Mycopathologia
DOI 10.1007/s11046-016-0056-x

Nano-LC-Q-TOF Analysis of Proteome Revealed Germination of *Aspergillus flavus* Conidia is Accompanied by MAPK Signalling and Cell Wall Modulation

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Received: 20 June 2016 / Accepted: 23 August 2016
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Abstract *Aspergillus flavus* is the second most leading cause of aspergillosis. The ability of *A. flavus* to adapt within the host environment is crtical for its colonization. Onset of germination of conidia is one of the crucial events; thus, in order to gain insight into *A. flavus* molecular adaptation while germination, protein profile of *A. flavus* was obtained. Approximately 82 % of conidia showed germination at 7 h; thus, samples were collected followed by protein extraction and subjected to nLC-Q-TOF mass spectrometer. Q-TOF data were analysed using Protein Lynx Global Services (PLGS 2.2.5) software. A total of 416 proteins were identified from UniProt *Aspergillus* species database. Orthologues of *A. flavus* was observed in *A. fumigatus*, *A. niger, A. terreus*, *A. oryzae,* etc. Proteins were further analysed in NCBI database, which showed that 27 proteins of *A. flavus* are not reported in UniProt and NCBI database. Functional characterization of proteins resulted majorly to cell wall synthesis and degradation, metabolisms (carbohydrate and amino acid metabolism), protein synthesis and degradation. Proteins/enzymes associated with aflatoxin biosynthesis were observed. We also observed Dicer-like proteins 1, 2 and autophagy-related proteins 2, 9, 18, 13, 11, 22. Expression of protein/enzymes associated with MAPK signalling pathway suggests their role during the germination process. Overall, the data present a catalogue of proteins/enzymes involved in the germination of *A. flavus* conidia and could also be applied to other *Aspergillus* species.

Keywords *Aspergillus flavus* · Germinating conidia · nLC-Q-TOF · Proteome · Cell wall · Anti-Aspergillus targets

Electronic supplementary material The online version of this article (doi:10.1007/s11046-016-0056-x) contains supplementary material, which is available to authorized users.

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Introduction

Aspergillus flavus is a unique fungus among *Aspergillus* species. It can cause infection/disease to humans depending on immunological status of the individuals [1]. Infection caused by *A. flavus* is seen across many phyla of animals known as aspergillosis [2]. A recent survey in Asia and middle-east showed out of 2117 isolates recovered from aspergillosis patient samples, and *A. flavus* was the predominant isolate (45 %) followed by *A. fumigatus* (32.4 %) [3]. On the other side, being cosmopolitan in nature, *A. flavus* is also a major responsible agent for aflatoxin contamination in several commodities such as maize, peanuts, cotton

Published online: 30 August 2016

which creates serious health risks and large economic burden [4]. Epidemiological studies related to aflatoxin are linked to cause hepatocellular carcinoma [5, 6].

Morphology of *Aspergillus* consists of mainly three stages, viz. conidia, mycelia and hyphae. The availability of *A. flavus* genome sequence showed that it contains 13,071 genes [7]. A 70-kb gene cluster comprising 24 structural genes was identified, and these genes are involved in the aflatoxin biosynthetic pathway under the control of *aflR* and *aflS* regulatory genes [8]. The first morphological change from conidia to germinating conidia showed isotropic growth which involves water uptake and decrease viscosity of cytoplasm along with metabolic activity [9, 10]. Polarized growth of the swollen conidia develops into germinating conidia that involves cytoskeleton, signalling pathways and vesicle trafficking system [11]. Pechanova et al. [12] recently analysed sub-cellular proteome at mycelia stage of *A. flavus* showing 538 mycelial proteins related to cellular metabolite and biosynthesis processes. Proteomic profiling of secreted proteins in *A. flavus* at mycelia stage showed a total of 51 unique secreted proteins [13]. Georgianna et al. [14] studied temperature dependent regulation of protein in *A. flavus*, which showed repression of transcription and biosynthesis repression of aflatoxin genes of *A. flavus* at 37 °C and upregulation at 28 °C. Protein data are available at conidia or mycelia stage in *Aspergillus* species and other fungal species [15–17]. Van Leeuwan et al. [18] has carried out the transcriptomic studies at germinating conidia of *A. niger* that showed germination is accompanied by major changes in protein synthesis and cell wall modulation, etc. In addition transcriptomic data of *A. fumigatus* at pre-germinating conidial stage (30, 60 and 90 min) revealed that dormant conidia showed transcripts from cellular protein metabolism, transporter activity, RNA metabolism and after 30 min of post-dormancy, the transcripts were from protein synthesis, carbohydrate metabolism, protein complex assembly and RNA binding protein [19]. However, there is lack of proteomic data at germinating conidial stage among *Aspergillus* species.

Major antifungal drugs for clinical use which target cell wall biosynthesis are echinocandins, azoles and polyenes [20]. Polyenes and azoles are mainly targeting ergosterol biosynthesis, and echinocandins is involved in the inhibition of β-1,3-glucan synthase, which shows that action of these antifungal drugs target fungal cell wall [21, 22]. Recently a proteomic study showed change in protein profile of *A. fumigatus* when exposed to itraconazole, which showed some of the candidate protein that may be targeted for new antifungal compounds [23]. Since most of the antifungal targets fungal cell wall biosynthesis, it is essential to understand the proteins/enzymes involved in the modulation of cell wall during germination of *Aspergillus* conidia.

Studies on transcriptome and protein data set of *A. flavus* at temperatures (28 °C and 37 °C) showed 664 differentially expressed proteins involved in translation, biosynthesis, metabolism and secondary metabolism. This finding also revealed less congruency between proteome and transcriptome data, which depicted that genes undergo post-transcriptional regulation possibly, influences biological pathways and secondary metabolites [24]. Thus, protein profiling may be an advantage to illustrate proteins/enzymes and functional pathway during germination of *A. flavus*. In this study, the proteome profile of *A. flavus* conidia at geminating stage was obtained using nLC-Q-TOF mass spectroscopy to elucidate the proteins/enzymes involved in germ tube formation. Our study demonstrated majority of proteins/enzymes were involved in protein synthesis, carbohydrate and amino acid metabolism, MAPK cell signalling and cell wall biogenesis.

Materials and Methods

Microorganisms and Growth Conditions

A. flavus conidia (MTCC9367), an environmental (soil) sample [25], was inoculated on Sabouraud dextrose agar (Himedia, India) and cultured for 7 days at 30 °C. Spores were harvested by gentle rinsing the colonies with phosphate-buffered saline (PBS) and 0.05 % Tween 20 (PBST), pH 7.4, centrifuged at 10,000 rpm for 10 min at 4 °C and washed with PBS twice, followed by a viability count (numbers of CFU/ml) on SDA plates. These conidial suspensions at a concentration of 1×10^6 cells/ml [26] were used as working conidial cells in our studies.

A. flavus Morphotypes

To check the morphogenesis of *A. flavus* at different time points, conidial growth (1×10^6 cells/ml) was performed at 30 °C in the rotary shaker (150 rpm). Samples were collected through 2–8 h at regular intervals till germinating conidial stage (conidia with germ tube) was achieved, followed by Lactophenol cotton blue stain (Himedia, India). Morphological stages were evaluated at 40× by Magnus MPS-USB (Olympus, India). All basic parameters such as inoculum size, media and growth conditions were set to achieve the maximum cellular homogeneity in two biological replicates. Morphological observation showed germinating conidial stage at 7 h, which were further picked for total protein extraction.

Large-Scale Culture for Protein Extraction

A. flavus spores (1×10^6 cells/ml) were inoculated in SD broth in culture flask, followed by incubation at 30 °C for 7 h (germinating conidial stage). Cells were scraped out, and morphological stage was analysed by haemocytometer count in triplicates. *A. flavus* cells were further pelleted down by centrifugation at 18,000 rpm for 10 min at 4 °C and stored at −70 °C until further use. The experiment was repeated twice, and these two biological replicates were used further for protein extraction.

Total Protein Extraction

Protein extraction from whole cells were carried out using the method described earlier [22], and extraction was performed for each biological replicate. *A. flavus* germ cells were grounded in liquid nitrogen (1.5 gm of wet mat), and total protein was extracted at 4 °C in cold lysis buffer (5 ml/gm) which contains 50 mM sodium phosphate buffer pH 7.0, with 1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA and 0.2 mM dithiothreitol (DTT) for 3 h with constant stirring. The protein extract was centrifuged at 12,000*g* for 20 min at 4 °C, and supernatant was collected to give final concentration of 5 % trichloroacetic acid and kept overnight at −20 °C for precipitation. The precipitate was washed with cold acetone (kept at −20 °C overnight), vortexed, again kept at −20 °C for 15 min and centrifuged at 12,000*g* for 10 min washing step was repeated thrice. Protein obtained was dissolved in rehydration buffer containing 8 M urea, 2 % 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and 25 mM DTT and estimated by Bradford's method [27].

SDS-PAGE Analysis

Protein sample separation was performed by SDS-PAGE, following the standard protocol of Laemmli [28]. Equal concentration of two biological replicates of concentrated protein sample was precipitated with phenol: chloroform (400:100 µl) and 300 µl distilled water followed by centrifugation at 10,000 rpm at 25 °C for 5 min. The supernatant was removed and vortexed in 400 µl methanol followed by centrifugation at 15,000 rpm at 25 °C for 10 min. Pellet was reconstituted in sample buffer containing SDS 10 %, Tris–Cl (pH 6.8) and glycine 20 % (2 ml). Bromophenol blue 0.02 % (w/v) and beta mercaptoethanol were added to make volume up to 100 µl at the time of use. Sample was heated at 95 °C for 5 min. Broad range molecular marker was mixed with sample buffer and heated at 95 °C. For SDS-PAGE, 12 % homogeneous polyacrylamide gel electrophoresis was carried. The gel was fixed for 2 h in fixing solution containing acetic acid (25 ml), methanol (100 ml) and distilled water (125 ml) and was stained in colloidal Coomassie stain overnight. All the chemicals used in this analysis were obtained from Bio-Rad, India.

Nano-LC-Q-TOF Mass Spectrometer

Sample Preparation

Two biological protein samples dissolved in 50 mM ammonium bicarbonate buffer were taken in required concentration (sample 1; 45 µl and sample 2; 55 µl) to a final concentration of 2.2 mg/ml. Sample volume of 100 µl was used for peptide digestion and subjected to nLC-Q-TOF for mass spectrometric analysis, analysed at Sandor Life sciences Pvt. Ltd, Hyderabad, India (sandorlifesciences.co.in). For analysis, 100 µl of protein sample was precipitated in acetone and kept overnight. Following day sample was treated with 10 mM DTT and incubated at 56 °C for 1 h. A total of 55 mM IDA treatment was provided at room temperature in dark for 45 min. The treated sample was then subjected to trypsin digestion and incubated overnight at 37 °C for efficient digestion.

Separation of Peptides

The tryptic digested peptides were vacuum-dried and dissolved in 0.1 % formic acid. To check and ensure the complete digestion, 1 µl of digested sample was loaded on SDS-PAGE, rest of the digested sample was centrifuged at 10,000*g* and the supernatant was injected on C18 Nano-LC column for separation of peptides.

MS and MS/MS Analysis

Peptide fractions separated on nano-liquid chromatography were analysed on a Synapt G2 equipped with a nano-flow high-performance liquid chromatography system (Waters, India Inc.) [29]. The column size was 75 µm × 150 cm × 1.7 µm BEHC18 column for 150 min. The separated peptides were ionized using electrospray ionization at a heated capillary temperature of 275 °C and spray volume of 2 kV. The mass spectra were operated in data-dependent mode to automatically switch between MS and MS2 acquisitions. Survey full-scan MS spectra (*m/z* 300–1150) were acquired in the Orbitrap with a MS resolution of 60,000 and MS/MS resolution of 15,000. The MS/MS fragmentation was achieved by HCD (higher energy collisional dissociation cell) mode in data-dependent manner (top 20) with an ion selection threshold of 5,000 counts, isolation window of 1.9 *m/z*, an MS accumulation time of 100 min and MS/MS accumulation time of 250 min with a normalized collision energy of 41. A lock mass of polysiloxane (*m/z* 445.120025) was used as internal calibration. Peptides with unassigned charge states, as well as with charge state <3 were excluded from fragmentation. Fragment spectra were acquired in the quadruple TOF analyser. The mass spectra were acquired and the parameters controlled by PLGS (Waters Inc) software. Peptide tol were 50 ppm, fragment tol were 100 pm, missed cleavage were 1 and each run was performed in triplicates.

Protein Identification

The data from nLC-Q-TOF were analysed using SEQUEST search algorithm against Mass Lynx 4.1 WATERS. The false discovery rate (FDR) was set to 0.01 for proteins, which had to have a minimum length of 6 amino acids. Peptide and proteins were identified with >95.0 %. The individual peptides MS/MS spectra were matched to the database sequence for protein identification on PLGS software, WATERS [30].

Protein–Protein Interaction and Pathway Prediction

To identify the association and interaction of identified proteins, proteins of *A. flavus* were examined by a computer-based database, viz. STRING [31] Version 10 (http://string-db.org/), on the basis of different parameters such as, species: *A. flavus,* confidence level: 0.400, active prediction method: all, input: UniProt accession IDs. The biological pathway of identified proteins were deduced from GO and KEGG (Kyoto encyclopaedia of gene and genome) of UniProt database.

FungiFun Software for Cell Wall Biogenesis Pathway Analysis

Proteins involved in carbohydrate metabolism pathway, which is a key function in fungal morphogenesis was analysed using FungiFun 2.2.8 BETA software (https://elbe.hki-jena.de/fungifun/fungifun.php) [32].

Results

Conidial Germination

Morphological studies showed the time required for *A. flavus* to germinate is 7 h. Polarized growth was observed at 4–7 h of germination, resulting to germ tube formation. Approximately, mean average of haemocytometer count showed more than 80 % cells in swollen conidia after 2 h of incubation (Fig. 1a). Approximately, 82 % cells were found to be in germinating conidial stage, after 7 h of incubation (Fig. 1b). The workflow of our study is shown in Fig. 2.

Analysis of Expressed Proteins of *A. flavus* at Germinating Conidial Stage

The cellular protein of *A. flavus* grown in SD broth was harvested after 7 h of incubation and analysed by SDS-PAGE as shown in Fig. 3, which determined all the visible cellular proteins. Classic nLC-Q-TOF mass

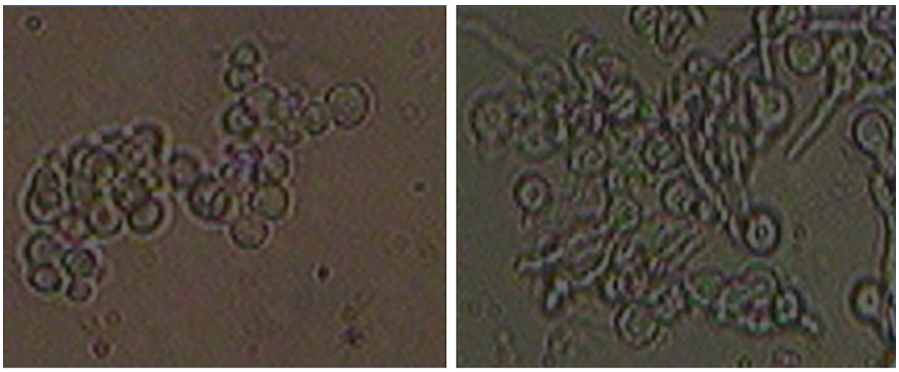


Fig. 1 Micrographic illustration of *A. flavus* morphotypes at two different time intervals, viz. 2 (swollen conidial stage) and 7 h (germinating conidial stage). Conidia are cultivated in Sabouraud dextrose broth. Cells are stained with lactophenol cotton blue, seen under ×40 magnifications

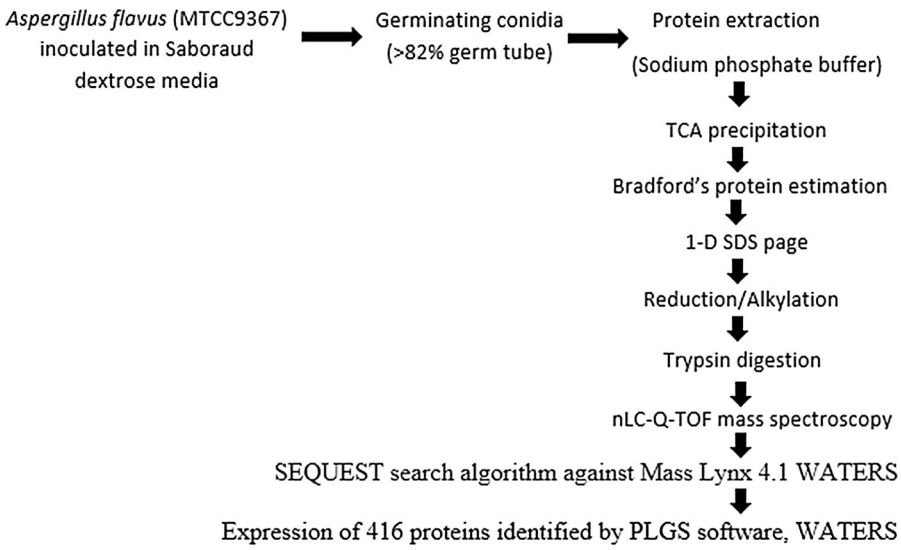


Fig. 2 Overall workflow for protein extraction at germinating conidial stage of *A. flavus* and nL-Q-TOF mass spectroscopic analysis

spectrometer was used to analyse the protein map of *A. flavus* at germinating conidial stage (7 h). A total of 416 proteins were identified using LC–MS/MS Q-TOF from *Aspergillus* species using UniProt database, which includes orthologues of several proteins of other *Aspergillus* species such as *A. niger, A. oryzae, Emericella nidulans*, *Neosartorya fumigata*, *N. fischeri*, *A. clavatus*, *A. terrus*, *Hypocrea jecorina*, *Saccharomyces pombe*, *Ustilago maydis*, *A. parasiticus* and *A. awamori* on the basis of PLGS score. All the expressed proteins are listed in Supplementary Table S1 (A) and further categorized on the basis of their GO functional classification are listed in Supplementary Table S1 (B). The number of proteins and their distribution for number of peptides matched, molecular weight, pI, % sequence coverage and PLGS score are listed in Supplementary Table S1 (B). Molecular weight ranged between 2.5–171.169 kDa, with pI value 4–11. The estimated sequence coverage for proteins ranged from 4 to 97 %, whereas PLGS scores from 2.34 to 2005, showing that proteins were reported with good confidence level.

A total of 416 proteins were further categorized on the basis of their assigned Gene Ontology (GO) on the basis of their biological process, molecular function and cellular component ontologies (Fig. 4). For cellular components proteins were clustered in 11 GO slim categories (Fig. 4a) including 21 % mitochondrial, 20.8 % nucleus, 13.72 % secretory protein and 12.8 % membrane proteins. However, only 3.9 %

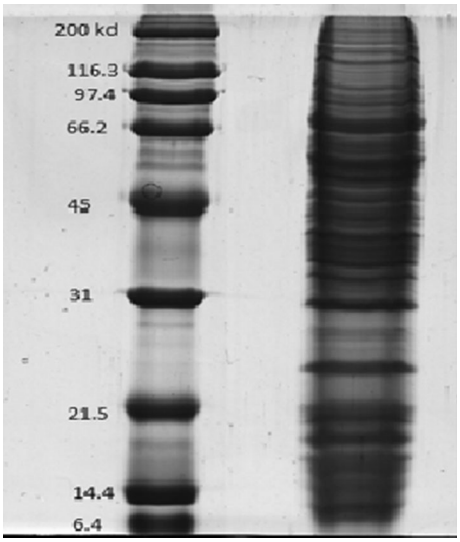


Fig. 3 SDS-PAGE separation of *A. flavus* protein extracts obtained by sodium phosphate lysis buffer at germinating conidial stage (7 h)

proteins were found in golgi complex. Classification on the basis of molecular functions was again annotated in 13 GO slim categories (Fig. 4b). General category hydrolase activity (36.04 %) was most active followed by transferase activity (14.63 %), nucleic acid binding (10.56 %). Antioxidant activity was seen up to 11.92 %. Some functions such as kinase activity (4.88 %), nucleotide binding (2.98 %), catalytic activity (2.71 %), isomerase activity (2.71 %), ligase activity (1.63 %), transporter activity (1.62 %) and electron carrier activity (1.08 %) were present in smaller number. Annotation for 416 identified proteins was categorized in 20 GO slim categories for biological functions (Fig. 4c). Most of the proteins were from metabolism process including carbohydrate metabolism (25.2 %), nucleic acid metabolism (12.7 %), amino acid metabolism (7.2 %), lipid metabolism (4.3 %) and secondary metabolic activity (9.3 %). Forty-one percentage of the proteins were associated with transport, and 32 % of proteins were associated with enzyme activity, making these two activities as predominant biological functions. A number of proteins belonged to biosynthesis process (11.2 %), translation (17.7 %), cell cycle (10.8 %), transcription (9.1 %), replication (6.2 %), protein transport (11.5 %) and sulphur, nitrogen and phosphorous utilization (23.5 %) were also identified. Lastly, a subset of terms was designated to stress response (11 %), autophagy (4.3 %), activator/repressor (3.6 %) and pathogenesis (2 %). The association of *A. flavus* proteins at germinating conidial stage with their GO categorization will be revealed as more functional data for future prospective.

Expressed proteins were also analysed against NCBI database for *A. flavus* protein homologues or for orthologues in *Aspergillus* species. The analysis showed homologues or orthologues of 416 proteins in *Aspergillus* species; however, 27 proteins of *A. flavus* did not show match in *A. flavus* database of UniProt and NCBI. These proteins are represented with its respective GO function in Table 1.

Germination of A. flavus Conidia Accompanied by MAPK Signalling Pathway

Proteome profile of *A. flavus* at germinating conidial stage resulted in proteins that are involved in morphogenetic processes of *Aspergillus* species. Carbohydrate metabolism enzymes such as enolase, hexokinase I, beta glucosidase, beta mannosidase, α-galactosidase, exopolygalactoneurase, rhamanogalacturonase, mannitol 2 dehydrogenase were overrepresented with categories involved in protein synthesis (FK506 binding protein 4, 40S ribosomal protein S1, FACT complex subunit spt16, LFACT complex subunit pob3, DnaJ homologue 1 mitochondria, tRNA uracil O 2 methyltransferase, peptidyl prolyl cis trans isomerase, eukaryotic translation initiation factor 3 subunit A, tRNA guanine N 7 methyltransferase non-catalytic subunit trm82, tRNA adenine 58 N 1 methyltransferase non-catalytic subunit trm6, etc.), cell cycle (separin, protein sds23, DNA ligase 4, F-box protein grrA, chitin synthase C, SCF subunit sconce, flap endonuclease 1, autophagy protein 5, regulatory protein abaA, cytokinesis protein sepA, kinesin-like protein bimC, E3 ubiquin ligase complex, negative regulator of mitosis, mitochondria ficompl 1 protein, protein transport protein sec31, SCF subunit sconB, stu1 cell division control protein, 48 cell division control protein, etc.) and proteins involved in signalling pathways (protein kinase C, mitogen-activated protein kinase mpkC, tyrosine protein phosphatase cdcA, serine threonine protein kinase MARK2, etc.). These all categories are the major functional categories involved in conidia germination, and identification of proteins related to this shows the accuracy of data. The diagrammatic representation of germination

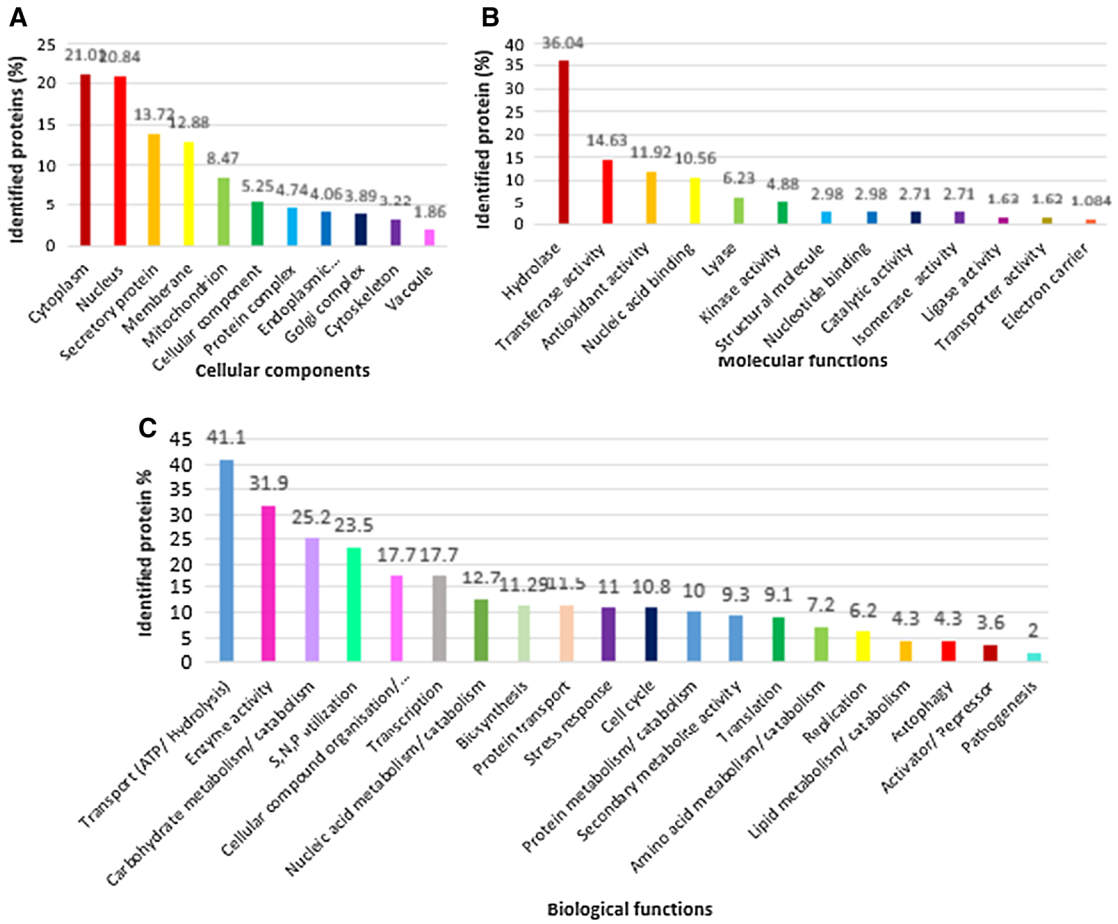


Fig. 4 Graphical representation of GO cellular component categories (**a**) and GO molecular functional categories (**b**) and GO biological categories (**c**) *in A. flavus* germinating conidial stage. The experimental data set is compared to entire *A. flavus* UniProt database followed by perceptual representation of identified protein of GO terms assigned for common GO categories

of *A. flavus* is shown in Fig. 5 which is drawn with the help of Coraldraw graphic suit, version X8 (www.coraldraw.com).

Cell Wall Modulation at Germinating Conidial Stage of A. flavus

Several proteins involved in cell wall modulation were also identified in protein data set which showed that cell wall modulation is an important identified step in germination of conidia. Proteins identified in this process are 1,3-β glucan synthase component FKS1, Arginine biosynthesis bifunctional protein ArgJ mitochondrial, chitin synthase C, exopolygalacturonase C, endopolygalacturonase B, GPI ethanolamine phosphate transferase 1, probable pectate lyase A, etc. Various glucan degrading enzymes were also observed such as β-glucosidase A, B, C, β-galactosidase, α, β-glucosidase agdC, endo-β-1,4 glucosidase and many more which showed cell wall biogenesis as a major process in germinating stages of *A. flavus*.

Expression of Aflatoxin Biosynthetic Pathway Proteins

Proteins involved in aflatoxin biosynthetic pathway were also observed. These proteins include aflatoxin biosynthesis regulatory protein, P450 monooxygenase AflN, versicolorin B desaturase, sterigmatocystin-8-*O*-methyltransferase, *O*-methyl sterigmatocystin oxidoreductase, norsolorinic acid reductase and averufin oxidase A. Additional proteins involved secondary

Table 1 List of proteins not reported in *A. flavus* according to UniProt and NCBI protein database with respective GO functions and annotated organisms

S. no.	Accession no.	Protein name	Reported organism	GO functions
1	Q078T0	3 hydroxyphenylacetate 6 hydroxylase	*E. nidulans*	Phenylacetate degradation, aromatic compound metabolism
2	A2R180	Actin cytoskeleton regulatory complex protein pan1	*A. niger*	Endocytosis, calcium ion binding
3	Q12732	Averantin hydroxylase	*A. parasiticus*	Aflatoxin biosynthetic process monooxygenase, oxidoreductase
4	A2QM49	Bifunctional lycopene cyclase phytoene synthase	*A. niger*	Carotenoid biosynthesis, intramolecular lyase activity, isomerase, transferase
5	Q5BBL4	Class E vacuolar protein-sorting machinery protein hse1	*E. nidulans*	Ascospore-type prospore assembly, membrane budding, protein transport
6	Q03149	Conidial yellow pigment biosynthesis polyketide synthase	*E. nidulans*	Heptaketidenaphthopyrone YWA1 biosynthesis, polyketide biosynthesis, pigment biosynthetic process, conidiation, sporulation
7	Q9Y7C8	Dihydromonacolin L monooxygenase LovA	*A. terreus*	Polyketide biosynthetic process, lovastatin biosynthesis, monooxygenase, oxidoreductase
8	Q5B6U3	DNA damage binding protein cmr1	*E. nidulans*	Cellular response to DNA damage stimulus
9	A1DA65	Fumitremorgin C monooxygenase	*N. fischeri*	Alkaloid metabolism, monooxygenase, oxidoreductase
10	A1CBF3	Increased rDNA silencing protein 4	*A. clavatus*	ATPase activity, coupled to transmembrane movement of substances
11	A1CLY7	Ketocytochalasin monooxygenase	*A. clavatus*	Monooxygenase, Oxidoreductase
12	P24686	Negative regulator of mitosis	*E. nidulans*	Ubiquitin protein transferase activity, cell cycle, cell division, Mitosis
13	A1C8C3	Ophiobolin F synthase	*A. clavatus*	Isoprenoid biosynthetic process, lyase, transferase, magnesium ion binding, terpene synthase activity
14	A2R919	Protein cft1	*A. niger*	mRNA processing,
15	Q9Y7B3	Protein dopey	*E. nidulans*	Protein transport, cell morphogenesis, cleistothecium development, conidium formation, endoplasmic reticulum organization
16	Q5BGR2	Protein mesA	*E. nidulans*	Establishment of cell polarity, hyphal growth
17	Q5BDB9	Protein OS 9 homologue	*E. nidulans*	ER-associated ubiquitin-dependent protein catabolic process, retrograde protein transport, ER to cytosol
18	A1D3V8	Protein sds23	*N. fischeri*	Protein serine/threonine phosphatase inhibitor activity, cellular response to glucose starvation, negative regulation of hydroxyl methyl glutaryl-CoA reductase (NADPH) activity, positive regulation of conjugation with cellular fusion, positive regulation of mitotic metaphase/anaphase transition
19	P24817	Ribosome inactivating protein beta momorcharin	*M. charantia*	Antiviral protein, hydrolase, Protein synthesis inhibitor, Toxin, rRNA N-glycosylase activity
20	Q2TWP5	Sensitive to high expression protein 9 homologue mitochondrial	*A. oryza*	Sterigmatocystin biosynthesis, mycotoxin biosynthesis, RNA polymerase II transcription factor activity
21	Q9UUZ9	Thiamine thiazole synthase	*A. oryzae*	Thiamine biosynthesis, mitochondrial genome maintenance, response to stress, thiazole biosynthetic process

Table 1 continued

S. no.	Accession no.	Protein name	Reported organism	GO functions
22	A2QFG8	Transcription activator of gluconeogenesis acuK	*A. niger*	Gluconeogenesis,Transcription, transcription regulation, activator
23	A2QJF9	Transcriptional activator of proteases prtT	*A. niger*	Positive regulation of pyrimidine-containing compound salvage, positive regulation of transcription, regulation of protein catabolic process
24	Q4WE58	tRNA adenine 58 N 1 methyltransferase non-catalytic subunit trm6		tRNA (adenine-N1-)-methyltransferase activity, tRNA processing
25	A1DA60	Tryprostatin B 6 hydroxylase	*N. fischeri*	Alkaloid biosynthesis, monooxygenase, oxidoreductase
26	Q5B288	Uncharacterized protein AN5342	*E. nidulans*	Methylation, stress response
27	A1CEE0	Vacuolar membrane-associated protein iml1	*A. clavatus*	Intracellular signal transduction, regulation of autophagosome assembly, GTPase activator activity

metabolic pathway were also identified and categorized on the basis of GO biological functions. Beta cyclopiazonate dehydrogenase, non-ribosomal peptide synthetase 13, neutral protease 2 homologue mep20, non-ribosomal peptide synthetase 10, Psi producing oxygenase A, non-ribosomal peptide synthetase 11.

Autophagy, Dicer-Like, Heat Shock Proteins

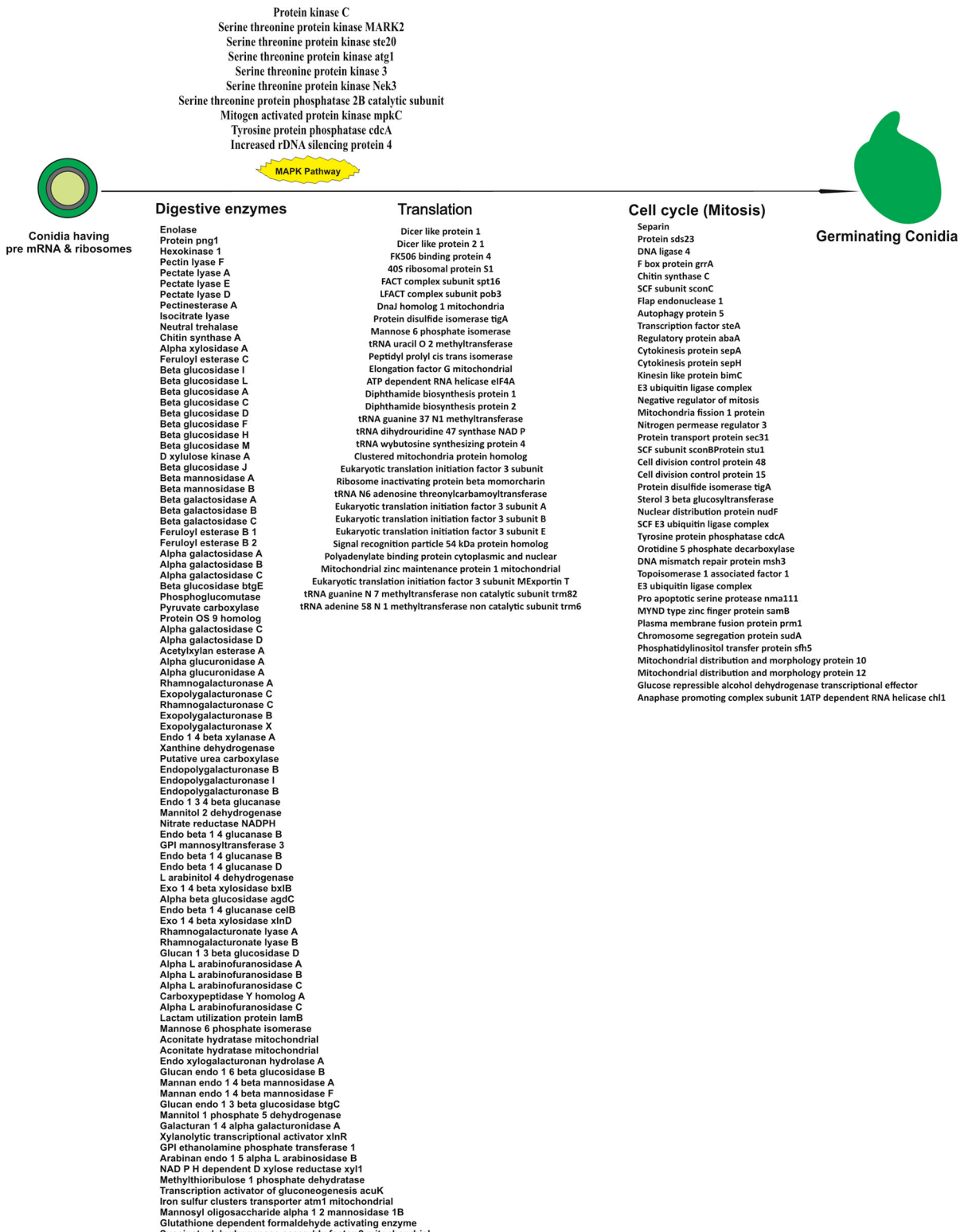
Expressed protein related to stress and defence response was also observed, which includes autophagy, dicer-like proteins and heat shock proteins. Autophagy-related protein 9, COPII coat assembly protein sec16, nitrogen permease regulator 3, metacaspase 1B and sterol 3 beta glucosyltransferase were certain protein involved in autophagy process responsible for cellular growth and development of *A. flavus*. Dicer-like proteins such as dicer-like protein 1, dicer-like protein 2 and dicer-like protein 22 observed to be expressed. Heat shock protein, 70 and 60 kDa, were also expressed in germinating conidial stage. Adenylyltransferase and sulfurtransferase uba4, catalase A, DnaJ homologue 1 mitochondrial, SCF E3 ubiquitin ligase complex F-box protein grrA and catalase peroxidase were additional important proteins expressed that are involved during stress response.

Protein–Protein Interaction Pathway

Protein's function is also influenced through interaction with other protein, so the protein–protein interaction was performed among *A. flavus* proteins using STRING database [31]. As the software allows only species specific protein–protein interaction at a time, *A. flavus* proteins matched in UniProt database were subjected for interaction using STRING software. Interactome map showing single interaction with other protein or no interaction was excluded. Proteins having interaction with more than 2 proteins were further subjected to protein–protein interaction profile (Fig. 6). On the basis of their functional categorization, interactome showed proteins involved in carbohydrate metabolism, amino acid biosynthesis, translation, transcription and cell wall biogenesis, etc., listed in Table 2.

Carbohydrate Metabolism Pathway

Carbohydrates mainly polysaccharides, viz. α and β (1, 3)-glucans, chitin, galactomannans are the main structural components of cell wall of *Aspergillus* species, responsible for fungal cell wall rigidity, which also includes various enzymes (glucanase, chitinase, transglycosidases) [33]. These interact directly with the host cells; thus, we have aimed carbohydrate metabolism-related proteins/enzymes for pathway analysis. In addition, cell wall biogenesis components are the important area to focus as antifungal targets or as diagnostics molecules [21]. Expressed proteins belonging to carbohydrate metabolic proteins (as categorized on the basis of GO biological functions) were further analysed using FungiFun 2.2.8 BETA software (https://elbe.hki-jena.de/fungifun/fungifun.php)


Protein kinase C
Serine threonine protein kinase MARK2
Serine threonine protein kinase ste20
Serine threonine protein kinase atg1
Serine threonine protein kinase 3
Serine threonine protein kinase Nek3
Serine threonine protein phosphatase 2B catalytic subunit
Mitogen activated protein kinase mpkC
Tyrosine protein phosphatase cdcA
Increased rDNA silencing protein 4
MAPK Pathway
Conidia having pre mRNA & ribosomes
Digestive enzymes
Enolase
Protein png1
Hexokinase 1
Pectin lyase F
Pectate lyase A
Pectate lyase E
Pectate lyase D
Pectinesterase A
Isocitrate lyase
Neutral trehalase
Chitin synthase A
Alpha xylosidase A
Feruloyl esterase C
Beta glucosidase I
Beta glucosidase L
Beta glucosidase A
Beta glucosidase C
Beta glucosidase D
Beta glucosidase F
Beta glucosidase H
Beta glucosidase M
D xylulose kinase A
Beta glucosidase J
Beta mannosidase A
Beta mannosidase B
Beta galactosidase A
Beta galactosidase B
Beta galactosidase C
Feruloyl esterase B 1
Feruloyl esterase B 2
Alpha galactosidase A
Alpha galactosidase B
Alpha galactosidase C
Beta glucosidase btgE
Phosphoglucomutase
Pyruvate carboxylase
Protein OS 9 homolog
Alpha galactosidase C
Alpha galactosidase D
Acetylxylan esterase A
Alpha glucuronidase A
Alpha glucuronidase A
Rhamnogalacturonase A
Exopolygalacturonase C
Rhamnogalacturonase C
Exopolygalacturonase B
Exopolygalacturonase X
Endo 1 4 beta xylanase A
Xanthine dehydrogenase
Putative urea carboxylase
Endopolygalacturonase B
Endopolygalacturonase I
Endopolygalacturonase B
Endo 1 3 4 beta glucanase
Mannitol 2 dehydrogenase
Nitrate reductase NADPH
Endo beta 1 4 glucanase B
GPI mannosyltransferase 3
Endo beta 1 4 glucanase B
Endo beta 1 4 glucanase D
L arabinitol 4 dehydrogenase
Exo 1 4 beta xylosidase bxlB
Alpha beta glucosidase agdC
Endo beta 1 4 glucanase celB
Exo 1 4 beta xylosidase xlnD
Rhamnogalacturonate lyase A
Rhamnogalacturonate lyase B
Glucan 1 3 beta glucosidase D
Alpha L arabinofuranosidase A
Alpha L arabinofuranosidase B
Alpha L arabinofuranosidase C
Carboxypeptidase Y homolog A
Alpha L arabinofuranosidase C
Lactam utilization protein lamB
Mannose 6 phosphate isomerase
Aconitate hydratase mitochondrial
Aconitate hydratase mitochondrial
Endo xylogalacturonan hydrolase A
Glucan endo 1 6 beta glucosidase B
Mannan endo 1 4 beta mannosidase A
Mannan endo 1 4 beta mannosidase F
Glucan endo 1 3 beta glucosidase btgC
Mannitol 1 phosphate 5 dehydrogenase
Galacturan 1 4 alpha galacturonidase A
Xylanolytic transcriptional activator xlnR
GPI ethanolamine phosphate transferase 1
Arabinan endo 1 5 alpha L arabinosidase B
NAD P H dependent D xylose reductase xyl1
Methylthioribulose 1 phosphate dehydratase
Transcription activator of gluconeogenesis acuK
Iron sulfur clusters transporter atm1 mitochondrial
Mannosyl oligosaccharide alpha 1 2 mannosidase 1B
Glutathione dependent formaldehyde activating enzyme
Succinate dehydrogenase assembly factor 2 mitochondrial
Translation
Dicer like protein 1
Dicer like protein 2 1
FK506 binding protein 4
40S ribosomal protein S1
FACT complex subunit spt16
LFACT complex subunit pob3
DnaJ homolog 1 mitochondria
Protein disulfide isomerase tigA
Mannose 6 phosphate isomerase
tRNA uracil O 2 methyltransferase
Peptidyl prolyl cis trans isomerase
Elongation factor G mitochondrial
ATP dependent RNA helicase eIF4A
Diphthamide biosynthesis protein 1
Diphthamide biosynthesis protein 2
tRNA guanine 37 N1 methyltransferase
tRNA dihydrouridine 47 synthase NAD P
tRNA wybutosine synthesizing protein 4
Clustered mitochondria protein homolog
Eukaryotic translation initiation factor 3 subunit
Ribosome inactivating protein beta momorcharin
tRNA N6 adenosine threonylcarbamoyltransferase
Eukaryotic translation initiation factor 3 subunit A
Eukaryotic translation initiation factor 3 subunit B
Eukaryotic translation initiation factor 3 subunit E
Signal recognition particle 54 kDa protein homolog
Polyadenylate binding protein cytoplasmic and nuclear
Mitochondrial zinc maintenance protein 1 mitochondrial
Eukaryotic translation initiation factor 3 subunit MExportin T
tRNA guanine N 7 methyltransferase non catalytic subunit trm82
tRNA adenine 58 N 1 methyltransferase non catalytic subunit trm6
Cell cycle (Mitosis)
Separin
Protein sds23
DNA ligase 4
F box protein grrA
Chitin synthase C
SCF subunit sconC
Flap endonuclease 1
Autophagy protein 5
Transcription factor steA
Regulatory protein abaA
Cytokinesis protein sepA
Cytokinesis protein sepH
Kinesin like protein bimC
E3 ubiquitin ligase complex
Negative regulator of mitosis
Mitochondria fission 1 protein
Nitrogen permease regulator 3
Protein transport protein sec31
SCF subunit sconBProtein stu1
Cell division control protein 48
Cell division control protein 15
Protein disulfide isomerase tigA
Sterol 3 beta glucosyltransferase
Nuclear distribution protein nudF
SCF E3 ubiquitin ligase complex
Tyrosine protein phosphatase cdcA
Orotidine 5 phosphate decarboxylase
DNA mismatch repair protein msh3
Topoisomerase 1 associated factor 1
E3 ubiquitin ligase complex
Pro apoptotic serine protease nma111
MYND type zinc finger protein samB
Plasma membrane fusion protein prm1
Chromosome segregation protein sudA
Phosphatidylinositol transfer protein sfh5
Mitochondrial distribution and morphology protein 10
Mitochondrial distribution and morphology protein 12
Glucose repressible alcohol dehydrogenase transcriptional effector
Anaphase promoting complex subunit 1ATP dependent RNA helicase chl1
Germinating Conidia

◀**Fig. 5** Overview of global development of *A. flavus* conidia into germinating conidial stages on the basis of protein profile of major functional categories that play important role in morphogenesis of *A. flavus*, using Coral draw software version X8 (www.coraldraw.com)

for their association in cellular, molecular and biological pathway for functional analysis of *A. flavus* proteins (Fig. 7). Gene Ontology (GO) was selected as input in the online software. The analysis revealed that the subjected proteins were associated with pathways related to metabolism including cell wall biogenesis, organic substance metabolic process, cellular metabolic process and macromolecule metabolism. The accession numbers of the selected proteins are listed in Supplementary Table S2.

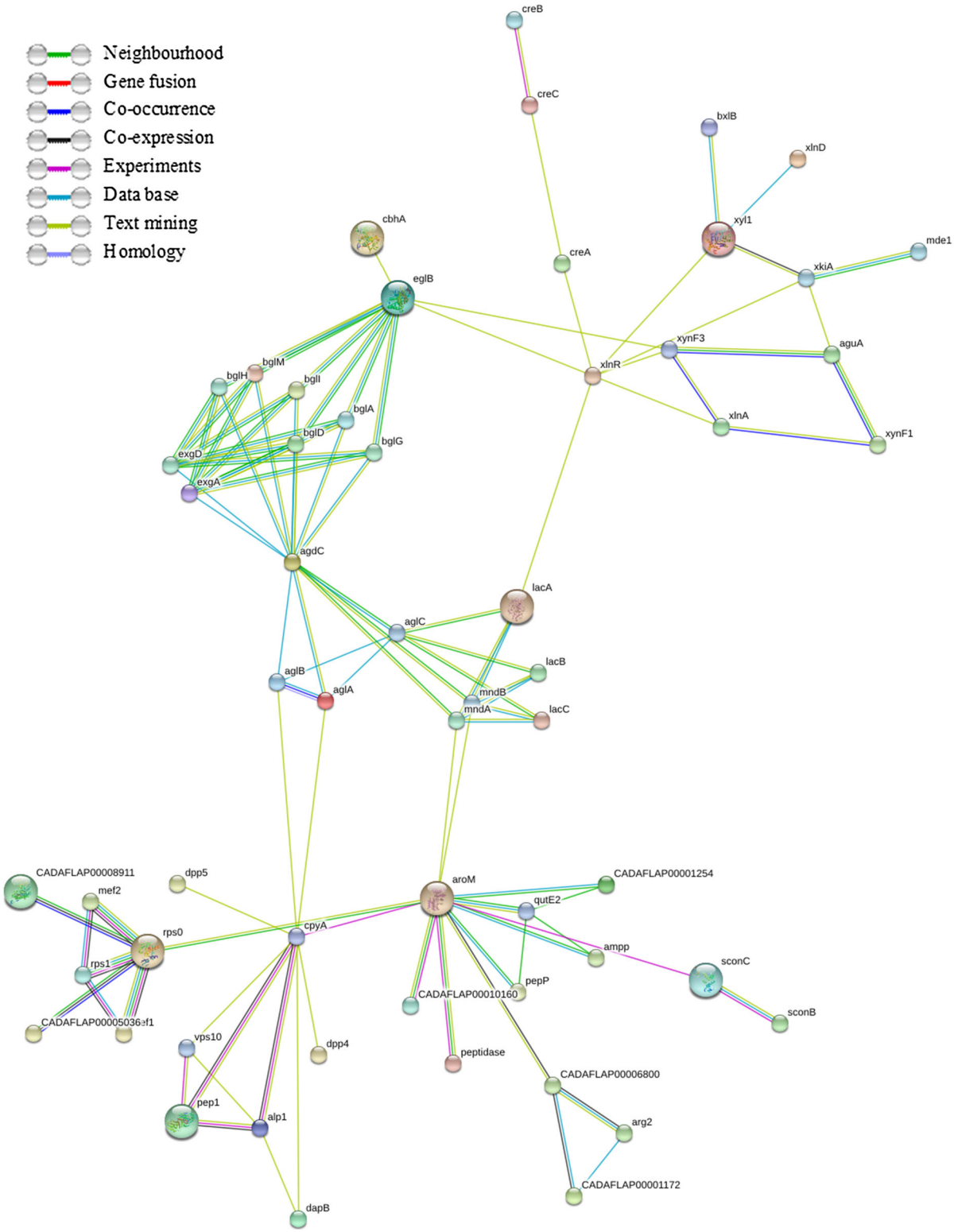
Discussion

Present study comprises of the proteome profile of *A. flavus* germinating conidia. To our knowledge, this is the first report describing the proteome profile at germinating conidial stage in *A. flavus*. Previously, Olga Pechanova et al. [12] reported protein profile of *A. flavus* at mycelial stage which predominantly involved protein from cellular metabolic process and aflatoxin biosynthesis pathway. Recently, van Leeuwen et al. [18] has carried out the transcriptome studies of *A. niger* at 8 h (germinating conidial stage) that showed germ tube formation is accompanied by polarized growth, including DNA processing, transcription, carbohydrate metabolism and cell wall biogenesis. Current data on proteome profile at germinating conidial stage of *A. flavus* showed that majority of proteins involved in carbohydrate metabolism, cell wall biogenesis, aflatoxin biosynthesis, MAPK signalling pathways, heat shock proteins, autophagy and dicer-like proteins. Some proteins were also found to be involved in pathogenesis and allergens.

We compared our data with previous studies on *A. fumigatus* proteomic data both at dormant stage (0 h) and germ tube formation stage (6 h) [15] and also with *A. niger* transcriptomic data both at dormant stage (0 h) and germ tube formation stage (6 h) [18]. Result showed that 22 expressed proteins from our data have similarity with *A. fumigatus* protein data and with that to *A. niger* at mRNA level data (Supplementary Table 3). We observed 2 proteins out of 22, viz. cytochrome c-oxidase subunit 1 and tubulin β-chain were not observed in dormant stage but observed to be expressed at germinating conidial stage. These two proteins are mainly involved in oxidative phosphorylation, energy metabolism and cytoskeleton formation. Histone H1 protein was not reported in both the dormant and germinating stages in *A. fumigatus,* but in our data it has been observed during germinating conidia.

Cell wall as an exoskeleton of fungal cells is a promising target for antifungal drugs, so it is important to elucidate the proteins involved in cell wall biosynthetic pathway and signal transduction. We observed β-1,3-glucansynthase (Fks1), which is a plasma membrane protein and deletion of this is supposed to be lethal for fungal species [34, 35]. However, in a recent research characterization of *A. fumigatus* Δ*fks1* mutant has been done and does not found to be lethal [36]. Chitin is the second most abundant polysaccharide in fungal cell wall, so far eight putative chitin synthase gene has been reported in *A. fumigatus* among which *csmA* and *csmB* are significantly important results in decreased colonial growth rate in *A. fumigatus* [21, 37]. The proteomic study of *A. flavus* at germinating conidial stage revealed involvement of two glycosylphosphatidylinositol (GPI) anchored proteins that is GPI monnositol transferase 3 and GPI ethanolamine phosphate transferase 1, which play major role in cell wall formation. Carbohydrate metabolizing proteins categorized on the basis of cellular component, molecular functions and biological functions from FungiFun software revealed that carbohydrate metabolizing proteins play major role in cell wall organization and structural organization. Increase in hydrolase activity and glucosidase activity is also seen at the time of carbohydrate metabolism.

Proteome profile of *A. flavus* at mycelia stage revealed the expression of proteins such as *O*-methyltransferase A, AflK/vbs/VERB synthase and aflatoxin B1-aldehyde reductase which are the key enzyme in aflatoxin production [12]. Our data also showed proteins related to aflatoxin biosynthesis such as *O*-methyl sterigmatocystin oxidoreductase, sterigmatocystin 8-*O*-methyltransferase, p450 monooxygenase AflN, versicolorin B desaturase, averufin oxidase A, averantin hydroxylase and noranthrone synthase which may suggest the involvement of aflatoxin production at germinating conidial stage.


Neighbourhood
Gene fusion
Co-occurrence
Co-expression
Experiments
Data base
Text mining
Homology
creB
creC
bxlB
xlnD
xyl1
mde1
xkiA
cbhA
creA
eglB
xynF3
aguA
xlnR
bglM
bglH
bglI
bglA
bglD
bglG
xlnA
xynF1
exgD
exgA
agdC
lacA
aglC
lacB
aglB
aglA
mndB
mndA
lacC
CADAFLAP00008911
dpp5
aroM
CADAFLAP00001254
mef2
qutE2
rps0
cpyA
ampp
sconC
rps1
pepP
CADAFLAP00010160
sconB
CADAFLAP00005036ef1
vps10
dpp4
peptidase
CADAFLAP00006800
pep1
alp1
arg2
CADAFLAP00001172
dapB

◀**Fig. 6** Protein–protein interaction between proteins differently expressed during *A. flavus* morphogenesis (7 h germination; germinating conidial stage). The diagram is generated using STRING, version 10.0 (http://string-db.org/), with the following parameters: species—*A. flavus*, confidence level—0.400, active prediction methods—all, input—UniProt accession numbers of expressed proteins. Details of interacting proteins are given in Table 1, categorized on the basis of GO biological functions

Fungal development and coordination of secondary metabolite production are controlled by MAPK module in *A. nidulans* [38].The identified proteins involved in signalling pathway were mitogen-activated protein kinase mpkC, protein kinase C, serine threonine protein kinase MARK2, serine threonine protein kinase atg1, tyrosine protein phosphatase cdcA and serine threonine protein kinase cbk1. MAPK pathway is activated by protein kinase C, which is a major pathway in filamentous fungi reported by Dirr et al. [39] and Valinate et al. [40]. This finding reveals that during morphogenesis of *A. flavus* MAPK signalling pathway plays major role in cell wall biogenesis and morphological transitions. In addition, it has been observed that MAPK pathway is associated with aflatoxin production [41]. Expression of two important secondary metabolite genes, viz. *laeA* and *aflR* involved in transcriptional process were found to be reduced in each mutant of MAPK module [38]. Thus, our data suggest MAPK pathway could be a crucial signalling pathway in germination of *A. flavus* conidia and secondary metabolite production.

Various antifungal studies in recent years depicted that *Aspergillus* species is becoming resistant to major drugs available in market (echinocandins, azoles, polyenes, etc.) by developing resistant strains and paradoxical effects [42]. In *A. fumigatus* upregulation of protein kinase C-encoding gene (*pkcA*) has been observed in response to echinocandin drug which also influenced calcineurin pathway, with increase in chitin synthesis, which showed the paradoxical effect of *A. fumigatus* [43]. Another class of protein identified in *A. flavus* that is p450 monooxygenase AflN and NADPH cytochrome P450 reductase involved in ergosterol synthesis pathway can act as efficient anti-*A. flavus* drug targets as it has been previously reported in studies conducted on *A. fumigatus* [21]. Camper et al. studied proteomic analysis of pathogenic fungus (*A. fumigatus*) that involved highly expressed conserved cell wall proteins. *A. fumigatus* proteins Bgt1, Gel1-4, Crf1, Ecm33, EglC showed homology with other fungal proteins and were found highly conserved and no homology with humans, among which Crf1 and Gel1 are being used as a promising vaccine candidate [44]. We observed Ecm14 and EglB in our study which is related to cell wall remodelling or carbohydrate metabolism processes. Thus, Ecm14 and EglB could also be explored for anti-*A. flavus* vaccine candidates. At germinating conidial stage, we also found allergen Asp fl 2 fragments found to be involved in IgG and IgE binding protein according to GO slim category functional characterization. Previous studies on other allergen showed that deletion in non-cytotoxic variants of *A. fumigatus* Asp f1 leads to reduced IgE binding [45]. As Asp fl 2 does not show homology with human counterpart, thus could be a potential candidate for vaccine studies.

Expression of heat shock proteins Hsp60 and Hsp70 suggests their role in *A. flavus* morphogenesis and osmotic stress conditions [46]. Transcripts encoding for Hsp70 protein was also observed in both aflatoxigenic and a toxigenic isolates of *A. flavus* at 30 °C [33]. Role of Hsp90 protein has been observed in germination and conidiation of *A. fumigatus* [47], and scarcity of Hsp90 protein (in *A. fumigatus*, *S. cerevisiae* and *C. albicans*) leads to increase in sensitivity against antifungal drugs [46, 48]. However, in the current data, we did not observed the expression of Hsp90 protein at germination conidial stages of *A. flavus*.

Our data analysis showed proteins such as autophagy-related protein 9, 11, 13, 18, 2, 22, COPII coat assembly protein sec16, nitrogen permease regulator 3, metacaspase 1B and sterol 3 beta glucosyltransferase are possibly involved in autophagy process. The role of autophagy process is in protein transportation and also involved in nutrient recycling that may lead to prolong cellular survival. It suggests that a nutrient recycling is the major phenomenon during development of conidia into germinating conidial stage. From previous studies, deletion of autophagy genes '*atg1*' or '*atg8*' resulted in altered fungal growth and development [49]. In addition, we also observed dicer-like protein 1, 2 and 21 in germinating conidia of *A. flavus*. Dicer-like proteins are involved in post-transcriptional regulation of transcripts [50]. This could be an interesting area to explore the role of post-transcriptional regulation during morphogenesis of *A. flavus*. Protein–protein interaction of 157 *A. flavus* proteins from STRING software revealed that

Table 2 Proteins involved in protein–protein interaction, categorized on the basis of their GO biological functions

Functional category	String ID	Protein name
Carbohydrate metabolism	xyl1	NADPH dependent D-xylose reductase xyl1
	cbhA	1,4-β-D-glucan cellobiohydrolase A
	xkiA	D-xylulose kinase A
	agalA	A-galactosidase A
	xlnD	Exo-1,4-β-xylosidase xlnd
	agdC	A-β glucosidase agdc
	eglB	Endo-β-1,4-glucanase B
	aglB	A-galactosidase B
	exgA	Glucan-1,3-β-glucosidase A
	lacC	β-galactosidase C
	bglM	β-glucosidase M
	lacA	β-galactosidase A
	bglI	β-glucosidase I
	aguA	α-glucuronidase A
	bglD	β-glucosidase D
	bglG	β-glucosidase G
	mndA	β-mannosidase A
	exgD	Glucan-1,3-β-glucosidase D
	bglH	β-glucosidase H
	bglA	β-glucosidase A
	mndB	β-mannosidase B
	aglC	A-galactosidase C
	xynF1	Endo-1,4-β-xylanase F1
	xlnA	Endo-1,4-β-xylanase A
	lacB	β-galactosidase B
	xynF3	Endo-1,4-β-xylanase F3
	bxlB	Exo-1,4-β-xylosidase bxlb
Amino acid biosynthesis	aroM	Pentafunctional AROM polypeptide
	arg2	Amino acid acetyltransferase mitochondrial
	CADAFLAP00006800	Arginine biosynthesis bifunctional protein argj mitochondrial
	mde1	Methylthioribulose-1-phosphate dehydratase
Amino acid metabolism	cpyA	Carboxypeptidase Y homologue A
	CADAFLAP00010160	Carboxypeptidase
	peptidase	Carboxypeptidase
	dapB	Dipeptidyl aminopeptidase B
	dpp4	Dipeptidyl peptidase 4
	dpp5	Dipeptidyl peptidase 5
	pepP	Xaa Pro aminopeptidase pepp
	CADAFLAP00001254	Xaa Pro aminopeptidase
Transcription	creC	Catabolite repression protein crec
	xlnR	Xylanolytic transcriptional activator xlnr
	ampp	Xaa Pro aminopeptidase P
	creA	DNA binding protein crea
	sconB	E3 ubiquitin ligase complex SCF subunit sconb
	creB	Ubiquitin carboxyl terminal hydrolase creb

Table 2 continued

Functional category	String ID	Protein name
Translation	rsp0	40S ribosomal protein S0
	mef1	Elongation factor G mitochondrial
	mef2	Ribosome releasing factor 2 mitochondrial
	rps1	40S ribosomal protein S1
Cell wall biogenesis	CADAFLAP00006800	Arginine biosynthesis bifunctional protein argj mitochondrial
	agdC	α,β-glucosidase agdc
	exgA	Glucan-1,3-β-glucosidase A
	exgD	Glucan-1,3-β-glucosidase D
Transport	vps10	Vacuolar protein sorting targeting protein 10
	xlnD	Exo-1,4-β-xylosidase xlnd
Proteolysis	alp1	Alkaline protease 1
Nucleic acid biosynthesis	CADAFLAP00001172	Adenylosuccinate synthetase
Cell cycle	sconC	E3 ubiquitin ligase complex SCF subunit sconc
Aromatic compound metabolism	qutE2	Catabolic 3 dehydroquinase 2

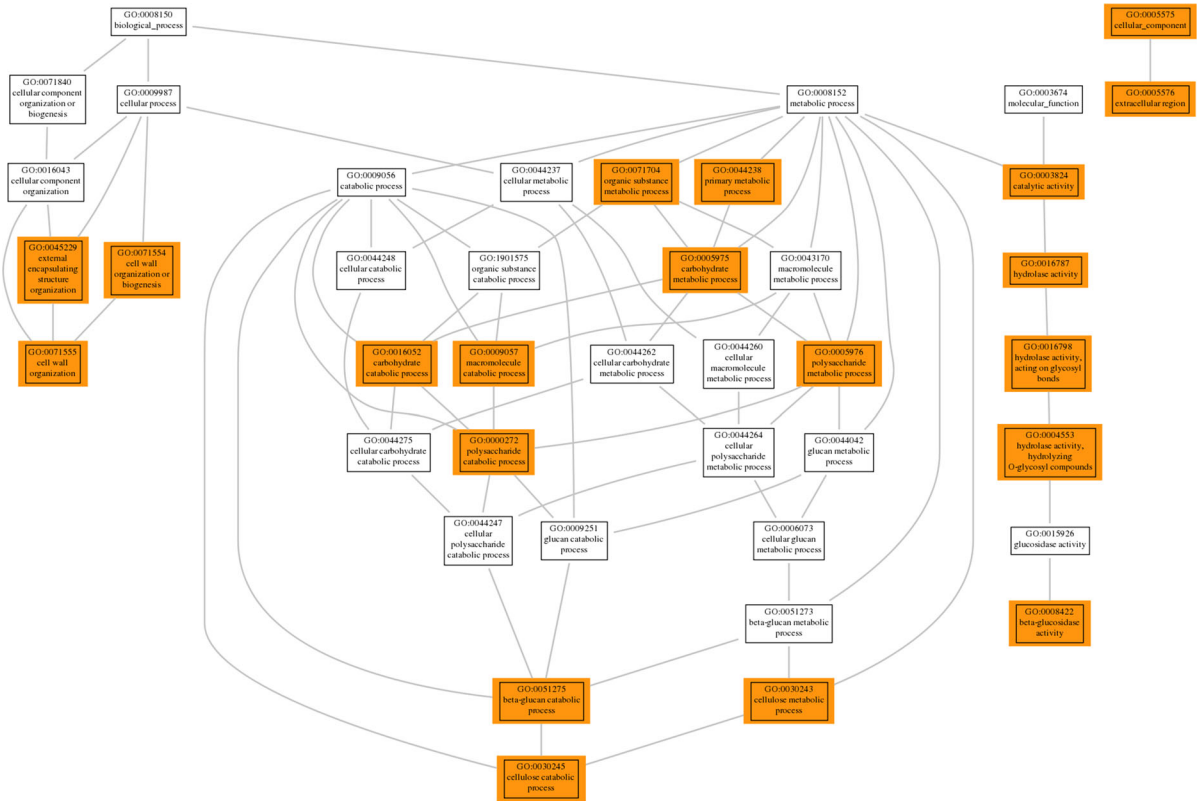


Fig. 7 Hierarchy representation of cellular, molecular and biological pathway of *A. flavus* proteins involved in carbohydrate metabolism pathway and their role in cell wall biogenesis at germinating conidial stage using FungiFun 2.2.8 BETA software (https://elbe.hki-jena.de/fungifun/fungifun.php), details enclosed in Supplementary Table S2

maximum number of interacting partners were observed for penta-functional AROM polypeptide (14 interactions) involved in amino acid biosynthesis α, β-gluclosidase agdC (12 interactions) involved in cell wall biogenesis, carboxypeptidase Y homologue A (11 interactions) involved in amino acid metabolism, xylanolytic transcriptional activator xlnR (7 interactions).

From the proteome profile, it has been observed that there are several proteins involved in fungal pathogenesis such as beta cyclopiazonate dehydrogenase, neutral protease 2 homologue mep20, non-ribosomal peptide synthetase 10, 11, 13, 3, Psi producing oxygenase A, and sphingomyelinase D. Beta cyclopenazole dehydrogenase is responsible to synthesize fungal neurotoxin alpha cyclopiazonic acid, which involves three enzyme pathways viz. CpaS, CpaD and CpaO [51]. *A. flavus* and *A. fumigatus* are involved in the production of two major types of proteases, serine protease and metalloproteases which is found to be involved in assimilation of proteinsious substrates such as histone and protamine [52]. Another class of protein found to be involved in pathogenesis, Psi producing oxygenase A, is a heme enzyme involved in the oxidation of linoleic acid, unsaturated fatty acids and regulation of secondary metabolism. This is also found to be involved in activation of human immune response by fungal development and resistance to host response [53]. Sphingomyelinase D is found to be involved in the hydrolytic cleavage of various lysophospholipids, viz. sphingomyelin for the production of choline, ceramide 1-phosphate and lysophosphatidic acid important for structural stabilization and pathogenesis in *A. flavus* [54].

In conclusion, the current study uses proteomics approach to decipher protein/enzymes involved in the germination of *A. flavus* conidia. Our study suggested that MAPK pathway could be the crucial signalling pathway involved in cell wall modulation and secondary metabolite biosynthetic pathway. Few of the proteins as discussed could be explored for anti-Aspergillus targets.

Acknowledgments Authors are thankful to Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, for providing facilities and Ph.D. fellowship to ST and RT.

Authors Contributions ST and JS conceived and designed the experiments. ST performed the experiments. ST, RT and JS analysed the data. GG and JS contributed reagents/materials/analysis tools. ST and JS contributed to writing the manuscript.

Conflict of interest The authors declare that they have no conflit of interests.

References

1. Thakur R, Anand R, Tiwari S, Singh AP, Tiwary BN, Shankar J. Cytokines induce effector T-helper cells during invasive aspergillosis; what we have learned about T-helper cells? Front Microbiol. 2015;6:429.
2. Guarro J, Xavier MO, Severo LC. Differences and similarities amongst pathogenic *Aspergillus* species. Aspergillosis: from diagnosis to prevention. New York: Springer; 2009. p. 7–32.
3. Chowdhary A, Sharma C, Kathuria S, Hagen F, Meis JF. Prevalence and mechanism of triazole resistance in *Aspergillus fumigatus* in a referral chest hospital in Delhi, India and an update of the situation in Asia. Front Microbiol. 2015;6:428.
4. Ramírez-Camejo LA, Zuluaga-Montero A, Lázaro-Escudero M, Hernández-Kendall V, Bayman P. Phylogeography of the cosmopolitan fungus *Aspergillus flavus*: is everything everywhere? Fungal Biol. 2012;116(3):452–63.
5. Bosetti C, Levi F, Boffetta P, Lucchini F, Negri E, La Vecchia C. Trends in mortality from hepatocellular carcinoma in Europe, 1980–2004. Hepatology. 2008;48(1): 137–45.
6. Hedayati M, Pasqualotto A, Warn P, Bowyer P, Denning D. *Aspergillus flavus*: human pathogen, allergen and mycotoxin producer. Microbiology. 2007;153(6):1677–92.
7. Yu J, Payne GA, Nierman WC, Machida M, Bennett JW, Campbell BC, et al. *Aspergillus flavus* genomics as a tool for studying the mechanism of aflatoxin formation. Food Addit Contam. 2008;25(9):1152–7.
8. Bhatnagar D, Cary JW, Ehrlich K, Yu J, Cleveland TE. Understanding the genetics of regulation of aflatoxin production and *Aspergillus flavus* development. Mycopathologia. 2006;162(3):155–66.
9. Van Leeuwen M, Van Doorn T, Golovina E, Stark J, Dijksterhuis J. Water-and air-distributed conidia differ in sterol content and cytoplasmic microviscosity. Appl Environ Microbiol. 2010;76(1):366–9.
10. Osherov N, May GS. The molecular mechanisms of conidial germination. FEMS Microbiol Lett. 2001;199(2):153–60.
11. Harris SD. Cell polarity in filamentous fungi: shaping the mold. Int Rev Cytol. 2006;251:41–77.
12. Pechanova O, Pechan T, Rodriguez JM, PaulWilliams W, Brown AE. A two-dimensional proteome map of the aflatoxigenic fungus *Aspergillus flavus*. Proteomics. 2013; 13(9):1513–8.
13. Medina ML, Haynes PA, Breci L, Francisco WA. Analysis of secreted proteins from *Aspergillus flavus*. Proteomics. 2005;5(12):3153–61.

14. Georgianna DR, Hawkridge AM, Muddiman DC, Payne GA. Temperature-dependent regulation of proteins in *Aspergillus flavus*: whole organism stable isotope labeling by amino acids. J Proteome Res. 2008;7(7):2973–9.
15. Suh M-J, Fedorova ND, Cagas SE, Hastings S, Fleischmann RD, Peterson SN, et al. Development stage-specific proteomic profiling uncovers small, lineage specific proteins most abundant in the *Aspergillus fumigatus* conidial proteome. Proteome Sci. 2012;10(1):1.
16. Kubitschek-Barreira PH, Curty N, Neves GW, Gil C, Lopes-Bezerra LM. Differential proteomic analysis of *Aspergillus fumigatus* morphotypes reveals putative drug targets. J Proteom. 2013;78:522–34.
17. Leng W, Liu T, Li R, Yang J, Wei C, Zhang W, et al. Proteomic profile of dormant Trichophyton Rubrum conidia. BMC Genom. 2008;9(1):303.
18. Van Leeuwen M, Krijgsheld P, Bleichrodt R, Menke H, Stam H, Stark J, et al. Germination of conidia of *Aspergillus niger* is accompanied by major changes in RNA profiles. Stud Mycol. 2013;74:59–70.
19. Lamarre C, Sokol S, Debeaupuis J-P, Henry C, Lacroix C, Glaser P, et al. Transcriptomic analysis of the exit from dormancy of *Aspergillus fumigatus* conidia. BMC Genom. 2008;9(1):417.
20. Ullmann AJ, Cornely OA. Antifungal prophylaxis for invasive mycoses in high risk patients. Curr Opin Infect Dis. 2006;19(6):571–6.
21. Valiante V, Macheleidt J, Föge M, Brakhage AA. The *Aspergillus fumigatus* cell wall integrity signaling pathway: drug target, compensatory pathways, and virulence. Front Microbiol. 2015;6:325.
22. Gautam P, Shankar J, Madan T, Sirdeshmukh R, Sundaram CS, Gade WN, et al. Proteomic and transcriptomic analysis of *Aspergillus fumigatus* on exposure to amphotericin B. Antimicrob Agents Chemother. 2008;52(12):4220–7.
23. Gautam P, Mushahary D, Hassan W, Upadhyay SK, Madan T, Sirdeshmukh R, et al. In-depth 2-DE reference map of *Aspergillus fumigatus* and its proteomic profiling on exposure to itraconazole. Med Mycol. 2016;54(5):524–36.
24. Bai Y, Wang S, Zhong H, Yang Q, Zhang F, Zhuang Z, et al. Integrative analyses reveal transcriptome-proteome correlation in biological pathways and secondary metabolism clusters in *A. flavus* in response to temperature. Sci Rep. 2015;29(5):14582. doi:10.1038/srep14582.
25. Anand R, Shankar J, Singh AP, Tiwary BN. Cytokine milieu in renal cavities of immunocompetent mice in response to intravenous challenge of *Aspergillus flavus* leading to aspergillosis. Cytokine. 2013;61(1):63–70.
26. Patel TK, Anand R, Singh AP, Shankar J, Tiwary BN. Evaluation of aflatoxin B1 biosynthesis in *A. flavus* isolates from central india and identification of atoxigenic isolates. Biotechnol Bioprocess Eng. 2014;19(6):1105–13.
27. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72(1–2):248–54.
28. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227:680–5.
29. Du L-Y, Zhao M, Xu J, Qian D-W, Jiang S, Shang E-X, et al. Identification of the metabolites of myricitrin produced by human intestinal bacteria in vitro using ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry. Expert Opin Drug Metab Toxicol. 2014;10(7):921–31.
30. Farrell A, Mittermayr S, Morrissey B, Mc Loughlin N, Navas Iglesias N, Marison IW, et al. Quantitative host cell protein analysis using two dimensional data independent LC–MSE. Anal Chem. 2015;87(18):9186–93.
31. Von Mering C, Jensen LJ, Snel B, Hooper SD, Krupp M, Foglierini M, et al. STRING: known and predicted protein–protein associations, integrated and transferred across organisms. Nucleic Acids Res. 2005;33(suppl 1):D433–7.
32. Priebe S, Kreisel C, Horn F, Guthke R, Linde J. FungiFun2: a comprehensive online resource for systematic analysis of gene lists from fungal species. Bioinformatics. 2015;31(3):445–6.
33. Bernard M, Latgé J-P. *Aspergillus fumigatus* cell wall: composition and biosynthesis. Med Mycol. 2001;39(1):9–17.
34. Henry C, Latgé J-P, Beauvais A. α1, 3 glucans are dispensable in *Aspergillus fumigatus*. Eukaryot Cell. 2012;11(1):26–9.
35. Firon A, Beauvais A, Latgé J-P, Couvé E, Grosjean-Cournoyer M-C, d'Enfert C. Characterization of essential genes by parasexual genetics in the human fungal pathogen *Aspergillus fumigatus*: impact of genomic rearrangements associated with electroporation of DNA. Genetics. 2002;161(3):1077–87.
36. Dichtl K, Samantaray S, Aimanianda V, Zhu Z, Prévost MC, Latgé JP, et al. *Aspergillus fumigatus* devoid of cell wall β-1, 3-glucan is viable, massively sheds galactomannan and is killed by septum formation inhibitors. Mol Microbiol. 2015;95(3):458–71.
37. Jiménez-Ortigosa C, Aimanianda V, Muszkieta L, Mouyna I, Alsteens D, Pire S, et al. Chitin synthases with a myosin motor-like domain control the resistance of *Aspergillus fumigatus* to echinocandins. Antimicrob Agents Chemother. 2012;56(12):6121–31.
38. Bayram Ö, Bayram ÖS, Ahmed YL, J-i Maruyama, Valerius O, Rizzoli SO, et al. The Aspergillus nidulans MAPK module AnSte11-Ste50-Ste7-Fus3 controls development and secondary metabolism. PLoS Genet. 2012;8(7):e1002816.
39. Dirr F, Echtenacher B, Heesemann J, Hoffmann P, Ebel F, Wagener J. AfMkk2 is required for cell wall integrity signaling, adhesion, and full virulence of the human pathogen *Aspergillus fumigatus*. Int J Med Microbiol. 2010;300(7):496–502.
40. Valiante V, Jain R, Heinekamp T, Brakhage AA. The MpkA MAP kinase module regulates cell wall integrity signaling and pyomelanin formation in *Aspergillus fumigatus*. Fungal Genet Biol. 2009;46(12):909–18.
41. Shankar J. An overview of toxins in *Aspergillus* associated with pathogenesis. Int J Life Sci Biotechnol Pharma Res. 2013;2(2):16–31.
42. Wiederhold NP. Paradoxical echinocandin activity: a limited in vitro phenomenon? Med Mycol. 2009;47(sup1):S369–75.
43. Fortwendel JR, Juvvadi PR, Perfect BZ, Rogg LE, Perfect JR, Steinbach WJ. Transcriptional regulation of chitin synthases by calcineurin controls paradoxical growth of *Aspergillus fumigatus* in response to caspofungin. Antimicrob Agents Chemother. 2010;54(4):1555–63.

44. Champer J, Ito JI, Clemons KV, Stevens DA, Kalkum M. Proteomic analysis of pathogenic fungi reveals highly expressed conserved cell wall proteins. J Fungi. 2016;2(1):6.
45. Garciá-Ortega L, Lacadena J, Villalba M, Rodríguez R, Crespo JF, Rodríguez J, et al. Production and characterization of a noncytotoxic deletion variant of the *Aspergillus fumigatus* allergen Aspf1 displaying reduced IgE binding. FEBS J. 2005;272(10):2536–44.
46. Tiwari S, Thakur R, Shankar J. Role of heat-shock proteins in cellular function and in the biology of fungi. Biotechnol Res Int. 2015;2015:132635. doi:10.1155/2015/132635.
47. Lamoth F, Juvvadi PR, Fortwendel JR, Steinbach WJ. Heat shock protein 90 is required for conidiation and cell wall integrity in *Aspergillus fumigatus*. Eukaryot Cell. 2012;11(11):1324–32.
48. Juvvadi PR, Lamoth F, Steinbach WJ. Calcineurin as a multifunctional regulator: unraveling novel functions in fungal stress responses, hyphal growth, drug resistance, and pathogenesis. Fungal Biol Rev. 2014;28(2):56–69.
49. Pollack JK, Harris SD, Marten MR. Autophagy in filamentous fungi. Fungal Genet Biol. 2009;46(1):1–8.
50. Dang Y, Yang Q, Xue Z, Liu Y. RNA interference in fungi: pathways, functions, and applications. Eukaryot Cell. 2011;10(9):1148–55.
51. Liu X, Walsh CT. Cyclopiazonic acid biosynthesis in *Aspergillus* sp.: characterization of a reductase-like R* domain in cyclopiazonate synthetase that forms and releases cyclo-acetoacetyl-L-tryptophan. Biochemistry. 2009; 48(36):8746–57.
52. Ramesh MV, Kolattukudy PE. Disruption of the serine proteinase gene (sep) in *Aspergillus flavus* leads to a compensatory increase in the expression of a metalloproteinase gene (mep20). J Bacteriol. 1996;178(13):3899–907.
53. Tsitsigiannis DI, Bok J-W, Andes D, Nielsen KF, Frisvad JC, Keller NP. Aspergillus cyclooxygenase-like enzymes are associated with prostaglandin production and virulence. Infect Immun. 2005;73(8):4548–59.
54. Dias-Lopes C, Neshich IA, Neshich G, Ortega JM, Granier C, Chávez-Olortegui C, et al. Identification of new sphingomyelinases D in pathogenic fungi and other pathogenic organisms. PLoS ONE. 2013;8(11):e79240.