ORIGINAL ARTICLE

Proteomic analysis of phytopathogenic fungus *Macrophomina phaseolina* identify known and novel mycelial proteins with roles in growth and virulence

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Abstract

Macrophomina phaseolina is a haploid, monotypic and clonally reproducing necrotrophic phytopathogenic fungus with widespread geographic distribution. Mycelia of this ascomycete play important role in propagation and pathogenicity providing insights into the mechanism of fungal growth and development. Despite its importance, proteomic analysis of *Macrophomina* mycelia is still not studied. Here, we have developed the first reference mycelial proteome map of *M. phaseolina* using 1-DE followed by MS/MS analysis. In total, 853 mycelial proteins were identified in three biological replicates. Gene ontology annotation depicted that most of these proteins function in biological processes related to translation and transcription initiation. In terms of molecular function, oxidoreductase activity represents the major biochemical reactions in fungal mycelia. KEGG pathway analysis further revealed that the mycelial proteins were primarily involved in metabolism, genetic information- and environmental information processing. Interestingly, 392 mycelial proteins were found to be involved in growth and virulence followed by 268 proteins in nutrient synthesis and translocation. Further, we examined the proteome data using network analysis that identified significant functional protein hubs centered on novel PCI domain-containing protein and known aconitate hydratase, oxidoreductase and pyruvate decarboxylase. This study reports the *Macrophomina* mycelial protein network that provides useful resource to further characterize mycelial proteins towards enhanced understanding of phytopathogenic fungal biology.

Keywords *Macrophomina phaseolina* · Mycelial proteome · 1-DE · Mass spectrometry

Abbreviations

LC–ESI–MS/MS	Liquid chromatography-electrospray ionization-tandem mass spectrometry
PMSF	Phenylmethylsulfonyl fluoride
CHAPS	3-((3-Cholamidopropyl) dimethylammonio)-1-propanesulfonate
DTT	Dithiothreitol
SDS-PAGE	One-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis
GO	Gene ontology
BLAST	Basic local alignment Search tool

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Introduction

Macrophomina phaseolina is a generalist and ubiquitous soil-borne fungus which infects nearly 500 species from 100 plant families. Taxonomically, it belongs to family Botryosphaeriaceae and class Dothideomycetes. It exhibits diverse host range, environmental persistence and is difficult to control due to genetic versatility and intraspecific variations among isolates. Based on the morphological and genomic characterization, no subspecies or races have been identified for *Macrophomina* (Crous et al. 2006). This fungus particularly inhabits tropical and subtropical region with arid to semi-arid climates and can result in severe crop losses (Islam et al. 2012). It is a causal agent of stem and root rot, charcoal rot and seedling blight diseases in diverse crop plants (Ghosh et al. 2018). Isolates of the fungus is largely variable differing in microsclerotia size and presence or absence of dark globose or flattened pycnidia. These pycnidia has simple, rod-shaped

conidiophores and single celled, hyaline and elliptic or oval conidia (Marquez et al. 2021). *M. phaseolina* undergoes asexual reproduction, parasexualism and formation of heterokaryons from different hyphae contributing to the variability among isolates (Almeida et al. 2003). Beside this, gray-black mycelia and sclerotia are produced in the various phases of fungal life cycle. During its interaction with host, mycelia and sclerotia plug the vessels preventing transportation of water and nutrients from the roots to aerial parts of plant.

Extensive studies have been conducted to identify genetic diversity among *M. phaseolina* isolates using molecular markers, including random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), rDNA sequencing and cluster analysis. Results depicted that variation in genetic diversity and pathogenicity of *Macrophomina* are associated with production of wall degrading and hydrolytic enzymes (Khan et al. 2017). To further identify and quantify *Macrophomina*, PCR, hybridization, internal transcribed spacer (ITS) regions of rDNA, loop-mediated isothermal amplification (LAMP)-based detection and real-time qPCR assay are standardized for various isolates (Babu et al. 2007; Burkhardt et al. 2018; Ghosh et al. 2017). Till date genomes of different strains of *M. phaseolina* isolated from jute, strawberry, alfalfa, and sorghum has been published, which provides an opportunity to examine the infection process and disease cycle at molecular level (Marquez et al. 2021). Very recently, proteomic investigation of *M. phaseolina* identified 2204 proteins associated with disease and virulence, metabolism, redox homeostasis and cell wall remodeling (Zaman et al. 2020).

Given the importance of mycelia in fungal propagation and interaction with host, it is interesting to understand protein dynamics using proteomic approaches. Fungal proteomic studies involving combination of gel-based and non-gel-based techniques followed by MS/MS analysis is imperative to study life-style, disease cycle and development of fungal pathogens. Many studies have been directed to catalog repertoire of fungal mycelial proteins across range of fungal species by developing reference proteome maps (Almeida et al. 2021; Barros et al. 2010; Elagamey et al. 2021; González-Fernández et al. 2014; Wang et al. 2021; Xu et al. 2019; Zhou et al. 2016). The work presented here aimed to address the question of how *Macrophomina* mycelia protein network relates to fungal biology. Attention was focused to investigate for the first time the physicochemical and functional dynamic of *Macrophomina* mycelia proteins. In silico analysis identified pathways related to fungal growth, development and virulence. Network analysis revealed associations involved in regulatory processes, related to fungal metabolism, protein turnover, redox homeostasis, mechanical rigidity and strength and pathogenicity.

Results and discussion

Establishment of *Macrophomina* mycelial proteome

M. phaseolina strain MS6 was grown in liquid potato dextrose medium at 30 °C for 7 days and mycelia were recovered from by two-layered miracloth (Islam et al. 2012). Mycelia of this strain appeared as grey-white thread like structure which subsequently turned dark black during maturity (Fig. 1). Proteins were extracted using the TCA/acetone–phenol/methanol precipitation method (Fernández and Novo 2013). Extracted proteins were resolved on 12.5% SDS-PAGE in three biological replicates, and the lane of each replicate containing the resolved proteins were lacerated from the gel and segmented into 32 slices. These slices from each replicate were subjected to trypsinolysis followed by LC–ESI–MS/MS analysis (Fig. 2a, Supplementary Information Fig. S1). Thus, in total 96 gel pieces from three biological replicates were analyzed to develop reference proteome map. The mass spectra were acquired for 96 samples and mapped on *Macrophomina phaseolina* protein sequences. Using 1-DE approach, 1704 proteins were detected, of which 853 proteins were identified and found to be common in all three biological replicates, whereas 481, 42, 167 proteins, respectively were exclusively detected in biological replicate 1, 2 and 3, respectively (Fig. 2b; Supplementary Information Table S1). Our data identified 244 proteins with two
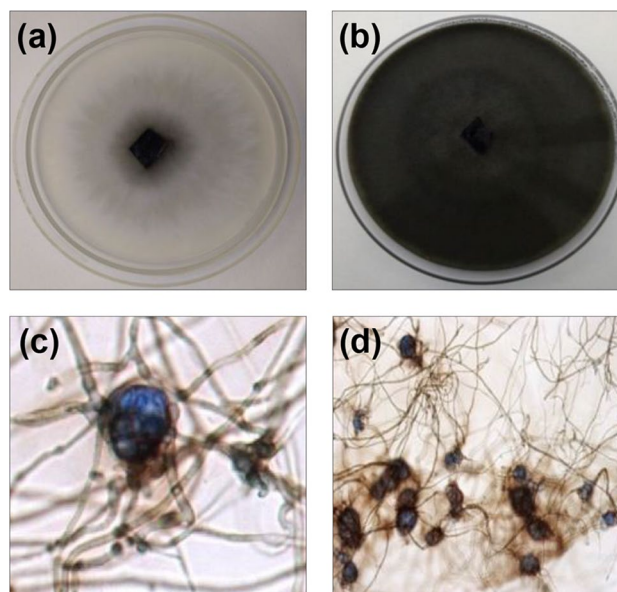

Fig. 1 Growth of *Macrophomina phaseolina* grown on potato dextrose agar media (PDA) (**a**) Day 3 (**b**) Day 7. Different stages of development (**c**, **d**) Hyphal network and sclerotia
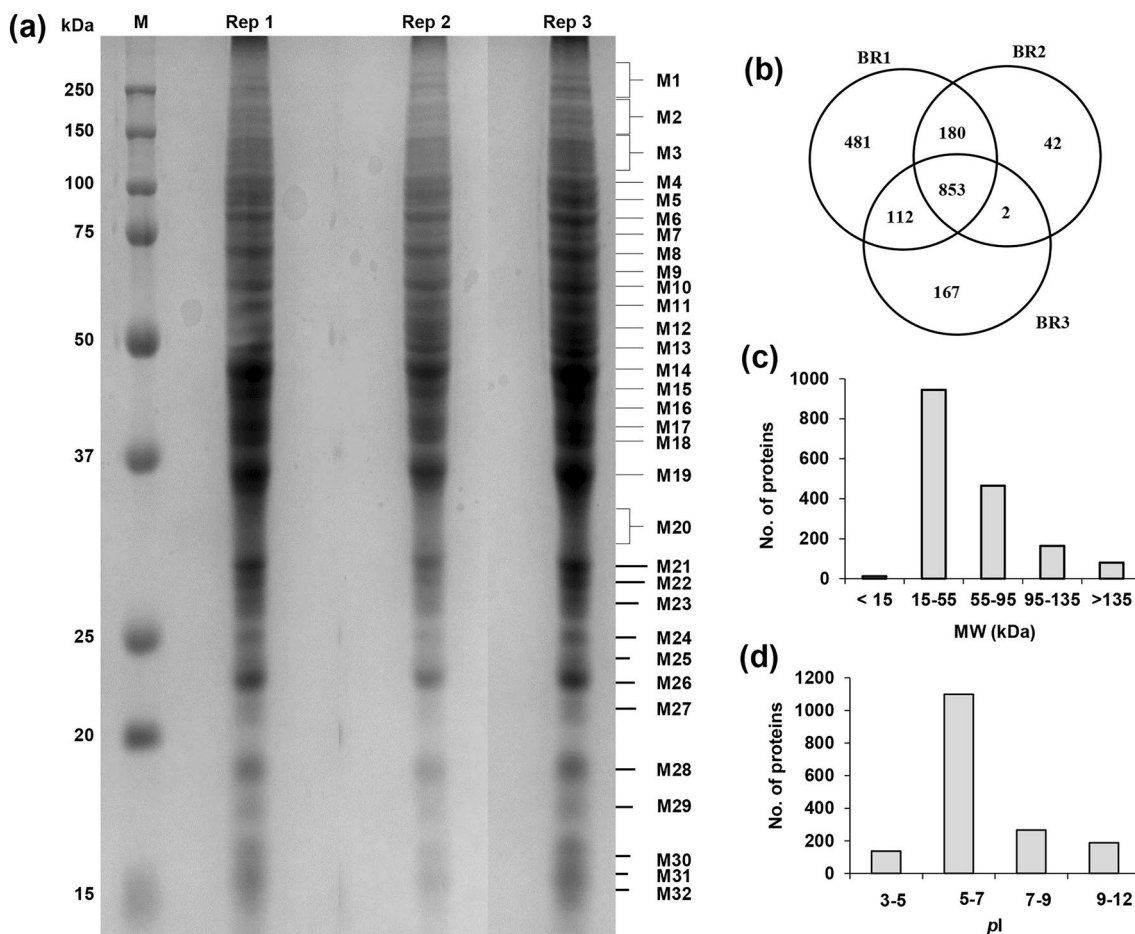
Fig. 2 Gel based proteomic analysis of mycelia (**a**) 1-dimensional gel electrophoresis (1-DE) of mycelial protein in three biological replicates (**b**) Venn diagram showing common and exclusive mycelial proteins in three biological replicates (**c**) Molecular weight (**d**) *p*I of identified mycelial protein

peptide hits. Of them, 180 proteins were identified in only 1 replicate. 56 and 8 proteins, respectively from 2 and 3 biological replicates were identified with 2 peptide hits. However, 98 proteins with single peptides were identified, which was not included for further analysis.

The mycelial proteins were found to vary in molecular weight ranging from 15 to 270 kDa (Fig. 2c). Majority of proteins (~ 55%) exhibiting a molecular mass in the range of 15–55 kDa. However, the isoelectric points (*p*Is) of the mycelial proteins varied from 3 to 12 with maximum proteins in the *p*I range of 5–7 (Fig. 2d). The acidic proteins predominated in mycelia that accounted for 1236 proteins in the *p*I range of 3–7. Many mycelial proteins had a *p*I of more than 8 (455 proteins), which corroborated the study of previous report (Fernández and Novo 2013). The identified mycelial proteins were then subjected to subcellular analysis by Protcomp v. 9.0 and 35% were found to be localized in cytoplasm followed by mitochondria (31%) and nucleus (13%) (Supplementary Information Fig. S2).

Functional cataloging of the mycelial proteins and pathway abundance

To understand cellular and molecular pathways, three main approaches were followed for functional cataloging of mycelial proteins. First approach involved categorization of mycelial proteins based on literature search. Proteins were sorted in to 12 distinct categories (Supplementary Information Fig. S1). The major functional category corresponded to nutrient synthesis and translocation (28%) followed by transcription and translation regulation (12.8%), protein turnover (9.4%), signaling (8.6%), redox homeostasis (6%), mechanical rigidity and strength (5.4%), secretion and transport (5.2%), DNA replication, recombination and repair (5.0%), growth and reproduction (4.4%), stress tolerance and virulence (4.3%) and metal binding (1%).

In the second approach, KEGG pathway analysis revealed that the mycelial proteins were associated with four major pathways, including metabolisms, genetic information

processing, cellular processes and environmental information processing with 833, 317, 304, 226 proteins, respectively (Fig. 3a). Metabolism pathway was enriched in amino acid, carbohydrate, energy, cofactor and vitamins, lipids, xenobiotics, nucleotide, secondary metabolites biosynthesis, glycan biosynthesis and metabolism and terpenoids and polyketides metabolism. Among these, proteins involved in aminoacid metabolism (219 proteins) predominated the mycelia proteome, indicating that aminoacids might constitute a major nutritional source for *Macrophomina* (Fig. 3b). Genetic information processing encompassed translation (150 proteins), folding, sorting and degradation (109 proteins), transcription (29 proteins) and replication and repair (29 proteins). In addition, cellular processes were represented by transport and catabolism (135), cell growth and death (110 proteins), cellular community (37 proteins) and
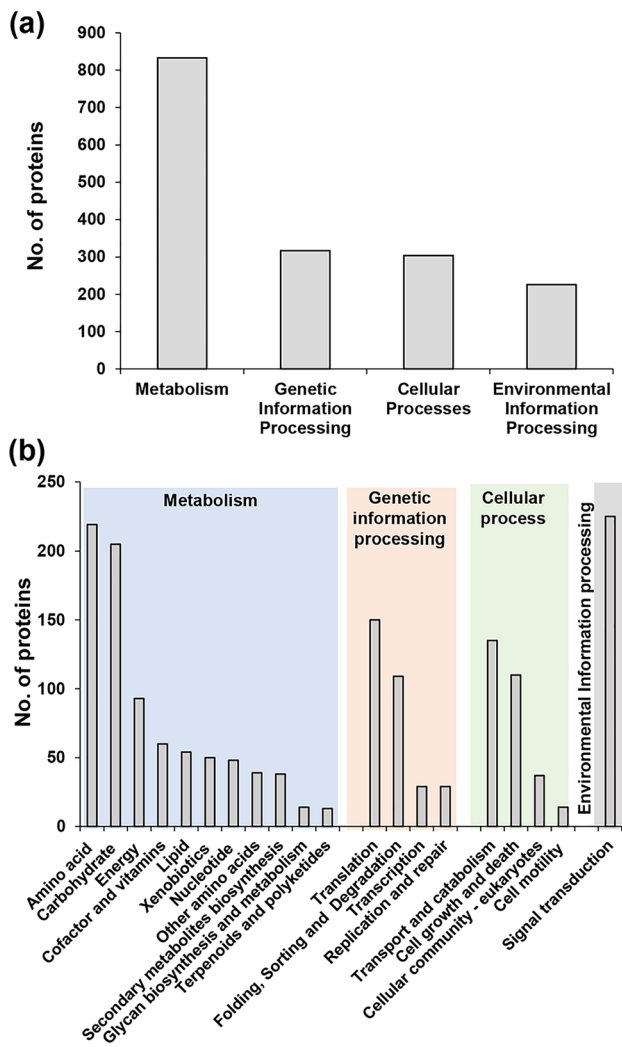
cell motility (14 proteins) related mycelia proteins. Majorly signal transduction related proteins were detected in environmental information processing pathway in the *Macrophomina* mycelia.

Finally, mycelial proteins were categorized into biological processes, molecular functions and cellular components
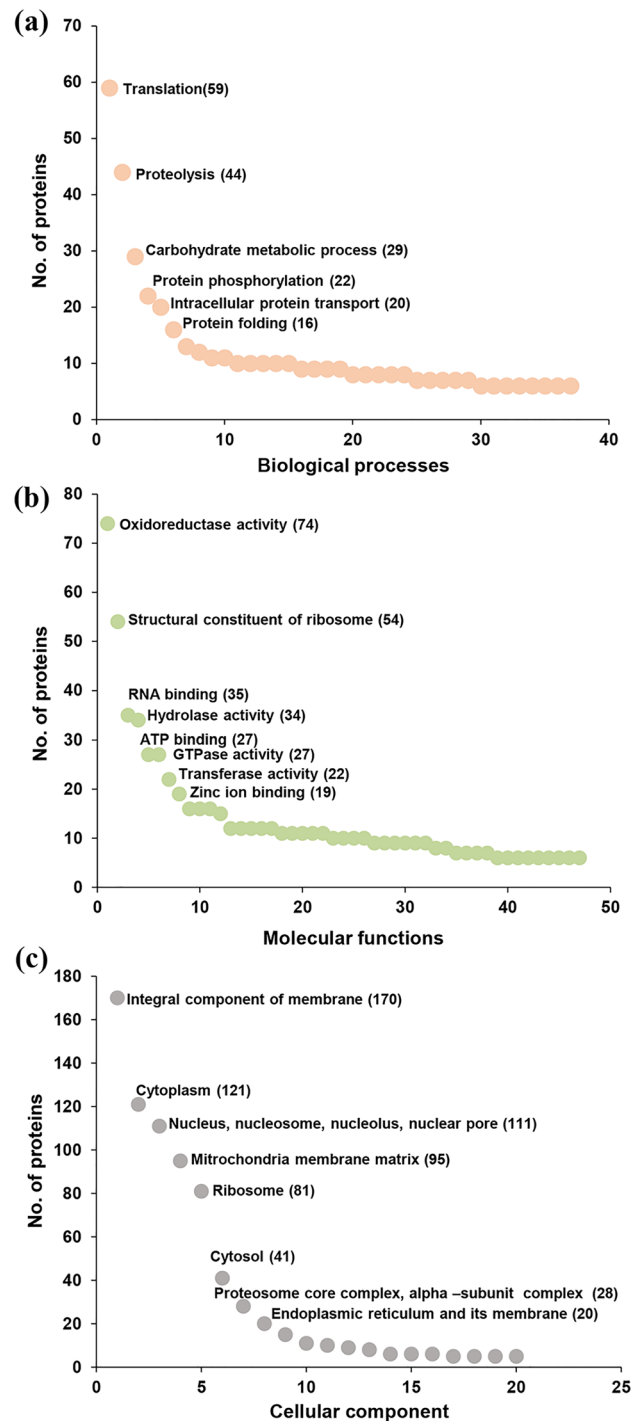

Fig. 3 Functional analysis of mycelial protein (**a**) KEGG analysis of identified proteins (**b**) Functional categorization of mycelial protein in the KEGG pathways

Fig. 4 Go ontology analysis of mycelial proteins (**a**) Biological processes (**b**) Molecular function (**c**) Cellular component

(Fig. 4; Supplementary Information Table S2). Among 1704 mycelial proteins, 1222 proteins were assigned to 536 different biological processes. Of them, translation process accounted for 59 mycelial proteins followed by proteolysis (44 proteins), carbohydrate metabolic process (29 proteins), protein phosphorylation (22 proteins) and intracellular protein transport (20 proteins). Rest other proteins were inconsequentially represented in other biological processes. Of the total, 1452 proteins were assigned to 602 different molecular functions, including oxidoreductase activity (74 proteins), structural constituent of ribosome (54 proteins), RNA binding (35 proteins), hydrolase activity (34), ATP binding (27 proteins), GTPase activity (27 proteins). Cellular component prediction analysis had depicted the mycelia proteins mainly localized in membrane, cytoplasm, nucleus, ribosome, mitochondria, microbody, proteosome complex, extracellular region, endoplasmic reticulum, golgi apparatus and nucleolus.

Mycelial protein–protein interaction network

Protein–protein interaction network could assist in building novel hypothesis about fungal growth and development and other biological processes. To identify set of molecular pathways, we designed and assembled interaction network based on experiment, database annotation, automated text mining and phylogenetic concurrence. The network consisted of 892 nodes and 2139 edges and was enriched in proteins involved in growth and reproduction, stress tolerance and virulence, nutrient synthesis and translocation, transcription and translation regulation, protein turnover, signaling, redox homeostasis, mechanical rigidity and strength, metal binding, secretion and transport, DNA replication, recombination and repair (Fig. 5).
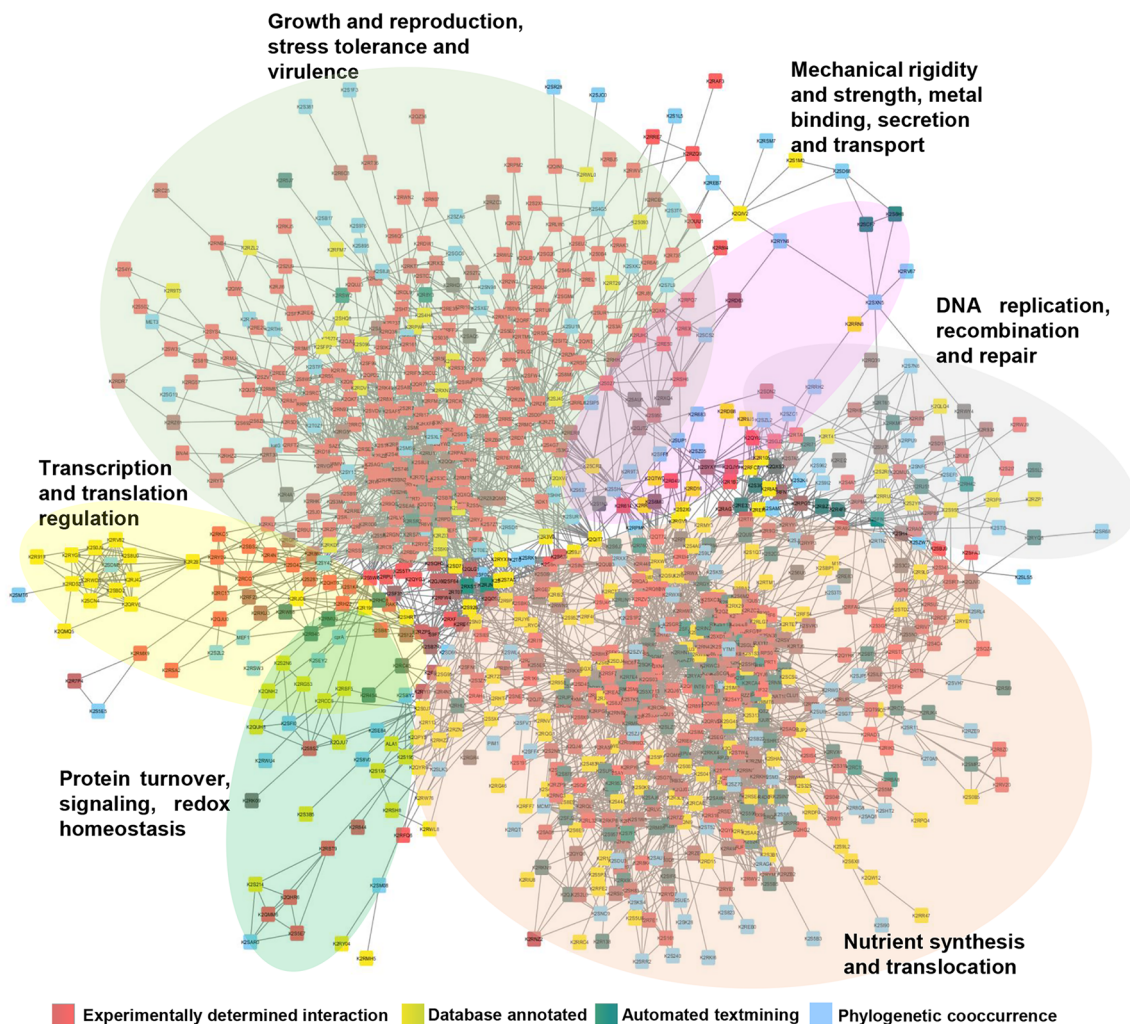

Fig. 5 Protein–protein interaction network of mycelial proteins

Role of nutrient synthesis and translocation related mycelial proteins

Nutrients are known to be taken by fungus in either active or passive routes and play important role in fungal growth and virulence (Breitenbach et al. 2015). Mycelia mediated nutrient translocation require coherent action of numerous proteins. It was observed that 489 *Macrophomina* mycelial proteins were found to be involved in nutrient synthesis and translocation in this study. We identified expected and novel nutrient translocation and synthesis related proteins. Some of the canonical proteins included ATP synthase, ATPase, 6-phosphofructokinase, Beta-ketoacyl synthase, bifunctional cytochrome P450/NADPH-P450 reductase, biotin carboxylase, biotin synthase, branched-chain-amino-acid aminotransferase, carboxyl transferase, CDP-diacylglycerol synthase, chalcone isomerase, choline/ethanolamine kinase, chorismate mutase, cystathionine beta-synthase, D-fructose-6-phosphate amidotransferase, dihydrolipoyl dehydrogenase, enoyl-CoA hydratase, formate dehydrogenase. While, carbohydrate kinase FGGY, carboxymuconolactone decarboxylase, Dak kinase, Dak phosphatase, dihydroorotase, diphosphomevalonate decarboxylase, FAR-17a/AIG1-like protein, GTP cyclo-hydrolase II RibA, acetokinase and hydroxymethylbilane hydrolyase appeared to be novel in fungal mycelia which are otherwise found to function in nutrient translocation in bacteria (Chao et al. 2014; Koh et al. 1996).

Effect of proteins related to growth and reproduction, metal binding, stress tolerance and virulence

Fungus initiates a series of processes during growth and reproduction, namely metal binding, stress tolerance and virulence. In this study 392 proteins were found to be involved in growth and virulence of this pathogen. Some of the proteins whose role in growth and virulence were earlier known and identified in this study are ABC transporter-like protein, Actin cytoskeleton-regulatory complex protein PAN1, actin-depolymerizing factor 1, actinin-type actin-binding conserved site, actin, AP complex subunit, arrestin, cell division protein GTP binding protein (Breitenbach et al. 2015). It was observed that carbohydrate degrading enzymes were identified whose role in growth and virulence is known in *Ustilago maydis*, including α-galactosidase, alpha/beta-glucosidase and alpha-mannosidase (Lakshman et al. 2016). However, this study also detected some novel proteins, including ENTH domain-containing protein, F-actin-capping protein subunit alpha, kynurenine 3-monooxygenase. Coordinated action of these proteins might govern mycelial growth and virulence of the fungus.

Redox homeostasis, signaling and protein turnover in *Macrophoomina*

Reactive oxygen species are short-lived and highly reactive to living cells, including fungi. It causes detrimental changes in macromolecules, such as proteins, lipids, polysaccharides, DNA, and RNA. Redox homeostasis and signaling events regulate protein turn over in fungi (Breitenbach et al. 2015). Some of the players having role in redox homeostasis were catalase, dienelactone hydrolase, glutaredoxin, glutathione S-transferase, glyoxalase, monooxygenase FAD-binding protein, thioredoxin, NAD-dependent epimerase/dehydratase, peroxin-1, superoxide dismutase and thiolase. *Macrophomina* mycelial proteins found to be involved in signaling events, were calcium-binding EF-hand, Ras GTPase, adenylate kinase, Rab GDP dissociation inhibitor and protein kinase domain-containing protein in G protein signaling, calcium signaling, AMP signaling, MAPK signaling and serine/threonine signaling. In addition, proteins involved in protein turnover were also identified, including 26S proteasome regulatory subunit, carboxypeptidase, CCT-beta, chaperonin, cullin, dipeptidyl peptidase, E2 ubiquitin-conjugating enzyme, E3 binding protein, F-box domain cyclin-like protein, heat shock protein DnaJ, heat shock protein Hsp20, peptidases, peptidyl-prolyl cis–trans isomerase, proteasome subunit alpha type, and ubiquitin.

Mycelial proteins related to DNA replication, recombination and repair

Data revealed that 87 mycelial proteins were found to be involved in DNA replication, recombination and repair. To protect against DNA-damaging processes fungal cells have evolved a set of conserved mechanisms referred as DNA damage response (DDR) to sense and repair damaged DNA, ensuring the fidelity of genetic information. This process included cell cycle checkpoints, chromatin remodeling, DNA repair and DNA-damage tolerance pathways (Zhou and Elledge 2000). Interestingly, we detected many proteins associated with above-mentioned processes, including DNA helicase, DNA polymerase, DNA repair protein, DNA replication licensing factor MCM7, DNA/pantothenate metabolism flavoprotein, DNA_MISMATCH_REPAIR_2 domain-containing protein, DNA-binding SAP, DNA-directed RNA polymerase subunit, ATP-dependent DNA helicase II subunit 1 and 2, chromatin-remodeling ATPase and DNA-directed RNA polymerase subunit beta. Many sensors, writer and modifier proteins were known to receive and relay the signal of damaged DNA and transfer to effector kinase to permit resumption of the normal cell cycle (Zhou and Elledge 2000). Our study detected high mobility group HMG1/HMG2, histone acetyltransferase type B, histone deacetylase, histone H2A, histone H2B, histone H4,

Rad50, regulator of chromosome condensation RCC1, UV excision repair protein RAD23, MMS19 nucleotide excision repair protein, nuclear pore complex protein, Nup85, nucleolar protein 58, Nup93/Nic96, nucleoporin Nsp1-like protein, nucleosome assembly protein (NAP), primosome PriB/single-strand DNA-binding protein, SNF5/SMARCB1/INI1 domain-containing protein, spindle assembly checkpoint component MAD1 and MRG domain-containing protein having role in signal transduction in response to DNA damage.

Conclusions

In summary, we explored firmly interconnected processes combining mycelial proteome analysis with information from functional annotation. Data identified distinct molecular events in necrotrophic fungal mycelia. Canonical proteins like primosome PriB/single-strand DNA-binding protein, SNF5/SMARCB1/INI1 domain-containing protein, and 14-3-3 are interesting candidates for future investigations. Novel findings included discovery of Dak kinase, Dak phosphatase, and FAR-17a/AIG1-like protein whose function in necrotroph pathogenesis is not known. Taken together, these data greatly improve our understanding of functional role of *Macrophomina* mycelial proteins and identified potential candidate for further characterization to provide new insight into *Macrophomina* biology, life style and its interaction with diverse crops.

Materials and methods

Fungal strain and growth condition

M. phaseolina strain MS6 was routinely sub-cultured from solid potato dextrose agar plates incubated at 30 °C. For isolation of fungal mycelia, the fungus was grown in liquid potato dextrose medium at 30 °C with agitation (120 rpm) for 7 days (Islam et al. 2012). Mycelia were harvested by filtration, frozen in liquid nitrogen. Frozen mycelia were ground to a fine powder using a mortar and pestle, and then freeze-dried by lyophilization. Lyophilized powder was used for further analysis.

Protein extraction and 1 dimensional gel electrophoresis

Macrophomina phaseolina mycelial proteins were extracted using TCA/acetone-phenol/methanol method (Fernández and Novo 2013). Briefly, fungal mycelia were collected from the liquid broth by two-layered miracloth. Then 2 g lyophilized mycelia powder was transferred to 10% (w/v)

TCA/acetone, mixed well by vortexing and sonicated for 5×15 s (50 W, amplitude 60) at 4 °C on the ice for 1 min. Further, the tube was filled with 10% (w/v) TCA/acetone, mixed, and centrifuged at 16,000 g for 5 min at 4 °C. The pellet was subsequently washed with 0.1 M ammonium acetate in 80% (v/v) methanol then with 80% (v/v) acetone, respectively by centrifugation at 16,000 g for 5 min at 4 °C and air-dried at room temperature. 1.2 ml of 1:1 phenol (pH 8): SDS buffer [30% (w/v) sucrose, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 0.1 M Tris–HCl pH 8.0] was added to the dried pellet and mixed vigorously, incubated for 5 min in ice and centrifuged at 16,000 g for 5 min. The upper phenol phase was transferred into a new 2-ml tube and 0.1 M ammonium acetate in 100% (v/v) methanol was added and allowed for precipitation overnight at -20 °C and centrifuged at 16,000 g for 5 min at 4 °C. The pellet was washed with 100% methanol then with 80% (v/v) acetone respectively, air-dried and resuspended in rehydration buffer [8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 20 mM DTT] at room temperature. Protein concentration was estimated using Bradford assay. The protein extracts were stored at -20 °C for further analysis. For 1 dimensional gel electrophoresis, 150 μg protein samples were loaded on 12.5% 1-D SDS-PAGE at constant current of 80 mA and gel electrophoresis were performed in three biological replicates according to the standard protocol (Laemmli 1970). The resolved proteins were visualized by Coomassie Blue R250 (Bio-Rad).

LC–MS/MS analyses

Protein bands were subjected to in-gel trypsin digestion. Digestion procedure involved resuspension, denaturation, and reduction of protein in 50 mM ammonium bicarbonate, and DTT for 1 h at 60 °C followed by alkylation with iodoacetamide for 30 min in dark. Trypsin digestion was performed in 1:25 (enzyme to substrate ratio) overnight at 30 °C on shaking. Digested peptides were dried *in vacuo*.

Analysis was performed on Nanospray III and TripleTOF 6600 (AB SCIEX) in information-dependent acquisition (IDA) mode. Peptides were desalted and resuspended in 0.1% formic acid and loaded into EkspertTM nanoLC (AB SCIEX) with nanoLC trap (ChromXP C18-CL 3 μm 120 Å, 350 μm × 0.5 mm) with flow rate of 300 nL/min on nanoLC column (75 μm × 15 cm, 3C18-CL-120, 3 μm, 120 Å). Mobile phases consisted of 0.1% FA and water (A), and 0.1% FA and ACN (B). Nonlinear gradient was employed and mass tolerance was set to 50 mDa, and detection limit was 120 cps. The MS was operated with a resolving power of 30,000 FWHM for TOF–MS scans. For IDA, survey scans were acquired in 250 ms and product ion scans were collected if exceeding a threshold of 120 counts per second (counts/s). MS spectra were acquired for 1 s with mass range of 400–1800 m/z in

which survey scan for peptides of charge state $2+-4+$ was performed in positive ion mode. Raw data generated by Analyst TF1.6 were converted to Mascot generic files by MS Converter and searched against uniprot *Macrophomina* database. Database search parameters were: peptide tolerance, 100 ppm; fragment mass tolerance, 0.4 Da; maximum allowed missed cleavage 1; fixed amino acid modification as carbamidomethyl and variable amino acid modifications; oxidation (M) and deamidated with targeted decoy search in within ProteinPilot Software with FDR < 0.05. The theoretical isoelectric point (*p*I) and molecular weight (MW) were computed using Compute *p*I/Mw Expasy tool (https://web.expasy.org/compute_pi/).

Functional annotation

Proteins were annotated using PANNZER2, BlastKO-ALA and PFAM (Kanehisa et al. 2016; Punta et al. 2012; Törönen et al. 2018). PANNZER 2 annotate proteins based on Z-scoRE, BlastKOALA performs KO (KEGG Orthology) assignments and PFAM domains were annotated using HMMER3 searches against PFAM 26.0 database. Subcellular localization of proteins was predicted by ProtComp v. 9.0 (http://www.softberry.com/berry.phtml?topic=protcompan&group=programs&subgroup=proloc).

Network analysis

Protein–protein interactions (PPI) network were searched and assembled from geneMANNIA, BAR, STRING, mentha, IntAct, DIP, APID, MINT, and BIND PPI databases and was visualized with Cytoscape version 3.9.1. Non-redundant mycelia proteins identified in this study was uploaded into Cytoscape and PPI was predicted for these proteins.

Declarations

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