of the protein is below 50 kDa. These findings are in line with the study of Bhadauria *et al*. (2010) which reported that more than half of the isolated proteins are below 50 kDa.

The identified protein spots were grouped into eight functional groups as depicted in Figure 2 using protein sequences from Pfam database (http://pfam.xfam.org/) and KEGG (http://www.genome.jp/kegg/). Figure 2 provides details of the eight groups and their percentages. Scores for these proteins ranged from 38 to 702 while E values were reported between 1.0 × 10-171 - 9.00E-53 for these proteins. The protein score is the combined score of all mass spectra that can be matched with the sequence of amino acids of the protein. Higher scores indicate higher confidence. The E value decreases exponentially when the match score increases. The value of E <0.1 and closer to zero gives a higher level of confidence. The score and E values obtained from this study indicates that over 95% of the identified proteins are within the range of high confidence level (Table 1).

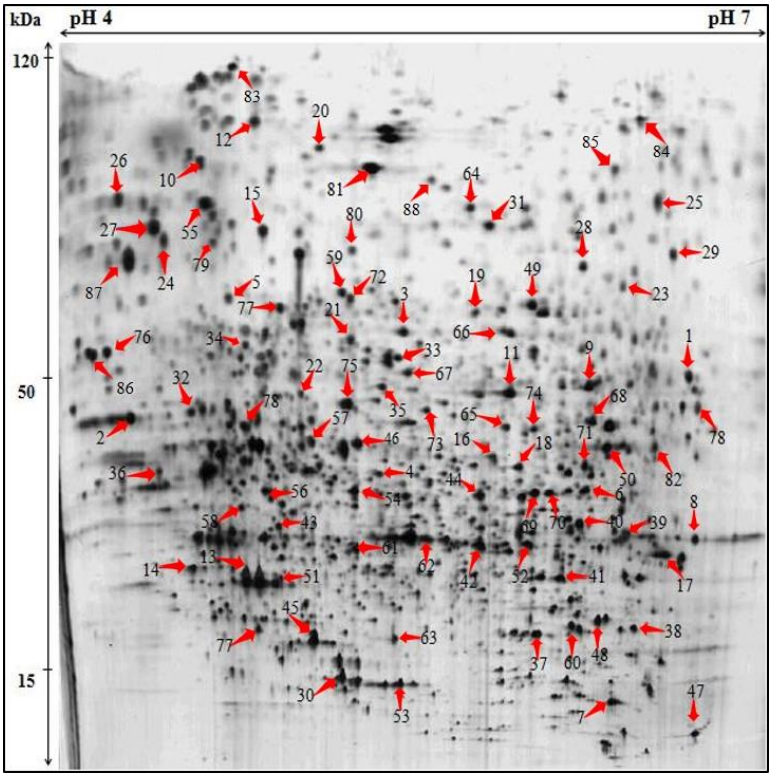

Figure 1: SDS-PAGE profile showing the protein spots derived from the total protein extract from *M. grisea* 7.6. These spots are references in Table 1.

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Table 1: Protein spots identified and segregated into eight functional groups based on KEGG.

Spot No	Loc No (GI)	Name	Score	E Value	Function
HYPOTHETICAL					
66	39942112	Hypothetical protein MG03136.4	283	1.00E-10	
77	39974503	Hypothetical protein MG00602.4	561	1.00E-158	
36	39945234	Hypothetical protein MG04599.4	300	6.00E-80	
25	39960644	Hypothetical protein MG09324.4	233	1.00E-118	
76	39943026	Hypothetical protein MG03593.4	586	1.00E-166	
57	39940654	Hypothetical protein MG04913.4	117	3.00E-39	
7	39962053	Hypothetical protein MG09588.4; MGCH7_ch7g15	184	8.00E-45	
67	39956960	Hypothetical protein MG09134.4; MGCH7_ch7g939	167	9.00E-53	
26	39977337	Hypothetical protein MG06571.4	140	8.00E-32	
88	39974523	Hypothetical protein MG00592.4	485	1.00E-135	
56	39941354	Predicted protein [*Magnaporthe grisea* 70-15]	58	7.00E-07	
55	39974153	Hypothetical protein MG00777.4	166	7.00E-73	
83	39973059	Hypothetical protein MG07824.4	264	8.00E-78	
60	39977527	Hypothetical protein MG06648.4	587	1.00E-166	
44	39945398	Hypothetical protein MG04681.4	180	7.00E-86	
13	39951779	Predicted protein	320	7.00E-86	
85	39977531	Hypothetical protein MG06650.4	293	1.00E-147	
82	39942498	Hypothetical protein MG03329.4	424	1.00E-117	
27	39939866	Hypothetical protein MG05307.4	494	1.00E-138	
38	39951929	Hypothetical protein MG01607.4	314	1.00E-162	
12	39940006	Hypothetical protein MG05237.4	306	1.00E-110	
54	39944992	Hypothetical protein MG04478.4	602	1.00E-171	
69	39965500	Hypothetical protein MG09952.4	308	9.00E-96	
2	39942216	Hypothetical protein MG03188.4	147	1.00E-33	
78	39970317	Hypothetical protein MG02625.4; Hypothetical protein MGCH7_ch7g349	200	2.00E-85	
29	39969253	Hypothetical protein MG10237.4; Hypothetical protein MGCH7_ch7g1005	496	1.00E-139	
70	39975769	Hypothetical protein MG06189.4; ECM33 [*Magnaporthe grisea*]	287	1.00E-124	
15	85091324	Hypothetical protein	165	4.00E-39	
84	39973387	Hypothetical protein MG01160.4; Hypothetical protein MG06293.4	189	2.00E-46	
81	85107523	Hypothetical protein [*Neurospora crassa* OR74A]	434	1.00E-120	
31	39951911	Hypothetical protein MG01598.4	377	1.00E-125	

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TRANSFERASE					
68	39944970	MG04467.4; protein like acidic ribosomal protein 60S	502	1.00E-150	
53	39973575	MG01066.4; protein like S23 ribosomal protein 40S	173	3.00E-72	
30	39940004	MG05238.4; protein like S14 ribosomal protein 40S	209	2.00E-52	
72	39970385	MG02659.4; protein like L14-A ribosomal protein 60S	206	2.00E-71	
52	39970561	MG02747.4; protein like S4-A ribosomal protein 40S	237	6.00E-61	
28	39939888	MG05296.4; protein like L34-B ribosomal protein 60S	126	1.00E-27	
1	39958032	MG09222.4; protein like S2 ribosomal protein 40S	303	1.00E-24	Resistance to antimicrobial agents (Das *et al*., 2013)
71	39978007	MG06888.4; Ribosomal Protein	483	1.00E-135	Regulation of RNA for the success of the infection process (Hafrén *et al*., 2013).
37	59803150	Protein like S28 ribosomal protein 40S [*Magnaporthe grisea*]	154	5.00E-36	
14	39957705	MG09194.4; protein like L17 ribosomal protein 60S	131	1.00E-54	
75	39977153	MG06479.4; protein like S22 ribosomal protein 40S	127	2.00E-37	
51	39968599	MG02392.4; protein like S30 ribosomal protein 40S	96	2.00E-18	
59	464706	S15 ribosomal 40S; protein S12 ribosomal cytoplasmic	128	5.00E-28	
74	5423321	50S ribosomal protein L27	67	2.00E-28	Resistance to antimicrobial agents (Das *et al*., 2013)
4	115399246	Peroxisomal carnitine acetyl transferase	8	1.00E-78	Formation of penetration hyphae during plant infection by *M. grisea* (Bhambra *et al*., 2006).
73	39943122	MG03641.4; protein like elongation factor 1-alpha	450	1.00E-125	Antimicrobial agents (Li *et al*., 2013).
50	39959465	MG09432.4; protein like elongation factor 3	459	1.00E-128	Pathogenic elicitors (PAMP) and induces defensive response in host (Kunze *et al*., 2004).
39	39944908	MG04436.4; protein like elongation factor 1-beta	386	1.00E-125	Required for fungi survival (Chakraburtty, 2001).
34	3265058	Combination monoubiquitin/carboxy [*Botryotinia fuckeliana*]	244	4.00E-68	Resistance to high temperatures, nutrient deficiencies, and production of radical oxygen species (ROS) (Finley *et al*., 1987).
35	85077292	Histone H4 [*Neurospora crassa* OR74A]	107	4.00E-44	
16	85097316	Protein [*Neurospora crassa* OR74A] Thiolase	493	1.00E-138	Role in fatty acid oxidation but not in virulence (Otzen *et al*., 2013).
ENERGY AND CARBON METABOLISM					
58	39940690	MG04895.4; isocitrate lyase (ICL)	376	1.00E-167	Lipid biosynthesis and turgor pressure for infiltration into host (Sexton and Howlett, 2006).
87	39970315	MG02624.4; protein like transaldolase	522	1.00E-147	Energy supply for growth and development for infection process (Ling *et al*., 2007).
3	39970315	MGG_03347; Transaldolase	70	2.00E-68	
49	85107523	Monosacccharide Transport protein [*Neurospora crassa* OR74A]	434	1.00E-120	Unknown
17	70999466	Aldolase fructose-bisphosphate, Class II [*Aspergillus fumigatus* Af293]	155	1.00E-100	Mutants impaired utilisation of pyruvate and malate and exopolysaccharide (EPS) production leading to inactivation of hypersensitive cell death and ROS (Thomas *et al*., 2002).
79	108862150	Expressed facilitator superfamily protein	66	2.00E-09	Unknown
47	39972043	MG07337.4; protein like oleate induced	137	6.00E-55	Protection from host defence systems (Gabaldón, 2010).

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		peroxisomal protein			Mutants loss of lipid utilisation, resistance to H_2O_2. And lack of melanin and turgor pressure (Jedd, 2011).
61	39970487	MGG-02710; Probable Peroxisomal Membrane Protein	56	1.00E-66	Glyoxylate cycle progression and is involved in mitochondrial metabolism (Kunze *et al*., 2006).
5	39977543	MG06656.4; protein like ADP/ATP carrier protein	368	1.00E-100	Induces appressorium formation (Irie *et al.*, 2003) and increases turgor pressure during proliferation (Thomas *et al*., 2002; Palmieri, 2013).
33	39945136	MG04550.4 ATPase	465	1.00E-129	Functional morphology and pathogenicity during infection. Cell wall integrity, aperture formation and melanin biosynthesis (Gilbert *et al*., 2006; Chen *et al*., 2013).
19	39974277	MG00715.4; glucose repressed gene – protein like protein	69	2.00E-23	Glucose to control pathogen growth
48	39942882	MG03521.4; protein like aconitate hydratase	702	0	Isomerisation process in Krebs cycle
86	39972007	MG07319.4; protein like white colar 2 protein	157	9.00E-169	Produces light inputs for adaptation and survival of fungi in the environment, and secondary metabolite formation (Bodor *et al.*, 2013).
32	39978185	MG06977.4; protein like beta succinyl-CoA ligase	457	1.00E-127	Important for the pathogenicity and survival of pathogens in the host (Sasikaran *et al*., 2014).
18	109940168	OXIDOREDUCTASE Superoxide dismutase [Cu-Zn]	45	0.006	Protects from plant host defence system. Deletion mutants showed reduction in pathogenicity (Lanfranco *et al*., 2005).
6	92870669	Aldo/keto reductase [*Medicago truncatula*]	137	1.00E-50	Associated with carbohydrate metabolism and glycerol production for proper organ formation and infection (Cobos *et al*., 2010).
40	968996	Glyceraldehyde-3-phosphate dehydrogenase	99	3.00E-19	Adhesion and evolution of organisms (Elkhalfi *et al*., 2013).
64	39973539	MG01084.4; protein like Glyceraldehyde-3-phosphate dehydrogenase	458	1.00E-127	Protects from the host defensive reaction (Zeng *et al*., 2006).
11	39946672	MG08564.4; NADPH and sacropine reductase 68 from *Magnaporthe grisea*	223	8.00E-97	Condenses α-aminodipat-δ-sepia aldehydes (AASA) with glutamic acid followed by a decrease with NADPH Schiff base to produce a perharopine L-lisin
41	145611200	MGG_00588; NADH:ubiquinone oxidoreductase	425	2.00E-40	Generation of a proton gradient used for ATP synthesis
80	39943078	MG03619.4; protein like FAD dependent oxidoreductase	702	0	
62	145608286	MGG_12749:glutatione disulfide reductase	421	1.00E-50	Stress adaptation in yeast (Grant, 2001)
21	39969787	MG10503.4; protein like manitol-1-phosphate dehydrogenase	486	1.00E-136	Many functions-carbohydrate storage, morphogenesis, conjugation, environmental protection and protection of ROS (Solomon *et al*., 2005).

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HYDROLASES					
22	39971571	MG07101.4; protein like mannan endo-1,6-alpha mannosidase	626	1.00E-178	Involved in cell wall degradation (Li *et al*., 2011; Ross-Davis *et al*., 2013)
42	39969609	MG10414.4; vacuolar triacylglycerol lipase	463	1.00E-176	Functions in lipid and glycerol lipolysis, turgor pressure and pathogenicity (Dean *et al*., 2005; Zechner *et al*., 2012).
65	1456022686	MGG_13009; Glycoside hydrolase,family 38	45	2.00E-90	Degradation of cell wall domain (Seidl *et al*., 2011; Geethu *et al*., 2013).
10	145608534	MGG_12798; Extracellular lipase, putative	83	2.00E-100	Lipolysis of lipid and glycerols in the vacuole to provide turgor pressure for penetration into host (Dean *et al*., 2005; Zechner *et al*., 2012)
63	39973165	MGG-07877; Esterase/lipase	82	5.00E-76	
		MOLECULAR CHAPERONES			
46	39969675	MG10447.4; Cyclophane	200	1.00E-104	Involved in signal transduction, protein clustering (Krücken *et al*., 2009), oxidative stress response and reconstructing receptor complex (Boldbaatar *et al*., 2008).
20	39973863	MG00922.4; protein like vacuolar protease A	579	1.00E-164	Reported in *M. oryzae* to retard entry and the infection process (Saitoh *et al*., 2009)
9	39944360	MG04191.4; heat shock like protein SSC1	602	1.00E-171	Role in fungal growth, melanin biosynthesis and temperatures sensitivities (Li *et al*., 2011).
43	145603594	MGG_113250; Nuetral protease 1	53	1.00E-125	Involved in protein folding (Oh *et al.*, 2008)
24	39975085	MGG-00311; Acid Protease	88	2.00E-100	
STRUCTURAL ORGANISATION					
45	39974653	MG00527.4; outer cell matrix protein	191	8.00E-97	Involved in germination and infection - supports spore attachment on plant surfaces.
8	39978189	MG06979.4; Synthetic YOP1 addition protein (SEY1)	330	1.00E-139	Survival in external harsh environment (Ngamskulrungroj *et al.,* 2012)
		KINASE			
23	39952359	MG01822.4; MAP kinase	253	3.00E-75	Responds to various stimuli such as mitogen, osmotic pressure, heat shock, profile regeneration, gene expression, cell growth, cell death, and apoptosis (Turrá *et al*., 2014).

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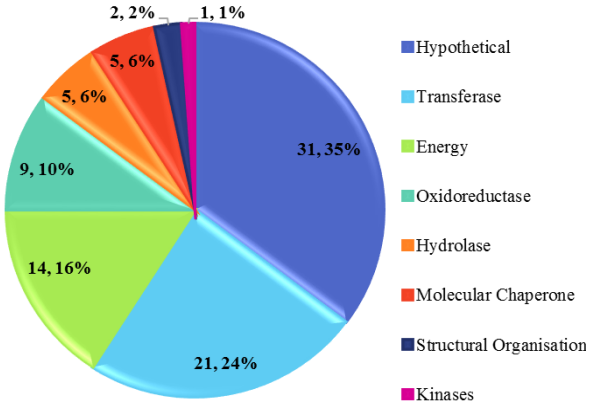

Figure 2: The segregation of the 88 identified proteins spots into functional groups based on analysis via KEGG (http://www.genome.jp/kegg/).

The functional groups

i. Hypothetical

Of the 88 proteins identified through 2D-PAGE and MALDI TOF methods, 31 of them are hypothetical proteins. This is 35% of the total protein identified from this study. Table 1 provides the Loc ID, scores and E values for all 31 proteins.

ii. Transferase

The second largest cluster is transferase with 21 proteins forming 24% of the identified proteins (Table 1). Bhambra *et al*. (2006) have shown in their studies that the transfer of acetyl carnitine is important in the functionality of the *M. grisea* appressorium. Transferase activity is controlled by the *PTH2* gene and the mutant without this gene lost its polarity, no transferase activity of acetyl carnitine and therefore could not use certain lipid substrates. Transferases are reported to be involved in the protection against chemical and oxidative stresses as well as resistance to antimicrobial substances (Allocati *et al*., 2008). The transferase group has ribosomal proteins, elongation factors, thiolases and ubiquitination proteins. The large amount of ribosomal proteins identified in this study may also be associated with resistance to antimicrobial agents (Das *et al*., 2013) and regulation of RNA as an external ribosomal protein which may be important in the infection process (Hafrén *et al*., 2013). The elongation factors (EFs) are important for the process of decomposition, purification and resistance to antimicrobial agents (Navarre *et al*., 2010; Li *et al*., 2013). EF3 is found in Ascomycetes and may be required by *M. grisea* 7.6 for its survival (Blakely *et al*., 2001; Chakraburtty, 2001). Further the ubiquitin complex has been shown to play a role in pathogenic resistance to high temperatures, nutrient deficiencies, catalytic decline and production of radical oxygen species (ROS) (Watt and Piper, 1997). Another protein that was found in this group is the ribosomal protein L27 which has been reported to be involved in 50S subunit assembly and the peptidyl transferase reaction. This protein is a secretome in *M. oryzae* and has a role in the fungus cellular function (Wower *et al.*, 1998). Peroxisomal carnitine acetyl transferase is required for elaboration of penetration hyphae during plant infection by *M. grisea* (Bhambra *et al*., 2006). Finally, thiolases are involved in fatty acid oxidation but not in virulence (Otzen *et al*., 2013) (refer to Table 1).

iii. Energy and carbon metabolism

The third largest cluster is energy and carbon metabolism (16%). This group has various members such as isocitrate lyase (ICL) which serves to catalyse the isocitric division into glyoxylate and succinate. This enzyme can synthesize C4 carboxylic acids from acetate through the modified tricarboxylic acid cycle (TCA). This cycle works for carbon assimilation from C2 compounds and allows microbes to refill TCA intermediates for gluconeogenesis and various other biosynthesis (Cozzone, 1998). During infection by *M. grisea,* ICL gene expression enhances the process of producing conidium, appressorium, mycelium and hyphae. Mutant studies by removing the *ICL1 M. grisea* gene results in reduction in the formation of appressorium, conidiogenesis, cuticle and reduction of general damage to rice and barley. Degradation of lipid storage contributes to turgor generation in the development of appressoria in *M. grisea* (Sexton and Howlett, 2006). The *icl1* mutants were found to fail to grow on fatty acids and acetate. Lesions are less on hosts and mutants fail to produce penetration (Idnurm and Howlett, 2002). All other members of this group are described briefly in following paragraphs and table 1.

Transaldolases (TALs) may function in energy supply for growth and development of *M. grisea*. TALs involvement in haustoria and infection structure development is indicated in *Blumeria graminis* f. sp. hordei and *Puccinia* f. sp. tritici (Broeker *et al*., 2006; Ling *et al*., 2007). In addition, TALs play a key role in a variety of life activities, development, and metabolism (Zhou *et al.*, 2016). The fructose 1.6 biphosphate aldolase (FBA) class II enzyme is critical for glycolysis or gluconeogenesis (Capodagli *et al*., 2013) in *M. oryzae* (Mandelc *et al*., 2009). In *Xanthomonas oryzae* pv. *oryzicola* FBA mutants were impaired in utilisation of pyruvate and malate and exopolysaccharide (EPS) production leading to loss of hypersensitive cell death and ROS (Thomas *et al*., 2002). Further, peroxisomes also contain oxidative stress enzymes such as H_2O_2 which may play a role in protection of *M. grisea* from host defence systems (de Duve, 2007; Gabaldón, 2010). The *PDE1* gene in *M. grisea* encodes a type of ATPase which is important for the maintenance of phospholipid asymmetry in the membrane and is responsible for production of penetration hypha during infection (Balhadère *et al*., 2001). MgATP2 is required for exocytosis process, root and foliar infections and the host defence activation. Vacuolar ATPase (V-ATPase) protein

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in *M. oryzae* is associated with functional morphology and pathogenicity during infection. It is also associated with cell wall integrity, aperture formation and melanin biosynthesis through cAMP (Gilbert *et al.,* 2006; Chen *et al*., 2013). Probable peroxisomal membrane protein has been implicated in the glyoxylate cycle progression and is also dependent upon mitochondrial metabolism. Metabolite transporters on the peroxisomal membrane are involved in this cycle (Kunze *et al*., 2006).

The suppressed glucose-like protein (GI39974277) is activated in the medium or environment where glucose is present. The suppression of glucose in the fungus reduces fungal growth. This gene is associated with energy and carbon mobilisation for fungal growth (McNally and Free, 1988). Another member, aconitate hydratase catalyses the isomerisation process in Krebs cycle (Beinert *et al*., 1993). Aconitase has been reported in various pathogens where it is involved in the production of oxalates for the Krebs cycle (Rio *et al*., 2008). The white-collar protein 2 (WCC) is an important component in producing light inputs for various types of blue light, including light chronobiology in the circadian rhythm, adaptation and survival of fungi in the environment, oxidative stress control, hypha development, and secondary metabolite formation (Rodriguez-Romero *et al.*, 2010; Ruger-Herreros *et al*., 2011; Bodor *et al*., 2013). Finally, we have Succinyl CoA that participates in the site of the itaconate degradation pathway, which is important for the pathogenicity, and survival of pathogens in the host (Sasikaran *et al*., 2014).

iv. Oxidoreductase

Seven proteins (9%) have been classified into oxidoreductase. Oxidoreductases are enzymes that catalyse the oxidation and reduction reactions that involve the transfer of electrons. Superoxide dismutase (GI 109940168) is responsible for the main line of defence against radical anoxic oxide and oxygen reactive species (Zelko *et al*., 2002). SOD protects *M. grisea* 7.6 from the plant host defence system. SsSOD1 deletion mutation studies showed an increase in heavy metal and oxidative pressures and reduction in pathogenicity of the pathogens (Lanfranco *et al*., 2005). The aldo / keto reductase (AR) (GI 92870669) are associated with carbohydrate metabolism and glycerol production in *M. grisea* to enable proper organ formation and infection (Cobos *et al*., 2010). Mannitol and its metabolism have many functions in the fungus which includes carbohydrate storage, NADPH regeneration, morphogenesis, conjugation, environmental protection and protection from ROS (Solomon *et al*., 2007). Glutatione disulfide reductases have been reported to be involved in stress adaptation in yeast (Grant, 2001). The NADH:ubiquinone oxidoreductase (Complex I), provides the input to the respiratory chain from the NAD-linked dehydrogenases of the citric acid cycle. The complex couples the oxidation of NADH and the reduction of ubiquinone, to the generation of a proton gradient which is then used for ATP synthesis. During biotrophic or necrotrophic fungal infections on plants, the level of mannitol was found to increase dramatically and was accompanied by increased gene expression mannitol-1-phosphate 5- dehydrogenase (*Mpd1*) (Solomon *et al*., 2005).

v. Hydrolase

The hydrolase group has two protein members (3%) (GI 39971571, 39969609). Pathogenic fungi such as *M. grisea* secretes various glycosides hydrolase, polysaccharides and esterase to degrade plant cell walls, form necrotic lesions and for conidiogenesis (Soanes, 2008; Cantu *et al*., 2008). Glycoside hydrolases (GH) are the most diverse group of enzymes used by microbes in the degradation of biomass. Over a hundred GH families have been classified to date (Murphy *et al*., 2011). Degradation of cell wall domains by cutinase, pectate lyase and hydrolase have been found in *M. grisea*, *Phytophthora* spp., *Fusarium oxysporum*, *Myrothecium verrucaria*, *Pythium myriotylum*, and *Verticillium alboatrum* (Seidl *et al*., 2011; Geethu *et al*., 2013). Similarly, mannan endo-1,6-alpha-mannosidase assist with colonisation through degradation of plant cell wall (Zerillo *et al*., 2013). Another member of this group, triacylglycerol (TAG) results in the lipolysis of lipid and glycerols in the vacuole to provide turgor pressure for penetration into host (Dean *et al*., 2005; Zechner *et al*., 2012). This function is also shared by the lipases in fungi.

vi. Molecular chaperones

Molecular chaperones which make up 4% of proteins are involved in protein-coordinated interactions (Kim *et al*., 2013). Cyclophilin is a peptidyl-prolyl-cis-trans isomerase that is involved in signal transduction, protein clustering (Krücken *et al*., 2009), oxidative stress response and reconstructing receptor complex (Boldbaatar *et al*., 2008). They have been shown to participate in growth, morphogenesis and evolution of plant pathogenic fungi such as *Botrytis cinera*, *Moniliophthora perniciosa* and *M. grisea.* The study on *M. grisea* mutants showed a reduction in the evolution and inhibition of penetration peg and pressure. The Lhs1p chaperone molecule is required by *M. oryzae* for pathogenesis, penetration and growth in the host (Vanghele and Ganea, 2010; Monzani *et al*., 2011). Another protein in this group, the vacuolar protease A (SPM1) has been reported in *M. oryzae* to retard entry and the infection process (Saitoh *et al*., 2009). Proteases are needed to assist with the proper folding of proteins. Finally, heat shock proteins (hsp) play a role in the process of conjugation and morphology of fungi on the media. Recently, another hsp (*MoSFL1* gene) was found in *M. oryzae*. Mutant studies on this gene shows the inhibition of fungal growth in the host, the reduction of melanin production and increased sensitivity to high temperatures (Li *et al*., 2011). Nascent polypeptides interact cotranslationally with a first set of chaperones, such as heat shock protein to prevent premature (mis) folding of proteins.

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vii. Structure organisation

The second smallest group is a structural organisation with two protein members (3%) which contains external cell matrix (ECM) and SEY1 protein. The external cell matrix is needed in the process of germination and infection. The ECM supports the spore attachment on plant surfaces. They SEY1 protein has GTPase activity and can assist with survival in external harsh environment (Ngamskulrungroj *et al*., 2012).

viii. Kinase

The smallest and most recent group is kinase with only one protein (1%). MAP kinase (GI 39952359) is a specific enzyme of the kinase class, which is the specific protein serine/ treonine/ tyrosine (Manning *et al*., 2002). These enzymes are involved in producing cell responses to various types of stimuli such as mitogen, osmotic pressure, heat shock, proinflamatory cytokines, profile regeneration, gene expression, cell growth, cell death, differentiation, mitosis, cell independence and apoptosis (Turrá *et al*., 2014). Xu and Hamer (1996) have shown the importance of MAPK in the formation of appressorium and *M. grisea* growth through the *PMK1* gene. These non-genetic mutants fail to form appressoria and grow inside the host and this proves the importance of MAPK in fungal pathogenesis. Gene *Mps1* (*M. grisea*) (Xu *et al*., 1998) and *CHK1* (*Cochliobolus heterostrophus*) are involved in appressoria formation.

Based on the functional groups and the proteins identified in this study, a diagrammatic representation of how these groups interact to enable the living, infection and proliferation of *M. grisea* has been illustrated in Figure 3. Through the proteins that were identified, we can conclude that almost all functional groups contribute collectively toward the infection and proliferation of the fungi into the host. This would include the contribution towards lipid and glycerol biosynthesis and metabolism which links directly to the provision of turgor pressure that is needed in the penetration and proliferation into the host. Some of these proteins are also involved in melanin biosynthesis that is connected to the sporulation and germination of the fungal spores. In addition, all groups except kinases, have been implicated in the fungal adaptation to stresses in environment, which could include nutrient stress and desiccation amongst others. Finally, an essential component of all cellular, molecular and biological would involve the process of signalling. Here the molecular chaperones and kinases are the two key players in executing this service. From previous reports by other researchers in the field, they have implicated hydrolase, transferases, energy and metabolism and molecular chaperones as possible targets that may be used in the development of fungicides. Meanwhile for mutational study candidates may be identified from all functional groups to generate defective mutant lines that may be utilised in understanding the function of the gene and also production of defective mutant lines that may be of use in the agricultural industry.

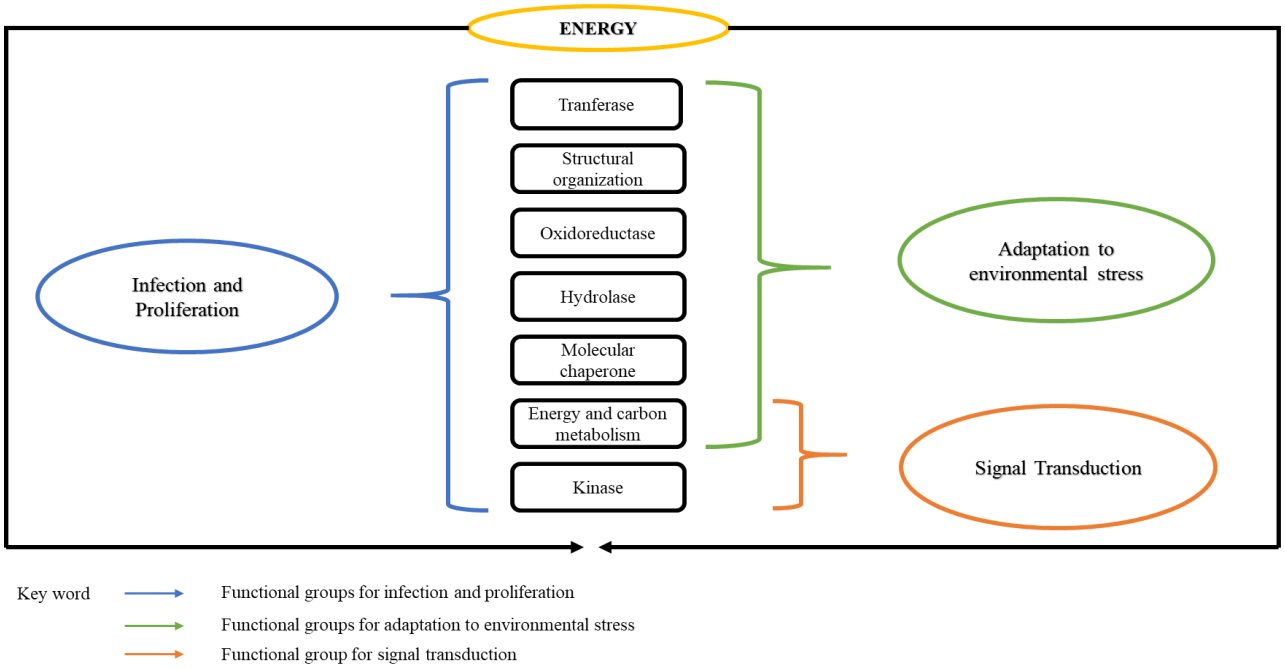

Figure 3: Pathway showing the connection between the functional groups and their functions and possible downstream application.

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CONCLUSION

In this study, total proteins were successfully extracted and identified from *M. grisea* 7.6. Eighty-eight proteins were classified into eight functional groups and the proteins and groups were linked into an interactive diagramme connecting each process towards their functionality in *M. grisea* 7.6. Proteins and pathways associated with such functionality may be targeted for the development of new fungicides or candidates for fungal mutagenesis studies. In our laboratory, mutational studies have been directed towards lipid biosynthesis and metabolism. Work is on the way to elucidate the function on key players in this pathway.

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