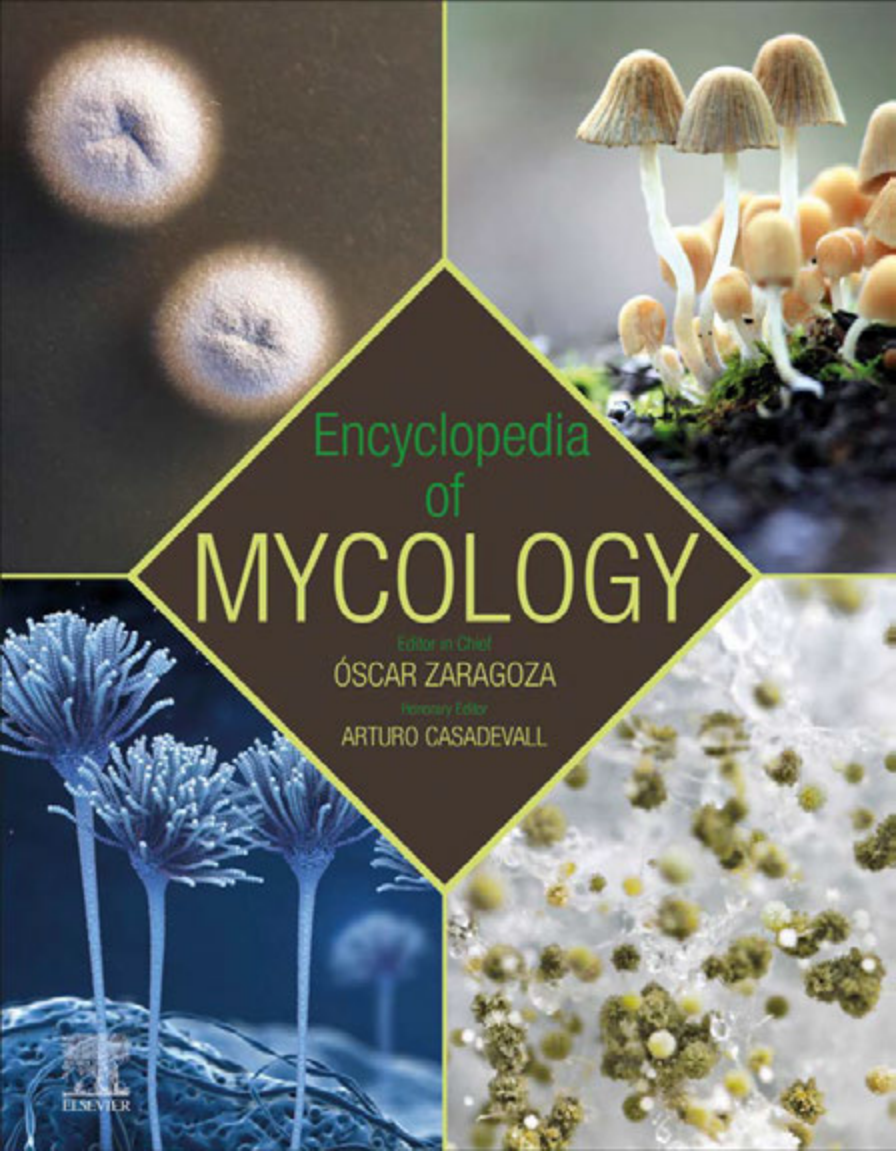
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EDITOR BIOGRAPHIES

Editor In Chief

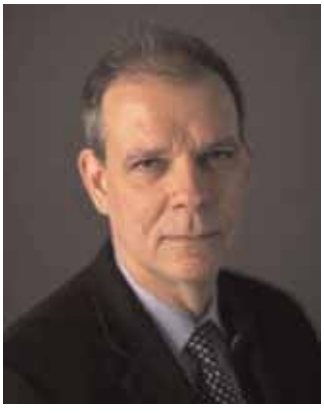
Dr Óscar Zaragoza received his degree in Biology in 1995, and in 1996 he started his scientific career, which has been focused on microscopic fungi. He started his PhD under the supervision of Dr Juana María Gancedo (Biomedical Research Institute, CSIC, Madrid, Spain) in 1996. He obtained his PhD in 2000, and in his thesis, he studied catabolite repression in the yeast *Saccharomyces cerevisiae*. During this period, and under the supervision of Dr Carlos Gancedo, Dr Zaragoza started working with human pathogenic fungi, using *Candida albicans* as model.

In 2001 he started his postdoctoral training in the laboratory of Dr Arturo Casadevall (Albert Einstein College of Medicine, New York), who is one of the most recognized scientists in the field of Clinical Mycology. During this period, he focused his research on human pathogenic fungi, and in particular, on *Cryptococcus neoformans*, which has a significant prevalence among HIV+ patients. In 2006, he returned to Spain, and began his work as Principal Investigator at the Mycology Reference Laboratory of the National Centre for Microbiology of the National Health Institute Carlos III (Madrid, Spain), directed by Dr Rodríguez-Tudela and Dr Cuenca-Estrella.

Dr Zaragoza's group main interest is the investigation of human fungal pathogens. These microorganisms affect mainly immunosuppressed patients, and they are associated with high mortality rates and a significant increase in the economic cost of patient treatment. In the last decade, he has directed two main research lines. One is focused on the mechanisms of fungal adaptation to the host, using the pathogen *C. neoformans* as model. In addition, the group is interested in the investigation of antifungal agents (action mechanisms and resistance). Dr Zaragoza is currently working at the Mycology Reference of the National Centre for Microbiology of the National Health Institute Carlos III, where he also supervises different tasks aimed to support the National Health System (identification and antifungal susceptibility of clinical isolates).

His research has authored more than 80 publications in some of the most important international journals of the field (*PLOS Pathogens*, *mBio*, *Infection and Immunity*, *mBio*, *Cellular Microbiology*, *Antimicrobial Agents and Chemotherapy*, among others), and several chapters in specialized books. Dr Zaragoza is also member of the editorial boards of *PLOS ONE*, *BMC Microbiology*, *Frontiers in Microbiology* (*Fungi and their development*) and *Mycopathologia*.

Honorary Editor



Arturo Casadevall, M.D., Ph.D., is a Bloomberg Distinguished Professor and Alfred and Jill Summer Chair of the Molecular Microbiology and Immunology at Johns Hopkins School of Public Health. He received his M.D. and Ph.D. degrees from New York University. He completed his internship/residency in internal medicine at Bellevue Hospital and specialized in Infectious Diseases at the Albert Einstein College of Medicine. The author of over 800 papers, books and chapters, his major research interests are in fungal pathogenesis and the mechanisms of antibody action. He is editor-in-chief of mBio, Deputy Editor of the Journal of Clinical Investigation and serves on several editorial boards. He has received numerous honors including election to the American Society for Clinical Investigation, American Academy of Physicians, American Academy of Microbiology, Fellow of the American Academy for the Advancement of Science, American Academy of Arts and Sciences and the National Academy of Medicine.

Section Editors

Raffaella Maria Balestrini. Biologist, PhD in Fungal Biology and Biotechnology. With CNR since 1998, Raffaella is currently a Research Director at CNR-IPSP. She has expertise on studies aimed to highlight the cellular and molecular bases of plant-soil microorganism interactions, mainly focusing on cell wall changes and nutritional exchanges. She mainly contributed to elucidate different aspects related to the interface creation and, by applying a laser microdissection (LMD) approach, she contributed to obtain new knowledge on the cell-specificity in arbuscular mycorrhizal (AM) roots. Thanks to the participation at diverse international consortia, she has contributed to highlight the genome features of different mycorrhizal fungi. Current research interests mainly address crop responses to environmental stresses and the role of root-associated microorganisms (e.g., mycorrhizal fungi) in improving tolerance. Scientific activities have been carried out within national and international projects including EU projects. Member of the editorial board for different scientific journals and she reviewed several project proposals for different national and international Institutions. She is member of diverse Editorial Boards of international peer-reviewed scientific journals. She has published over 100 papers in peer-reviewed indexed journals and many book chapters.

Miia R. Mäkelä is Principal Investigator and Adjunct Professor at the Department of Microbiology, University of Helsinki, Finland. Her main research interest is to understand fungal plant biomass conversion and degradation at the molecular level and apply this knowledge in biotechnology.

She obtained her PhD in Microbiology from the Faculty of Agriculture and Forestry, University of Helsinki. Her postdoctoral projects addressed various aspects of fungal lignocellulose degradation, including genome mining and characterization of novel enzymes for plant biomass conversion and valorization. During her position at Westerdijk Fungal Biodiversity Institute, The Netherlands, she focused on regulation of plant biomass conversion and metabolic engineering in ascomycete fungi. She received the prestigious Academy of Finland Research Fellowship position in 2017, which allowed her to develop a research line on the basidiomycete regulatory systems controlling plant biomass conversion process. Functional genomics and post-genomic approaches as well as genome editing are integral to her research.

She has (co-)authored over 100 peer-reviewed publications and has been a guest-editor for two special issues of the journal Fungal Genetics and Biology. She is currently an editorial board member for Biotechnology Letters, and an associate editor for Frontiers in Fungal Biology and Frontiers in Microbiology.

Josh Nosanchuk, MD is Professor of Medicine (Infectious Diseases) and Microbiology & Immunology as well as Senior Associate Dean for Medical Education at Albert Einstein College of Medicine in New York City. In his Dean position, Dr Nosanchuk strives to integrate basic, clinical and health system science across the curriculum of the medical school. Dr Nosanchuk's laboratory focuses on pathogenic fungi and novel therapeutics. Major pathogenic fungi studied include *Histoplasma capsulatum*, *Candida parapsilosis*, *C. auris*, *C. albicans*, and *Cryptococcus neoformans*. In particular, the laboratory investigates 1) how antibody can modify disease outcomes, 2) the role of melanin production on pathogenesis, and 3) the effects of extracellular release of vesicles from fungi, which contain numerous products associated with virulence. Novel therapeutics developed in the Nosanchuk laboratory include melanin-binding antibodies that have been used in a clinical trial for melanoma and pre-clinical compounds such as nitric oxide-releasing nanoparticles and siRNA targeting fidgetin-like 2, a microtubule severing enzyme that regulates wound healing.

Carla Viegas is a full Professor at Lisbon School of Health Technology, Director of the Occupational Health Master's course and researcher at H&TRC- Health & Technology Research Center from ESTeSL-IPL, NOVA National School of Public Health, Public Health Research Centre, Universidade NOVA de Lisboa and Comprehensive Health Research Center (CHRC). She is graduated in Environmental Health from Lisbon School of Health Technology – Polytechnic Institute of Lisbon has a Masters degree in Safety and Ergonomics from Lisbon University and PhD in Occupational and Environmental Health from New University of Lisbon. Her major field of study is occupational and environmental mycology leading and participating in several national and international projects about both areas of expertise. Special interests are occupational exposure to fungi in highly contaminated settings and complementarity of culture based- methods and molecular tools to assess fungal contamination. Additionally, she has expertise in sampling campaigns performed in different occupational environments using wide sampling and analyses approach to assess multiple microbiologic agents. Carla has authored has several publications/communications in the referred areas of specialization.

Alfredo Vizzini is a biologist with over 30 years' experience in Systematic Mycology. He graduated with a PhD in Biology and Biotechnology of fungi from the Department of Plant Biology of Torino, Italy. He is now an Associate Professor of Systematic Botany at the University of Torino (Department of Life Sciences and Systems Biology) where he teaches biodiversity of fungi and terrestrial plants. His research interests focus on mushroom taxonomy, especially agaricoid and boletoid species, using morphological and molecular approaches.

He is the author of over 200 publications on peer-reviewed international journals. Alfredo is currently associate/section editor of the journals Ascomycete.org, Biodiversity Data Journal, Bulletin of Environmental and Life Sciences, Italian Botanist, MycoKeys, Mycosphere, Phytotaxa, Studies in Fungi, Taxonomy, and a curator of the webpages Basidiomycota (https://basidio.org/), and Faces of Fungi (https://www.facesoffungi.org/).

Ronald P. de Vries. Group Leader Fungal Physiology at Westerdijk Fungal Biodiversity Institute (The Netherlands) and Professor in Fungal Molecular Physiology at Utrecht University (The Netherlands).

His research addresses the conversion of biomass (plant and marine) by fungi, focusing on the extracellular enzymes, the intracellular metabolic pathways and the transcriptional regulators that control these processes, as well as the application of fungi for the biobased economy. It contains a strong (post-)genomic component combined with modern and established experimental methodologies, such as genome editing, enzymology, metabolic engineering and physiology.

He graduated at Wageningen University (The Netherlands) and spent some time there and at Institut Pasteur (Paris, France) as a postdoc, after which he moved to Utrecht University as a postdoc and senior scientist, before taking up his current position. He obtained the prestigious Dutch VIDI (2005) and VICI (2013) grants and became an honorary member of the Hungarian Microbiological Society in 2017. He is a visiting professor at University of Helsinki (Finland) and a member of the fungal advisory board of the Joint Genome Institute, USA.

PREFACE

Fungi are organisms that have a profound significance in multiple processes of great importance for our daily life. But, apart from their influence in human affairs, fungi are fascinating organisms to study in themselves. They comprise both macroscopic and microscopic organisms, and it is estimated that there are somewhere between one and five million species of fungi. Fungi are found in most of ecosystems and are in constant contact with multiple other organisms, being involved in many commensal, symbiotic and parasitic interactions.

From a biological point of view, fungi are heterotrophic eukaryotic organisms that have a cell wall that contains chitin and several other polysaccharides (glucan, galactomannan, etc). They can reproduce by both sexual and asexual cycles. Despite the great variation among fungal morphology (macroscopic and microscopic), genetic tools reveal close relationships from an evolutionary point of view, and are closer to animals than to plants. But still, the Fungal kingdom contains a great variability in structure, shape, colour, reproduction and biology.

Most readers are already familiar with macroscopic fungi (mushrooms). Their colours and shape could be considered as one of the beautiful creations of nature, and many people look forward to those seasons of the year when they can enjoy viewing them in fields and forests. But in addition, macroscopic fungi have been enigmatic organisms for centuries. Some of them are edible, others poisonous or hallucinogenic, and still others have medical properties. Nowadays, they have multiple applications, not only as a delicious food source, but also in biotechnology, and there is a great evidence of their role in geochemical balance in the environment.

A major part of mycology involves microscopic fungi, which are divided in two main classes: yeasts and filamentous fungi (or moulds). And although invisible to the eyes, their biology and effects are also fascinating. In fact, we could express the importance of microscopic fungi by remembering one of the most famous quotes from the "The little Prince", when the Fox revealed that the essential things in life are invisible to the eyes. When Antoine de Saint-Exupéry wrote this quote, he did not refer to microscopic fungi, but when we consider all the effects that they have on our world, we could certainly consider them as essential. How would the world be today without fungi? We will never know, but the fact that fungi are found in most ecosystems and establish interactions with all kind of living organisms indicate that they have played a role in evolution to select and shape our current world and way of living. Even in the case of humans, fungi have influenced the evolution of our immune systems, microbiota and other aspects such as body temperature. For thousands of years, we have taken advantage of fungal biology to produce cheese, wine, bread, beer and many other products. Paradoxically, we have not understood the basis of their action until relatively recently in our history, towards the end of XIXth century, and nowadays they are a powerful tool in today's biotechnology. Fungi triggered a revolution in the medicine when Alexander Flemming found that they can be the source of antibiotics. Since the discovery of penicillin from the fungus *Penicillium notatum*, the search for many other compounds with biological activities produced by any kind of microorganism has been continuous and successful. Today fungi produce numerous important drugs including antimicrobial drugs and statins. In addition, fungi are used as microscopic factories to produce many compounds of interest or acquire new metabolic functions that provide multiple biotechnological applications.

Research in microscopic fungi has been classically promoted by their important role in biotechnology. This interest began during the XXth century as the industry developed and some species became important model organisms (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* or *Neurospora crassa*, and *Aspergillus nidulans*), which allowed studies that led to the unravelling of key aspects in metabolism, genetics, cell cycle regulation, circadian cycles and many others are process. Even the first eukaryotic microorganism that was fully sequenced was the yeast *S. cerevisiae*, which highlight the importance of these microorganisms in research.

Despite their innumerable beneficial effects, the interaction of fungi with other living organism such as plants and animals can also result in deleterious outcomes and disease. We could explain the role of fungi as potential pathogens by revealing the secret that the letter "F" hides in the name Fungi, since they can be considered as either Friends or Foes. In the case of humans, microscopic fungi can cause many different diseases. Some of these pathogenic fungi live as inoffensive commensal in our body, but in some cases, depending on multiple factors, mainly related to our immune system, they can produce damage and disease. In some way, we could envision them as the main character from the classic written by Robert L. Stevenson, so these fungi could resemble to the kind and nice Dr. Jekyll that sometimes turns into the hideous and evil Mr. Hyde. In other cases, fungi that cause disease are acquired from the environment and they do not belong to our microbiota. This poses the challenge of how environmental changes (global warming, release of antimicrobial, use of antifungals, environmental interactions, etc) affect the virulence of the fungi that we constantly acquire from external sources. Most of fungal diseases are superficial and non-life threatening, but they can cause great discomfort. The incidence of these types of fungal diseases, such as infections of the skin (i.e., athletes foot), eye and mucosae such as the vagina (mainly vaginal candidiasis) or oral cavity is enormous, reaching around one hundred millions of people per year. In addition, some fungal species can be the causative agents of invasive disease, mainly among immunocompromised patients. As historical curiosities, the first cases of HIV+ was originally diagnosed with invasive fungal diseases. And even more anecdotic, fungi were in some moment linked to the mythical "Tutankhamun's Curse" and related to the deaths of some of the discoverers of the tomb of the young Pharaoh. Nowadays, it is estimated that around 1.5 million people die every year due to fungal pathogens, a number that is unacceptably high. Moreover, beyond humans, fungi can also cause devastating plagues in the environment in animals and plants. As examples of their negative effects, the economic impact of fungi as crops contaminants is estimated in billions of dollars every year. And among animals, many fungi are common causes of disease

in multiple species, both vertebrates and invertebrates, and there are some species of amphibians have been driven to extinction by fungal diseases. In the United States the so called 'white nose syndrome' fungal disease threaten to drive several bat species to extinction.

In conclusion, the biology of fungi and their multiple effects in our world justified the organization of this Encyclopaedia, which has been possible to develop thanks to the dedicated work of the editors that have organized the five sections in which this work is organized: **Dr. Carla Viegas** (Lisbon School of Health Technology/Polytechnic Institute of Lisbon, Portugal, editor of Environmental Mycology), **Dr. Alfredo Vizzini** (University of Torino, Italy, editor of Macroscopic fungi), **Dr. Raffaella Ballestrini** (Fungal Biology), **Dr. Miia R. Mäkelä** (University of Helsinki, Finland) and **Dr. Ronald P de Vries** (Westerdijk Fungal Biodiversity Institute/Utrecht University, both editors of the Fungal Biotechnology section) and **Dr. Josh Nosanchuk** (Einstein College of Medicine, Ney York, editor of Medical Mycology). This work has been also possible due to the engagement of a large number of authors who are leading experts in their respective fields. We would like to stress that the development of this project took place during COVID19 pandemic, which has all our lives. We are aware of how difficult it has been for many authors to continue with their regular work (academia, research, health assistance, etc) with the restrictions caused by the pandemic. We are sure that for all of them, participation in this Encyclopaedia required a great deal of extra effort, and for this, and we are very grateful for their dedication and engagement in this enterprise. We also acknowledge and thank the Elsevier staff for their support and their assistance with such a challenging project. We warmly thank all the people involved in the completion of this Encyclopaedia, and we are proud that the final result provides a nice and comprehensive work on one of the most exciting topics in Biology.

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Next Generation Sequencing: Transcriptomics

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The Rise of NGS Transcriptomics in Fungi

Genomics and its applications are some of the most advanced areas of current biological research. However, the genome sequencing represents just the first step toward a broad knowledge of an organism. The integration of functional, structural and cellular approaches that, all together, form the so-called *post-genomic* activities, is necessary for the deeply understanding of the role and the network of genes/proteins, and to the assignment of functions to the whole gene dataset discovered into organisms. Transcriptomics is one of the most developed fields in this current *post-genomic* era. By mirroring the differential gene expression during development and different phases of organisms life cycle, as well as during their responses to biotic and abiotic factors, the transcriptome constitutes the dynamic link between the static genome of an organism, a tissue or a cell, and its phenotype, at a specific time point (Meijueiro *et al.*, 2014; Stark *et al.*, 2019). In fact, morphogenetic changes of organisms might often depend on corresponding changes of activity of large numbers of genes. Several studies on single transcripts were carried out many years before any transcriptomics approaches were available. Traditional techniques included cDNA Amplified Fragment Length Polymorphisms (cDNA-AFLP), low-throughput Sanger sequencing of random transcripts from complementary DNA (cDNA) libraries [namely, expressed sequence tags (ESTs)], and PCR-based techniques, i.e. retro-transcriptase quantitative PCR (RT-qPCR) (Bhadauria *et al.*, 2007; Lowe *et al.*, 2017). Nevertheless, only after the development and the improvement of higher-throughput techniques it was possible to investigate how an organism transcriptome as a whole is finely regulated.

Fungal transcriptomics is a rising field which has gained more and more attention in the last two decades. The first fungal transcriptome, based on serial analysis of gene expression (SAGE), was released in 1997 for yeast (Velculescu *et al.*, 1997), and after that, thanks to the development and the availability of modern large-scale molecular tools, an exponential increasing number of fungal transcriptomes was published (Nowrousian, 2013; Meijueiro *et al.*, 2014). Currently, even if some other recent promising techniques has been proven to be useful in transcriptomics i.e., high throughput-SuperSAGE (HT-SuperSAGE) (Soanes *et al.*, 2012), the two most popular large-scale transcritptomic approaches applied in fungi remained microarrays (Nowrousian, 2007) and RNA sequencing (RNA-seq) (Wang *et al.*, 2010b). Microarrays refers to a collection of thousands of oligonucleotide spots, defined as probes, attached to a solid surface, which can hybridize a cDNA, defined as target, under high-stringency conditions (Schulze and Downward, 2001). The cDNA sequences, each of them representing a single transcript, are labeled and hybridize to the corresponding gene sequences on the array, and relative abundance of cDNA in the target is quantified by fluorescence detection and quantification (Schulze and Downward, 2001). This technology allowed for the first time the simultaneous analysis of thousands of transcripts. Pioneering fungal microarray studies were performed in yeast more than 20 years ago (DeRisi *et al.*, 1997; Lashkari *et al.*, 1997). Microarrays have been produced for several species of filamentous fungi, mainly by using PCR amplicons from EST libraries (Breakspear and Momany, 2007). The application of microarray technology to decipher transcriptomic profiles of fungi subjected to different conditions has proven to be useful for extending the knowledge on how fungi face with their surrounding environment. For example, *Cryphonectria parasitica* cDNA microarrays were used to monitor the changes in transcriptional profile when infected by a hypovirus (Allen *et al.*, 2003). A significant reprogramming of the *C. parasitica* transcriptome was observed and differentially expressed genes (DEGs) related to stress responses, carbon metabolism, and transcriptional regulation were detected (Allen *et al.*, 2003). Another classical example is represented by microarrays for *Aspergillus nidulans,* fabricated by using PCR products from a EST libraries generated during conidial germination, and used to assess the transcriptomic response of the fungus to different nutrient sources (Sims *et al.*, 2004). In the symbiotic Ascomycete *Tuber melanosporum*, microarrays allowed to track the transcriptomic changes occurring in its life cycle, in particular during the shift from free living mycelium to the formation of ectomycorrhizas and fruit bodies (Martin *et al.*, 2010).

However, it should be noted that microarray-based technology can be affected by some important limitations. First, for the customization of arrays, a reference genome sequence or a comprehensive set of EST sequences must be available, and second, non-specific or repeated cDNA sequences with high similarity hybridization to targets might strongly affect the dynamic range of the recorded signal (Lowe *et al.*, 2017). Moreover, not characterized transcripts (e.g., non-coding transcripts) and RNA species missing in EST libraries or in genome annotations cannot be detected by using this technology which is based on the availability of well-known sequences (Lowe *et al.*, 2017).

Currently, the emergence and the democratization of high throughput next-generation sequencing (NGS) techniques as substitute to Sanger sequencing for large-scale genomic analysis provided an opportunity to further explore and understand the differential gene expression at an exceptional resolution (Marconi *et al.*, 2014). In contrast to traditional Sanger sequencing, NGS allowed high throughput parallel sequencing of over millions of DNA nucleotides from multiple samples at reduced cost and time (Metzker, 2010). International sequencing activities, such as the ongoing "1000-fungal genomes" project (see "Relevant Websites section") of the U.S. Department of Energy (DOE) Joint Genome Institute (JGI) (Grigoriev *et al.*, 2014), are aimed at increasing the dimension of fungal genome sequence databases by covering all fungal taxonomic units through sequence characterization of the most representative isolates. Remarkably, for almost all genome projects currently finished or in progress, a related large scale gene expression dataset is provided (Kuske *et al.*, 2015). Dedicated databases containing manually annotated fungal transcript sequences (e.g., see "Relevant Websites section"; Stajich *et al.*, 2012) are also in ongoing development.

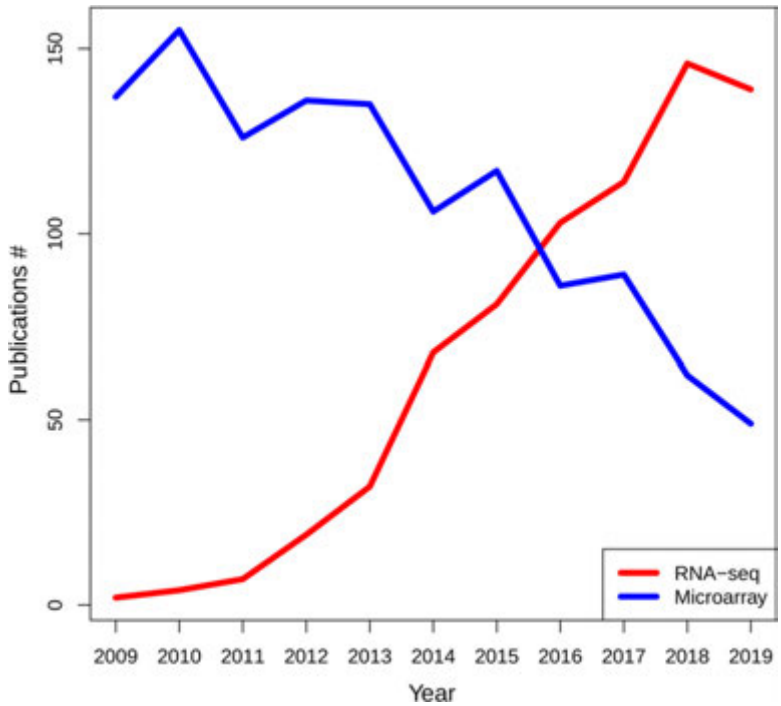

Fig. 1 Results of a search on Scopus database (www.scopus.com) with the terms "microarray AND fungi" and "RNA-seq AND fungi". Number of publications and years were reported.

In this line, RNA-Seq refers to the sequencing, through NGS, of transcripts converted into cDNA sequences, in which abundance is directly proportional to the number of counts from each transcript (Stark *et al.*, 2019). Compared to microarrays, RNA-seq does not require a priori knowledge of the genome sequence and this method can potentially be used for the detection of quantification of each RNA species present in samples, including non-coding or uncharacterized RNAs. RNA-seq overcomes microarray technology because of its intrinsic potential to discover transcript variants, i.e., transcripts showing SNPs and mutations (Stark *et al.*, 2019). Comparative transcriptomics analyses using RNA-seq provided high accurate determination of quantitative expression levels, the identification of tissue-specific transcript splicing variants and isoforms, and the detection of small and large non-coding RNAs involved in gene expression regulation (Stark *et al.*, 2019). For all these reasons, RNA-seq has become the gold standard for whole-transcriptome gene expression quantification (**Fig. 1**).

Transcriptomics Through RNA-Seq

The first step of a RNA-seq, as for microarray, includes the isolation of RNA from the samples, either from single fungal isolates and from environmental matrices. Commercial kits available for plant so far have started to be optimized also for fungal materials, and some protocols for extraction of high-quality RNA with sequencing purposes have been published (Patyshakuliyeva *et al.*, 2014; Cortés-Maldonado *et al.*, 2020). This step is crucial since fungal cells, in some cases even more than plant cells, may contain nasty chemicals such as melanin and phenolic compounds as well as high levels of polysaccharides which can interfere with the downstream analysis, especially those PCR-based. In addition, the yield of fungal RNA in some samples such as plant or food samples is often very low (Argumedo-Delira *et al.*, 2008). Optimal starting amount of RNA for RNA-seq may range from 100 ng to 1000 ng, but there are kits available for ultra-low RNA input such as 10 pg–10 ng (Tariq *et al.*, 2011). Once RNA is isolated, purified and its quality is checked, several NGS platforms are available. One of the first NGS technology serving transcriptomic research was pyrosequencing, a method involving sequencing-by-synthesis followed by detection of pyrophosphate release during the activity of DNA polymerase through chemiluminescent enzymes, such as luciferases (Ronaghi, 2001). Pyrosequencing was licensed by 454 Life Sciences and allowed to obtain sequences of approximately 500 bp in size, but, as becoming noncompetitive for costs, the platform was discontinued in 2013. Currently, the two most popular and established NGS platforms used for RNA-seq in fungi are manufactured by Illumina (MiSeq and HiSeq) and Ion Torrent (Lahens *et al.*, 2017). The two platforms mainly differ for the basic sequencing technologies: while for Illumina amplified individual library DNA molecules were spotted on a solid surface and a fluorescence-based system for reading the bases in a nucleotide sequence is used (sequencing by synthesis), for Ion Torrent emulsion PCR libraries were used and detection is achieved by electronic sensors reading bases through the H^+ ion generated during nucleotide incorporation (Lahens *et al.*, 2017). Despite these NGS methods, defined as of second generation (the first included Sanger and Maxwell sequencing methods), proven to be powerful, they still retain some pitfalls, including the generation of short reads, i.e., ranging from 100 to 500 bp, which often might hinder the correct reconstruction of

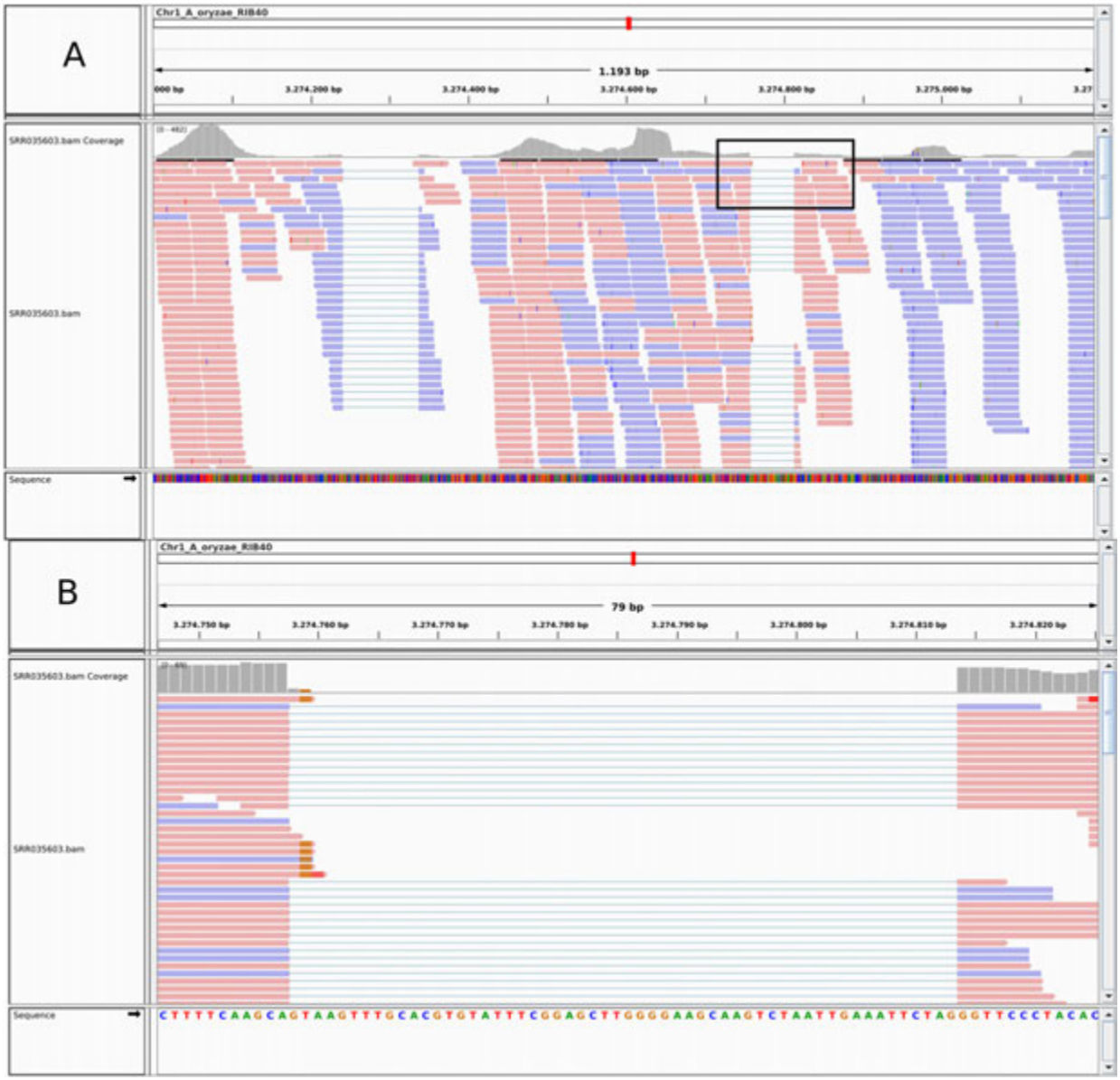

Fig. 2 Example of visualization of aligned reads from a RNA-seq experiment in IGV. In (A), the chromosome 1 of *Aspergillus oryzae* isolate RIB40 is used as reference for the alignment and the pileup of reads embedded in a BAM file (SRR035603.bam) is showed. Coverage is represented as gray histogram on the top of the alignment. Red and blue arrow-blocks represent first strand and complementary paired-end reads, respectively. Black squared section is detailed in (B). In (B), structure of an intron is showed. The intronic region was not covered by reads from the transcript, and reads over-spanning the exon boundaries. A cyan line, representing the intron, links the two exons. Data for this figure were obtained from Nowrousian, M., 2013. Fungal gene expression levels do not display a common mode of distribution. BMC Research Notes 6, 55, and freely available in SRA database.

full-length transcripts (Lowe *et al.*, 2017). Recently, the so called third-generation/long-read methods appeared on the market. Third generation sequencing works by reading the nucleotide sequences at the single molecule level, in contrast to existing methods which require breaking DNA strands into small fragments and the subsequent amplification (Van Dijk *et al.*, 2014). In this scenario, the company PacBio developed the sequencing platform of single molecule real time sequencing (SMRT), where signals of fluorescent light emission from each nucleotide incorporated by a DNA polymerase bound to the bottom of a chip, characterized by an optical waveguide (zero-mode waveguide), were detected and sequences were resolved (Roberts *et al.*, 2013).

Whereas each platform requires specific protocols of library preparation and nucleotide detection, the second generation sequencing platforms are characterized by the need to prepare amplified sequencing libraries before proceeding to the sequencing of the amplified cDNA clones (Lowe *et al.*, 2017). A standard RNA-seq protocol shared among all platforms include the cDNA library preparation, which consists of nucleic acid fragmentation, cDNA synthesis from RNA using random or oligo (dT) primers, adapter ligation, fragment size selection, and PCR amplification. By contrast, third generation sequencing platforms does not need

the prior amplification of cDNA (Van Dijk *et al.*, 2014). It is worth noting that transcriptomics primary focus on coding RNA translated into proteins (mRNA) which typically represent the 2%–3% of the total RNA in a cell. Other RNA species are indeed present into cells, including ribosomal RNA (rRNA) and tRNA (which are approximately 95% of a cell), and non-coding RNA such as snRNA, siRNA, snoRNA and microRNA (Meijueiro *et al.*, 2014). Several strategies of enrichment of a specific RNA population can be used to trigger the transcriptomic analysis depending on the focus of the experiment. For example, mRNA is usually isolated and converted into cDNA by using reverse transcriptase and oligodT primers, whereas noncoding RNA species can be included in libraries by using immunoprecipitated RNA-binding proteins or by selectively ligating 3′ and 5′ adapters before the reverse transcription to cDNA (Lowe *et al.*, 2017).

Irrespective of NGS platform used, transcripts deriving from RNA-seq are provided as short (less than 1.5K bp) sequences called *reads*, which can be aligned against a reference genome or *de novo* assembled into longer sequences when a reference genome is not available. However, Illumina and Ion Torrent platforms showed some differences in the generated data: for the first one all reads generated in a single run have the same lengths, while the read lengths of the second are variable (Lowe *et al.*, 2017). Paired end reads, i.e. reads from both ends of a fragment, can be also produced if the utilized NGS technology allowed it. Reads are typically provided as unsorted FASTQ files, a file format similar to FASTA but comprehensive of all the information about base-calling and sequence quality (Marconi *et al.*, 2014). Reads with low quality levels, i.e., low *phred* scores, or with sequence errors should be purged and sequences of vectors, adapters, tags, and tails that were added experimentally during the preparation of the libraries should be trimmed by specific bioinformatic tools (Kulski, 2016). Filtered reads are then assembled into longer sequences called *contigs* representing complete transcripts, their fragments or isoforms (Kulski, 2016). Presence of isoforms and alternative splicing in fungi has been indeed demonstrated so far (Grützmann *et al.*, 2014). Several bioinformatic tools are used to map the reads to genomic sequences, chromosomes and scaffolds. Compared to aligners of DNA sequences, RNA-seq mappers need to cope with intronic sequences which lead large gaps in the alignment. In fungi, the most established read mappers for this purpose include TopHat (Trapnell *et al.*, 2009), STAR (Dobin *et al.*, 2013), Trinity (Haas *et al*, 2013), and MapSplice (Wang *et al.*, 2010a). Commercial softwares such as CLC Genomics Workbench (CLC bio, Cambridge, MA, USA) and Newbler (Roche 454 Life Science, Branford, CT, USA) are also available. A common output of the read alignment process is a sortable SAM (Sequence Alignment/Map) file or its compressed binary version BAM. Barcodes, read quality, amplicon tags and other information are embedded in this type of files (Li *et al.*, 2009). These format files can be used as input for the popular software SAMtools (Li *et al.*, 2009), which not only allows at calculating read coverage by performing read pileup on reference genome, but also can be useful for statistics and filtering purposes (Li *et al.*, 2009). Visualization of reads aligned to reference sequences can be achieved by using Integrate Genomic Viewer (IGV; Thorvaldsdóttir *et al.*, 2013) which has been developed as an open source easy to understand tool for the dealing of NGS data, with a fashionable graphical user interface (GUI) (**Fig. 2**).

On the other hand, as stated before, without the availability of a reference genome, reads need to be assembled into longer contigs through a *de novo* assembly process. This strategy is generally based on the generation of De Bruijn graphs, where overlapping sequences are represented as paths which overlap, in order to reconstruct the original transcript sequences (Grabherr *et al.*, 2011). This approach is also recommended as complementary analysis of reference-guided mapping, in order to identify transcripts missed by alignment processes and to correct errors that may occur during the annotation (Hölzer and Marz, 2019). *De novo* assembly of RNA-seq data is more challenging than *de novo* genome assembly because of difference on gene expression, potentially masking low expressed loci, and presence of splicing events, making the reconstruction of exons in genes in the assembly paths complicated (Hölzer and Marz, 2019). Several powerful free tools such as Trinity (Grabherr *et al.*, 2011), Oases (Schulz *et al.*, 2012), and SoapDeNovo (Xie *et al.*, 2014) were developed for *de novo* transcriptome reconstruction. Completeness of *de novo* assembly can be verified through different bioinformatic tools included in specific pipelines, such as BUSCO (Simão *et al.*, 2015). This step is pivotal because of presence of large gaps and missing transcripts are know to likely occurring during *de novo* assembly processes.

Transcript sequences deriving from RNA-seq analysis may allow to strongly improve *in silico* annotations of putative genes and gene structures, as well as to train bioinformatic tools like gene predictors (e.g., AUGUSTUS; Stanke *et al.*, 2006). Thanks to alignment of reads on the available genomes, assembled transcripts are utilized to detect intron-exon boundaries in predicted/annotated genes (Lowe *et al.*, 2017), thus reducing annotation errors. A study in *Laccaria bicolor* highlighted that approximately the 69% of predicted gene models deviated from the real transcript sequences derived by RNA-seq data (Larsen *et al.*, 2010). Characterization of transcriptome can be carried out through gene ontology (GO) mapping of sequences, via specific tools such as Blast2Go (Conesa *et al.*, 2005). Basically, the GO mapping processes result in the assignment of defined GO terms, which provide information on involved biological process, molecular functions and predicted cellular components, of annotated transcripts, in order to characterize them (Conesa *et al.*, 2005).

In addition to the *in silico* characterization of transcripts, the second main goal of RNA-seq experiments is to compare the expression level of genes in two or more different conditions. Quantification of gene expression can be achieved since the total number of reads per transcript is directly proportional to the level of a transcript multiplied by transcript length (Haas *et al.*, 2013). However, it is worth noting that transcripts with same expression levels may have different chance to be sequenced depending on their size. In order to reduce this issue, expression levels are frequently normalized by counting the number of reads or fragments per kilobase per million reads (RPKM and FPKM, respectively) (Mortazavi *et al.*, 2008). Normalizing data into RPKM also provide an indication of relative expression levels between transcripts in a single library or between two libraries showing different sequencing depth (Lowe *et al.*, 2017). Quantitative differences in transcriptional levels can be also normalized and calculated on raw read count through the use of dedicated tools, including the two R packages DESeq2 (Love *et al.*, 2014) and

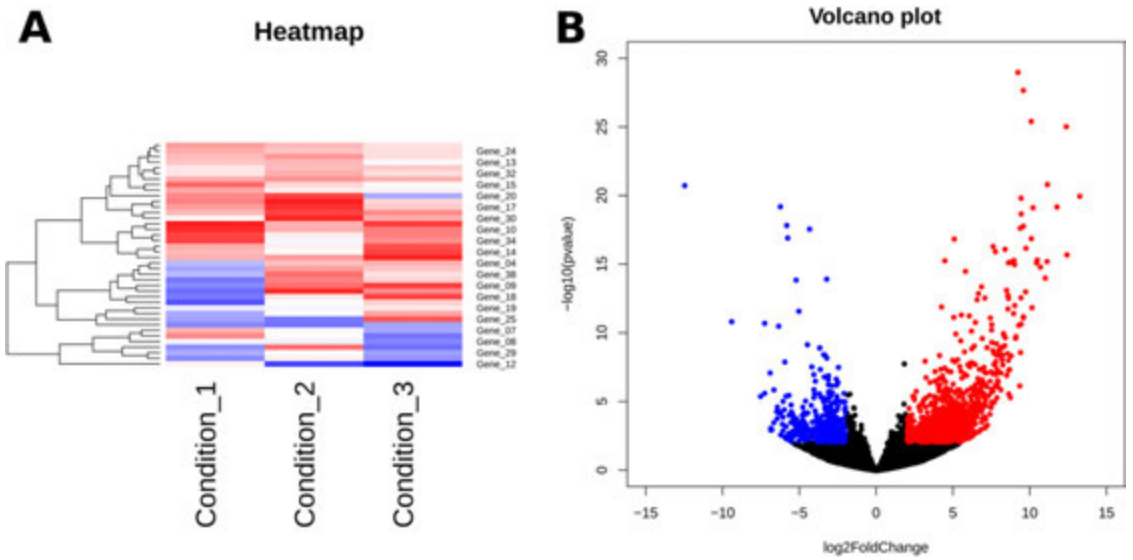

Fig. 3 Example of visualization output of RNA-seq data. In (A), example of a heatmap. Gene expression pattern of each sample/condition is represented in columns, while rows represent expression of each specific gene. Transcript abundance is indicated by different color: high-expression (red), average expression (white) and low or no expression (blue), e.g., expression of Gene_20 is high on Condition_2, and very low in Condition_3. Genes with similar expression profiles can be hierarchical clustered. In (B), a volcano plot showing DEGs. Significant up-regulated genes were represented by red dots (log2fold change > 2, p-value < 0.05), while down regulated genes were represented by blue dots (log2fold change < -2, p-value < 0.05). Black dots represent genes slightly regulated and/or not significantly expressed. Both p-value and fold changes were log2-transformed, as usually carried out for this type of data. Data for this figure were random generated.

edgeR (Robinson *et al.*, 2010), as well as the software Cuffdiff (Trapnell *et al.*, 2010), which test for differential expression using parametric approaches based on a negative binomial distribution model. The suite of tools Cufflinks embedded the algorithms for assembling transcripts, estimating their abundances, and assessing for differential expression (Trapnell *et al.*, 2012). The package *limma*, intended to be used for microarray data, has recently upgraded in order to perform differential gene expression analysis using RNA-seq data (Ritchie *et al.*, 2015). Recently, other softwares, e.g., Salmon, were developed to quantitatively and statistically assess DEGs by RNA-seq data (Patro *et al.*, 2017).

The outcomes of comparative analysis of differences in gene expression are often visualized as heatmaps, in which a color gradient for each gene represents its specific expression level in a determined condition/time/treatment (**Fig. 3**). Hierarchical clustering of genes showing similar patterns of expression may also be performed, allowing at fast tracking transcriptional changes at cluster level and at identifying gene co-expression patterns across different samples/conditions (Ben-Dor *et al.*, 1999; Lowe *et al.*, 2017). Volcano plots representing the whole set of DEGs are also commonly utilized to show a broad picture of the transcritpomic data (**Fig. 3**).

The raw sequence data from RNA-seq analysis are mostly submitted to dedicated sequence databases such as the NCBI Sequence Read Archive (SRA) database and the Gene Expression Omnibus and ArrayExpress, in order to obtain a database accession number useful for tracking data in publications (Shumway *et al.*, 2010). The number of accessions of RNA-seq studies in fungi in SRA database is increasing day by day (**Fig. 4**).

It should be mentioned that results from a large scale transcriptomic approach may be intrinsically prone to errors and attention should be focused during characterization of absolute gene expression levels. Several researchers agree that data from RNA-seq analysis have to be eventually validated using a target approach, such as the quantitative RT-PCR, by considering specific reference genes for normalization of expression, by following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines, and by possibly using additional biological replicates (Bustin *et al.*, 2009; Fang and Cui, 2011).

NGS Transcriptomics to Identify Changes During Development and Growth Under Different Environmental Conditions

Experiments performed in fungi by using RNA-seq techniques allowed primarily to expand the knowledge on their basic biology, especially on shifts between the different phases of their life cycle. For instance, the cellular and molecular remodeling of mycelia to form complex reproductive structures such as fruit bodies have been studied in the light of changes in transcriptional landscapes. Several Basidiomycetes producing macroscopic fruit bodies were used as models to elucidating the mechanisms of the development of the reproductive structures. Through a comparative analysis of transcriptional patterns among six Agaricomycetes species, it has been demonstrated that, in fungi, fruit bodies development involves a major reprogramming of gene expression (Krizsán *et al.*, 2019). In *Schizophyllum commune*, for example, approximately 60% of total genes were expressed during specific developmental stage, including fruit body formation (Ohm *et al.*, 2010), while in *Auricularia polytricha*, about 9% of the total transcript catalogue were significantly differentially expressed during the shift from free living mycelium to fruit body

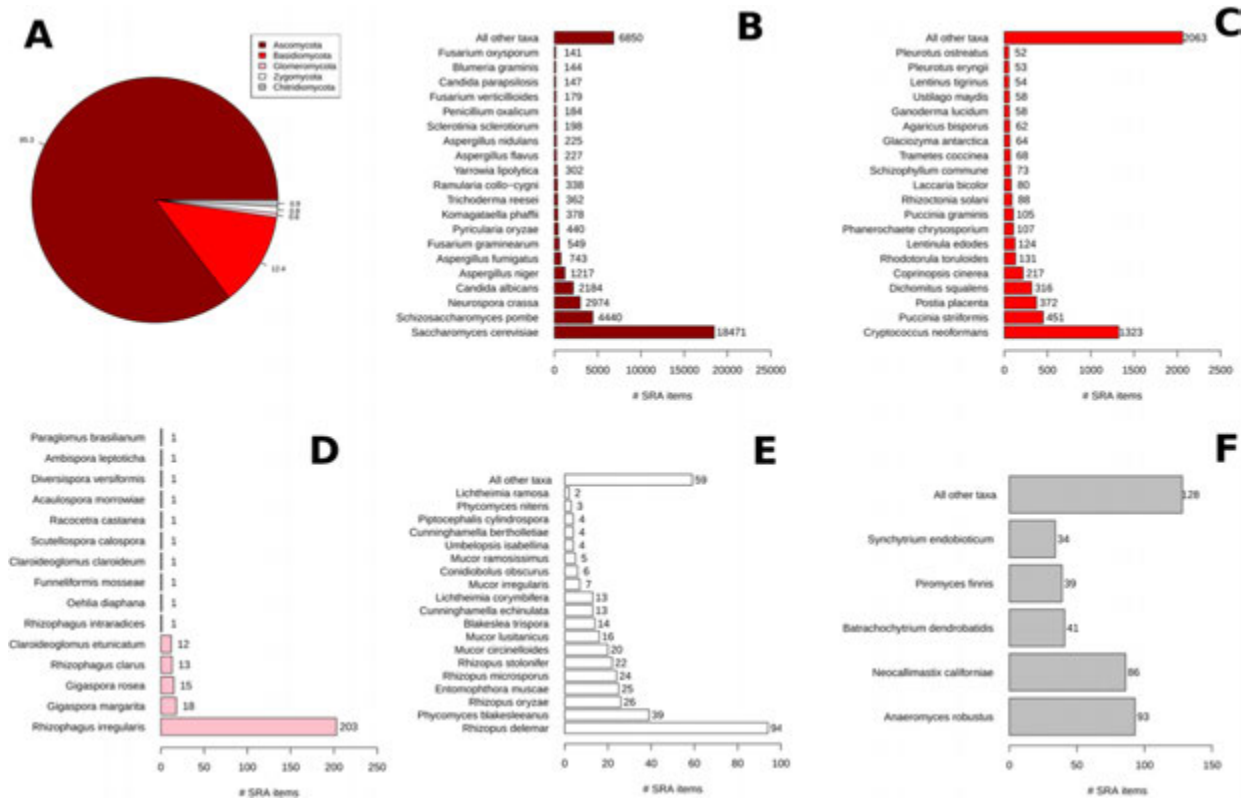

Fig. 4 Outcomes of a search on NCBI SRA database using the words "RNA-seq" and "organism: fungi" (search performed on March 2020). In (A), distribution of the main fungal phyla in the whole dataset of SRA accessions. Percentage (%) for each phylum is reported. In (B), (C), (D), (E) and (F) list of Ascomycota, Basidiomycota, Glomeromycota, Zygomycota, and Chitridiomycota species, respectively, with high number of accessions in NCBI SRA database.

(Zhou *et al.*, 2014). Common families of DEGs detected among Basidiomycetes during fruit body production are represented by genes coding for Carbohydrate Active Enzymes (CAZymes) affecting main components of the fungal cell wall, i.e., chitin and glucans, glycoside hydrolases (GH), hydrophobins, expansin-like proteins, and cerato-platanins (Krizsán *et al.*, 2019). The displacement of genes encoding cell wall related proteins seem to be required during the transition from simple to complex multicellular structures like macroscopic fruit bodies, which implied a dramatic cell wall remodeling. RNA-seq data also provide evidence of the crucial role in sporulation of several conserved key multicellularity-related genes including transcription factors, which are developmentally regulated in fruiting bodies (Pelkmans *et al.*, 2017; Krizsán *et al.*, 2019), and species-specific defense-related genes, suggesting that also chemical defense might be a function involved in a fruiting body production (Krizsán *et al.*, 2019). In *Morchella importuna*, the gene expression profile detected by RNA-seq analysis showed that carbohydrate catabolism and energy metabolism related pathways were finely regulated during fruit body formation, as well as genes encoding for heat shock proteins, thus suggesting a role of environmental temperature in sporulation (Hao *et al.*, 2019). From RNA-seq data, it was also observed that antisense transcripts may play a role in the formation of reproductive structures of *Coprinopsis cinerea* (Muraguchi *et al.*, 2015). A comparative large-scale analysis among three related smut species, i.e., *U. maydis* (common smut of corn), *Ustilago hordei* (covered smut of barley), and *Sporisorium reilianum* (head smut of corn), suggested that antisense RNAs could have a genome-wide influence on gene expression in smut fungi (Donaldson *et al.*, 2017). Antisense expression of genes appeared to have a role in growth and development of *Schizophyllum commune* (Ohm *et al.*, 2010), in *Aspergillus nidulans* (Sibthorp *et al.*, 2013), in the rice blast pathogen *Magnaporthe grisea* (Gowda *et al.*, 2006), and in *Neurospora crassa* (Arthanari *et al.*, 2014).

Sexual development transcriptional profiles were studied by high-throughput transcriptomic approach also in several Ascomycetes. Using *Botrytits cinerea* as a model system, whole transcritpome of apothecia was obtained and results allowed at inferring a possible priming role of gene involved in plant infection during fruit body generation (Rodenburg *et al.*, 2018). Comparative analysis of transcriptomic levels of orthologous gene repertoire between closely related species allowed at elucidating other ecological and evolutionary aspects of fungal development. For example, by coupling morphological characterization with RNA-seq analyses, it was possible to provide useful information on the ecological differences between two plant pathogenic fungi, *Fusarium verticillioides* and *F. graminearum* (Sikhakolli *et al.*, 2012). The comparison of the regulation of orthologous genes in a time-point experiment highlighted the different role of fruiting bodies in the ecology and epidemiology of the two pathogenic species (Sikhakolli *et al.*, 2012). A comparative RNA-seq analysis of several different truffle species (*Tuber* spp.) demonstrated that volatile organic compounds of their edible fruit bodies rely on the differential expression of a plethora of existing genes not strictly specific to *Tuber* species (Murat *et al*,

2018). More recently, a transcriptomic profiling of the edible Ascomycete *Tuber magnatum* Pico (white truffle) has been obtained from fruit bodies collected under different environmental conditions, and a link between RNA-seq data and metabolomic profile has been performed (Vita *et al.*, 2020). Developmental transitions during the life cycle of *Tuber melanosporum*, i.e., free living mycelium, fruit body production and ectomycorrhizae, was also studied by RNA-seq (Tisserant *et al.*, 2011).

During adaptations to diverse environments, fungi seems to finely regulate different fractions of their transcriptomic pattern (Meijueiro *et al.*, 2014). For example, the genes and pathways associated to protein production in different culture media were identified in *A. nidulans* and *A. niger* by a RNA-seq analysis (Pullan *et al.*, 2014; Brown *et al.*, 2016). A RNA-seq study on the entomopathogenic fungus *Beauveria bassiana* suggested that the fungus adapted to different environmental niches by activating well-defined gene sets: for instance, when locust hind wings were used as substrate for growth, proteases were found to be induced, whereas the use of corn root exudates enhanced the expression of carbohydrate hydrolases (Xiao *et al.*, 2012). Colonization of wood by the brown rot agent *Postia placenta*, explored through a time course RNA-seq and coupled with enzymatic assays, allowed at detecting a unique fungal "pretreatment" strategy acting during the first phases of wood degradation, providing insights on a natural efficient conversion of woody plant materials into cellulosic compounds (Zhang *et al.*, 2016). In *Grosmannia clavigera*, RNA-seq data highlighted that the fungus can use products of detoxification of terpenoids as a carbon source, resulting in a transcriptional reprogramming of this species when subjected to environmental stresses (DiGuistini *et al.*, 2011).

NGS Transcriptomics to Decipher the Molecular Mechanism of Interactions With Other Organisms

Transcriptomic approaches based on NGS and aimed at dissecting the molecular mechanisms of interaction between fungi and other organisms has been successfully used in many different research fields. Given that the output of RNA-seq simultaneously comprises reads deriving from transcripts of both partners, and the comparison of these sequences against dedicated databases allow at distinguishing the origins of each transcript inside the RNA pool (Westermann *et al.*, 2012), this method was applied to study with high accuracy the pathways involved in the interaction dual experimental systems.

In the last years, large scale transcriptomics contributed to address important questions on human, animal and plant pathology, as well as on symbiosis between fungi and plants. A remarkable example is represented by a study on the opportunistic pathogenic yeast *Cryptococcus neoformans*, which causes human meningitis and it is responsible for thousands of deaths among immunodepressed people every years (Janbon *et al.*, 2014). By using RNA-seq, it was analyzed and compared the gene expression of two strains of *C. neoformans* collected from the cerebrospinal fluid of infected patients, and pathways that were crucial for the survival of *C. neoformans* in the central nervous system were identified. This allow at elucidating the genetic basis of the fungal disease (Chen *et al.*, 2014). Other important transcriptomic analysis performed on this basidiomycetous pathogenic yeast provided insights on *C. neoformans* response to stress, mating efficiency, and virulence (Janbon *et al.*, 2014; Yu *et al.*, 2020).

Plant disease were also studied from a fungal transcriptomic point of view. Transcriptomic profile of predicted secreted proteins strongly improved the ability to identify putative genes coding for small molecule selectively binding to plant proteins and regulating their biological activity, called effectors. It has been well documented that effectors play a key role during infection processes in compatible/incompatible interactions and in susceptibility/resistance to diseases, as well as in symbiosis (Alfano, 2009). Dual RNA-seq analysis on Norway spruce trees naturally infected by the forest pathogens *Heterobasidion* spp. revealed a specific repertoire of effector-like genes up-regulated during host colonization, in addition to genes encoding carbohydrate- and lignin-degrading enzymes (Kovalchuk *et al.*, 2019). The transcriptomic profile by NGS of the rice blast agent *Magnaporthe oryzae* during host infection allowed at identifying 240 transcripts encoding putative secreted proteins probably involved as effectors (Kawahara *et al.*, 2012). Up-regulation of genes encoding glycosyl hydrolases, cutinases and LysM domain-containing proteins were observed in the blast fungus, while expression of pathogenesis-related and phytoalexin biosynthetic genes was observed in rice (Kawahara *et al.*, 2012). Candidate effectors were discovered through dual RNA-seq in several important plant pathogens, e.g., *Cronartium ribicola* (Liu *et al.*, 2015), *Erysiphe pisi* (Gupta *et al.*, 2020), *Parastagonospora nodorum* (Jones *et al.*, 2019), *Puccinia striiformis* f. sp. *tritici* (Dobon *et al.*, 2016), and the number of new reported putative effector-like genes in different pathosystems is still rising.

Symbiosis between plants and fungi has been deeply studied through transcriptomic analysis. By using 454 pyrosequencing of transcripts isolated from the orchid mycorrhizal fungus *Tulasnella calospora* and its plant host *Serapias vomeracea*, an inventory of plant and fungal genes expressed in mycorrhizal protocorms was generated and the symbiotic process was characterized from both host and fungal sides (Balestrini *et al.*, 2014). The candidate effectors involved in the mutualistic symbiosis between the ectomycorrhizal fungus *Laccaria bicolor* and its hosts were also identified thanks to a large scale transcriptomic approach, and numerous small secreted proteins (SSPs) putatively involved in the establishment of the symbiosis were characterized (Martin *et al.*, 2008; Plett *et al.*, 2015). RNA-Seq data from fully formed ectomycorrhiza between *L. bicolor* and poplar roots were also used to predict the ectomycorrhizal metabolome (Larsen *et al.*, 2011). Large scale transcriptomics have been performed on other important ectomycorrhizal fungi, including *T. melanosporum* and *Paxillus involutus* (Kohler and Tisserant, 2014). Arbuscular mycorrhizal (AM) fungi has been also characterized through deep transcriptome sequencing. A RNA-Seq approach on the AM fungus *Rhizophagus irregularis* was performed, allowing at exploring the gene expression profile upon symbiosis with the plant *Medicago truncatula* (Tisserant *et al.*, 2013) . About 4,7% of its genes were induced in colonized roots, and most of them were characterized as involved in signal transduction, energy production and conversion, secondary metabolism, transport and metabolism (Tisserant *et al.*, 2013). As for ectomycorrhizal fungi (Martin *et al.*, 2008), several genes coding for SSPs were found to be highly expressed during AM symbiosis interaction (Tisserant *et al.*, 2013).

Perspectives of NGS Transcriptomics in Fungi

Thanks of the exponential availability of specific protocols and reagents, in the last years NGS methods have been coupled to other novel molecular approaches in order to improve the resolution of analyses. A recent tool which has been proven to be useful when coupled to RNA-seq is laser microdissection (LM) technology. Laser microdissection is a powerful technology that allows the rapid isolation of selected cell populations from a section of heterogeneous tissues in a way conducive to the extraction of several cellular compounds, including nucleic acids, proteins and metabolites (Balestrini *et al.*, 2009). LM in fact combines the use of the microscope and the application of a manual guided (PC assisted) laser to separate different cytological components from specimen sections on a microscope slide. This technology was used in studies on mycorrhizal fungal symbioses in order to isolate cortical cells from roots of several plant species colonized by arbuscular mycorrhizal fungi in combination with gene expression target approaches (Balestrini and Fiorilli, 2020 for a review) and large scale transcriptomics (Gaude *et al.*, 2012) or from truffle/hazelnut ectomycorrhizae (Hacquard *et al.*, 2013), and in studies on plant pathogenic fungi in order to dissect the tissue-specific responses of plants to the infection (Hacquard *et al.*, 2010; Chandran *et al.*, 2010). Recently, one interesting study performed on the Ascomycete *Sordaria macrospora* has revealed the high potential of this tool for elucidating the various transcriptomic patterns of different fungal tissues (Teichert *et al.*, 2012). By selectively isolating RNA from protoperithecia and free living mycelia of *S. macrospora* through a LM approach, and by analyzing it with RNA-seq, significant difference in gene expression between the two components were detected (Teichert *et al.*, 2012).

Despite LM is useful for tissue-specific analysis, and tailored RNA isolation protocols are available for extraction of nucleic acid from chemically-treated samples like paraffine-embedded specimens (Takahashi *et al.*, 2010), a high number of cells are needed as starting biological material. In some cases, e.g., to investigate on differential expression of a spore population with different or similar genetic background, the RNA sequencing of a single cell [Single-cell RNA-seq (scRNA-seq; Saliba *et al.*, 2014)] is required. Several methods to isolate single cell from tissues and cell populations, i.e., multiparametric flow cytometry and sorting based on a fluorescence gating strategy, micromanipulation with a glass pipette, optical tweezers laser-based, were successfully used in several studies (Saliba *et al.*, 2014). In the last years, thanks to SMRT provided by third generation sequencing technology, scRNA-seq has started to be an established method for uncovering the individual transcriptomic complexity within populations of yeasts (Gasch *et al.*, 2017; Nadal-Ribelles *et al.*, 2019; Saint *et al.*, 2019). Results were promising and it is likely that this approach will be applied in future for other microorganisms, including filamentous fungi.

An emerging and promising field taking advantage by the rapid development of NGS tools and aimed at expanding the knowledge on the identification of multipartite metabolic interactions occurring between two or more organisms is represented by metatranscriptomics. Metabarcoding studies on DNA from environmental samples demonstrated without doubt to be very useful to study fungal community in the field. However, metabarcoding surveys may be biased by PCR amplification and not all species present in the samples have the same chance to be detected (Tedersoo *et al.*, 2015). Moreover, both dead and living organisms are detected by DNA sequencing, potentially masking the real structure of the living populations in some particular samples, e.g., in stool or soil samples, where contaminant DNA cannot be distinguished from DNA of the living microbial community (Marcelino *et al.*, 2019). The sequencing by RNA-seq of transcripts, representing the whole activity of genes of all organisms living in the samples, may circumvent this issue, helping researchers in ecological studies of complex fungal community (Kuske *et al.*, 2015; Marcelino *et al.*, 2019). In addition, as for dual RNA-seq, metatranscriptomics can allow to decipher the interactions among fungi and other organisms. Metatranscriptomics of some mycorrhizal communities have been recently performed, allowing a deeper understanding of the functional roles of these fungi in nature (Gonzalez *et al.*, 2018; Liao *et al.*, 2014). However, as for transcriptomics of a single organism, the interpretation of RNA-seq results from metatranscriptomics is still challenging, because of differences in transcript turnover rates and of limitations of using homology-based gene assignments during function prediction (Kuske *et al.*, 2015). The increased availability of annotated transcriptomes of fungal species in validated databases will aid to fill the missing information needed by future metatranscriptomics studies (Kuske *et al.*, 2015).

Conclusions

In the last years, thanks to NGS strategies, fungal transcriptomics has improved the understanding of how genomes are expressed during growth, development and under environmental stresses, what genes were involved in pathogenic and symbiotic interactions, and what is the role of different RNA species, such as non-coding RNA and antisense transcripts, in fungal life cycles. The growing availability of large-scale transcriptomics data allowed comparative analyses aimed at addressing important questions on functional, ecological and evolutionary aspects of fungi. Both small scale laboratories and big consortia have access to these NGS data, and thanks to the reduction of costs of NGS technology, they will be able to perform new transcriptomics studies in order to improve the databases and to strength the network between transcriptomics and other *omics* approaches. The new challenge for high throughput NGS transcriptomics, in fact, will be the growing request of computational resources for analyzing and storing big data coming from RNA-seq. Future improvement of sequencing technologies and bioinformatic tools will be pivotal to keep this positive trend, along with the proportional enhancement of bioinformatic facilities and sequence databases.

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The Cell Wall of Medically Relevant Yeasts and Molds

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Introduction

Thus far, an estimated 1.5 million fungal species have been classified, and 300 out of there affect human beings (Fisher *et al.*, 2020). Fungal infections are acquiring relevance in the last years because most of them affect individuals already with serious underlying illnesses and are challenging to treat. The main risk factors for developing a fungal infection include anti-cancer, immunosuppressive and antibiotic therapies, solid organ and hematopoietic stem cell transplantations, HIV infection, diabetes mellitus, and the use of intravenous lines (Lanternier *et al.*, 2013). Moreover, the socio-economic and geo-ecological characteristics of a population can increase fungal disease risk factors, incidence, and prevalence (Bongomin *et al.*, 2017).

The fungal cell wall (CW) is a key player in the pathogenic process, being important in the fungal adhesion to the host, and contains pathogen-associated molecular patterns that can interact with the pattern recognition receptors of the host immune cells. The fungal CW is located above the plasma membrane, acts as a permeability barrier, contributes to cell shape and protection from osmotic and mechanic stresses, and is essential for cell integrity and viability (Díaz-Jiménez *et al.*, 2012). The fungal cell growth can be radial or polar, and this defines the final cell morphology of yeast and filamentous fungi, respectively. Molds grow by polarized and apical extension, forming filamentous and vegetative cells known as hyphae, whose continued growth forms the hyphal network name as mycelium. Thus, the hyphae's biological traits, including the CW structure and organization are closer to that found in molds.

The *Candida albicans* Cell Wall

Candida albicans is a dimorphic and opportunistic organism, and one of the leading etiological agents of nosocomial fungal infections (Brown *et al.*, 2012). The *C. albicans* CW is likely the most thoroughly studied fungal structure from medically relevant fungal species and will be used here as an example of CW composition, organization, and synthesis (**Fig. 1**). Its wall is a layered structure, with a homogeneous inner layer of about 100 nm and the outermost layer of about 180 nm and composed of proteins (Klis *et al.*, 2001). The main components of the CW are carbohydrates, contributing to 80%–90% of the CW dry weight (Díaz-Jiménez *et al.*, 2012; Mora-Montes *et al.*, 2009). These saccharides are chitin, β-1,3- and β-1,6-glucans, and mannose oligosaccharides (mannan) covalently associated with proteins (Mora-Montes *et al.*, 2009). Proteins and lipids contribute to about 6%–25% and 1%–7% of the CW dry weight (Chaffin *et al.*, 1998) (see **Fig. 1**).

Chitin and β-glucans are the main components of the wall inner layer, are covered by the outer layer components, except in the budding scars and the hypha primary septum (Perez-Garcia *et al.*, 2011), and are the wall skeleton that provides physical strength (Chaffin *et al.*, 1998). Chitin is a linear polymer and is arranged in an antiparallel fashion, associated with each other by hydrogen

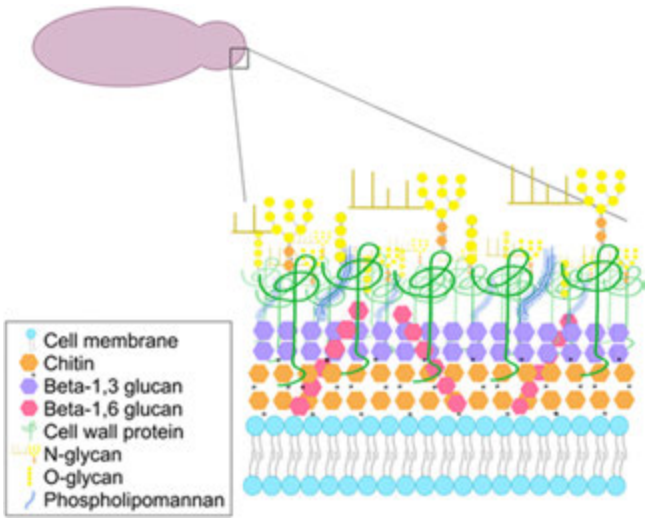

Fig. 1 Representation of *C. albicans* cell wall. The inner layer of the cell wall (close to the cell membrane) is composed of antiparallel chitin chains, followed by β-1,3- and β-1,6-glucans. The outermost cell wall layer is composed of a thick mannoproteins layer that contains *N*-linked and *O*-linked mannans, and phospholipomannans.

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bonds (Shepherd, 1987); while β-1,3-glucans are aligned and kept together by hydrogen bonds (Klis *et al.*, 2001). The β-1,6-glucans can be of variable lengths and distribution and act as molecular linkers between wall proteins and β-1,3-glucans and chitin (Klis *et al.*, 2001; Garcia-Rubio *et al.*, 2020). The outermost layer is composed of mannoproteins, which account for about 20%–30% of the total CW weight (Mora-Montes *et al.*, 2009), and may contain glycosylphosphatidylinositol (GPI) anchors that cross-link them to β-1,6-glucans (Garcia-Rubio *et al.*, 2020). They also contain *O*-linked and *N*-linked oligosaccharides attached to serine/threonine or asparagine residues, respectively (**Fig. 1**). The phospholipomannan is currently the main glycolipid characterized in this CW and contains β-1,2-oligomannosides linked to phytoceramide associated with phytosphingosine and hydroxy fatty acids (Trinel *et al.*, 2002).

Chitin Synthesis

Chitin is the second most abundant organic compound on earth, after cellulose, and is synthesized by a wide variety of organisms from different taxonomic groups, including pathogenic fungi (Merzendorfer, 2011). This is a homopolymer of N-acetylglucosamine (GlcNAc) that is linked by β-1,4- glycosidic bonds and folds on itself to form antiparallel chains of about twenty units. This arrangement allows compaction in strong microfibrils, more resistant than any other molecule in nature (Lenardon *et al.*, 2010).

A chitin proportion found in pathogenic fungi is deacetylated to chitosan by one or more chitin deacetylases (Klis *et al.*, 2006). In *C. albicans*, 5% of the chitin is deacetylated to chitosan, while in *Cryptococcus neoformans* more than 60% chitin is deacetylated (Baker *et al.*, 2007). Chitin synthesis is highly conserved in fungi and the process involves a defined number of enzymatic reactions that convert different sugars into a GlcNAc polymer. The sugar main source is glucose or its storage compounds -glycogen or trehalose (François and Parrou, 2001). The synthesis route is divided into three main reactions, the first leads to GlcNAc formation (**Fig. 2**), the second one follows a variant of the Leloir route that gives rise to uridine diphosphate (UDP)-GlcNAc (**Fig. 2**), and the last reaction involves chitin polymerization using UDP-GlcNAc as the activated sugar donor (**Fig. 2**). The first two reactions occur in the cytoplasm; while the third step takes place in specialized microdomains of the plasma membrane (Merzendorfer, 2011).

The presence of glutamine-fructose-6-phosphate amidotransferase, UDP-GlcNAc, and chitin synthase (CHS) are determinants for chitin synthesis. The CHS activity is specifically associated with chitin biosynthesis, and is divided into seven classes (I-VII); although the functional importance and presence of all these classes seem to be species-specific (Niño-Vega *et al.*, 2004) (**Table 1**). Class I and II enzymes produce only a small chitin fraction but are essential for the primary septum formation (Munro *et al.*, 2001). Class IV, V, and VII enzymes often produce considerable chitin amounts and share sequence homology; whereas class V and some class VII enzymes contain myosin-like domains (Niño-Vega *et al.*, 2004). Class III, V, VI, and VII enzymes have only been identified in filamentous fungi and are absent in *C. albicans* (Lenardon *et al.*, 2010) (**Table 1**). The multiplicity of CHS enzymes in various organisms suggests that they may have redundant functions in chitin synthesis.

The CHS are membrane proteins and the catalytic domain, which faces the cytoplasm, contains conserved saccharide- and nucleotide-binding sites (Merzendorfer, 2011). For this reason, nascent chitin chains must translocate through the plasma membrane before they assemble into microfibrils and are deposited on the cell surface. The chitin translocation process is thought to be a CHS intrinsic property that involves transmembrane segments of the C-terminal region (Lenardon *et al.*, 2010; Merzendorfer, 2011).

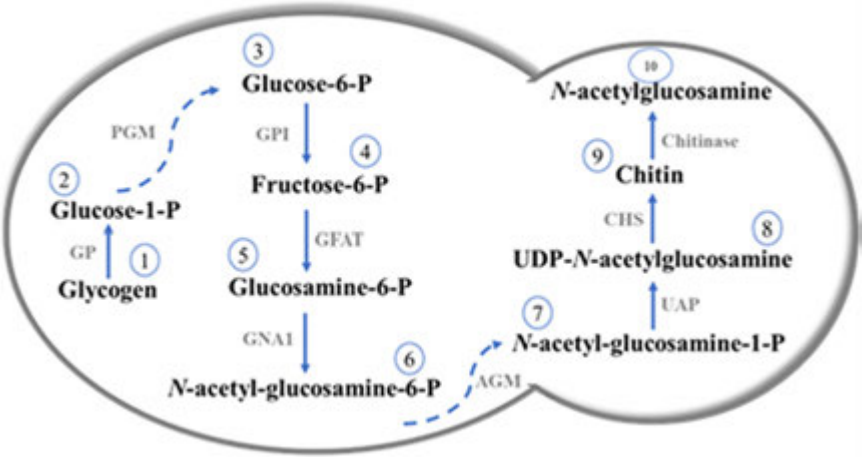

Fig. 2 Simplified chitin synthesis pathway in fungi, starting from glycogen. The acronyms in the figure refer to Glycogen phosphorylase (GP), phosphoglucomutase (PGM), glucose-6-phosphate isomerase (GPI), glutamine-fructose-6-phosphate amidotransferase (GFAT), glucosamine-6-phosphate-*N*-acetyltransferase (GNA1), phosphoacetylglucosamine mutase (AGM), UDP-*N*- GlcNAc pyrophosphorylase (UAP), chitin synthase (CHS) and chitinase.

Table 1 Classification of chitin synthases in fungi of medical interest

	CHS classes							
Organism	*I*	*II*	*III*	*IV*	*V*	*VI*	*VII*	*Function*
C. albicans	Chs2 Chs8	Chs1	–	Chs3	–	–	–	*Chs1*: primary septum synthesis *Chs2 and Chs8*: protection of the nascent cell wall during polarized growth *Chs3*: synthesizes the majority of chitin found in the cell wall as well as the chitin ring at division sites (Lenardon *et al.*, 2010)
A. fumigatus	ChsA	ChsB	ChsC ChsG	ChsF	CsmA	ChsD	CsmB	*ChsA and ChsC*: cell wall chitin compensation *ChsB*: function at polarized growth sites and forming septa during hyphal growth and conidia development *CsmA and CsmB*: these enzymes appear to localize themselves to sites of polarized cell wall expansion in an actin-dependent manner *ChsD, ChsG, ChsF*: unknown functions (Lenardon *et al.*, 2010)

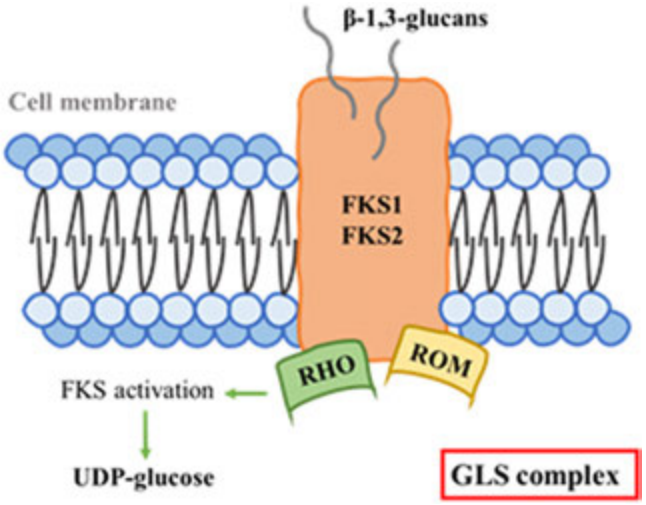

Fig. 3 Schematic representation of the GLS complex, which is involved in the β-1,3-glucan synthesis. The FKS subunit catalyzes the elongation of growing β-glucan chains by the addition of UDP-glucose monomers. RHO is a GTP-dephosphorylase protein that regulates Fks1 and Fks2 by GDP. ROM is a cell wall-associated GDP-GTP exchange protein responsible for the GTP regeneration.

β-Glucans Synthesis

Most of the fungal β-glucans are made up of β-1,3-linked glucose units with varying amounts of β-1,6-linked branches. The β-1,3-glucans are the most important and abundant polysaccharides in the fungal CW, reaching around 65%–90% of total glucan content (Bowman and Free, 2006).

The initial step in the β-1,3-glucan synthesis is the cytoplasmic generation of the precursor UDP-glucose (UDP-Glc), used as a sugar donor by glucan synthases. In fungi, the GLS membrane protein complex (UDP-Glc:1,3-β-D-glucan 3-β-D-glucosyltransferase), is in charge of catalyzing the reaction of β-1,3-glucan synthesis, using UDP-Glc as a substrate (Latgé, 2007). The elongation continues with the addition of UDP-Glc monomers at the non-reducing end of the growing glucan chain, and each new polymer is transported from the plasma membrane to the CW, through a channel of transmembrane domains. The GLS complex is composed of two subunits, a catalytic FKS unit and a regulatory RHO unit with guanosine triphosphatase activity (Beauvais *et al.*, 2001) (**Fig. 3**). The gene encoding for Fks1 of the FKS subunit is expressed in vegetative growth under optimal conditions; while *FKS2* is induced by environmental stress or during sporulation. Various evidence indicates that Fks1 and Fks2 are the β-1,3-glucan synthase catalytic subunits (Dijkgraaf *et al.*, 2002; Nogami and Ohya, 2009).

It is known that RHO is a regulatory G protein, which fulfills the function of activating FKS by GTP dephosphorylation. A Rho1 post-translational modification is necessary for β-1,3-glucan synthesis, the C-terminal end of RHO-type GTPase is modified with a geranylgeraline group by the type I geranylgeranyltransferase enzyme, this modification is required for binding and activation of Fks1 (Ohya *et al.*, 1993; Nogami and Ohya, 2009). The ROM protein is also involved in this process, since allows GDP-GTP exchange in the wall and modulates RHO activity (Teparić and Mrsa, 2013). The cytosolic domains of FKS are responsible for

Table 2 Genes involved in the synthesis of β-1,3- and β-1,6-glucans in medically relevant fungi

S. cerevisiae gene[a]	*C. albicans*	*A. fumigatus*	*Function*
KRE5	*KRE5*	AFUA_2602360	UDP-glucose: glycoprotein glucosyltransferase, 1,6-β-D-glucan biosynthesis
KRE6	*KRE6*	BA78_1578	Essential β-1,6-glucan synthase subunit
SKN1	*SKN1*	BA78_1578	Protein with a role in β-1,6-glucan synthesis; probable *N*-glycosylated type II membrane protein
KRE9	*KRE9*	CDV57_05718	Protein of β-1,6-glucan biosynthesis; required for hyphal growth.
KNH1	*KNH1*	CDV57_05718	Protein involved in cell wall β-1,6-glucan synthesis, similar to Kre9
KRE1	*KRE1*	Not found	Cell wall glycoprotein; β-glucan synthesis
RHO1	*RHO1*	RHO1	Small GTPase of Rho family; regulates β-1,3-glucan synthesis activity
FKS1	*MEQ_00226*	FKS1	Essential β-1,3-glucan synthase subunit
FKS2	*GSL2*	FKSP	Protein similar to β-1,3-glucan synthase
ROM2	*ROM2*	ROM2	Putative GDP/GTP exchange factor

[a]*Notes*: The synthesis of β-glucans has been extensively studied in the fungus *Saccharomyces cerevisiae* and the genes involved in this synthetic pathway are almost completely characterized. Therefore, using a blast analysis, taking as reference the *S. cerevisiae* genes, a search was carried out to determine the presence of these genes in the selected organisms. Gene nomenclature corresponds to accession codes of the GeneBank database (https://www.ncbi.nlm.nih.gov/genbank/).

catalysis, and therefore β-glucans synthesis takes place in the cytosol. Subsequently, the biomolecules go to the periplasmic space, where they are incorporated into the CW (Reverberi *et al.*, 2004; Bowman and Free, 2006). Other proteins, such as Pma1 have been found near the GLS complex, and this particular protein maintains an acidic environment in the CW closer to the membrane, which is important for glucan synthesis (Schimoler-O'Rourke *et al.*, 2003) (see **Table 2**).

The β-1,6-glucan synthesis is likely to occur on the cell surface, although the mechanism behind is not well dissected yet. The first step in its synthesis may involve an endoplasmic reticulum (ER) hypothetical protein acceptor, followed by the synthesis of polysaccharide chains and branching in later stages of the secretory pathway on the cell surface. However, the activity of the identified gene products remains unknown, so it is not clear how and to what extent they are involved in the synthesis of this polymer (Nogami and Ohya, 2009). Thus far, a cell surface β-1,6-glucan synthase has not been found, but several proteins of the Golgi apparatus and ER seem to be involved in this synthetic pathway in an unknown way (Shahinian and Bussey, 2000). The *KRE5*, *CWH41*/*GLS1*, *ROT2*/*GLS2*, and *CNE1* encode ER proteins that participate in the β-1,6-glucan synthesis (Shahinian *et al.*, 1998). In the case of Kre5, this has a similarity to UDP-glucose: glycoprotein glucosyltransferase and has been suggested that may play a role that indirectly contributes to glucan synthesis (Shahinian and Bussey, 2000) (see **Table 2**).

Glycoproteins Synthesis

The carbohydrate part of glycoproteins is known as glycans and are linked to the polypeptide backbone in a process known as protein glycosylation pathway. This posttranslational modification plays an important role in protein structure and function, affecting solubility, folding, stability, and intracellular trafficking (Helenius and Aebi, 2001). Some glycoproteins are covalently attached to the CW matrix and are known as "integral CW proteins", which often are produced as GPI-anchored proteins; while the "non-integral" proteins are weakly associated with the wall (Castillo *et al.*, 2008).

According to the glycan-binding site on the protein, there are two types of glycans, the *N*-linked and *O*-linked glycans. The *O*-linked glycans are short and simple oligosaccharides attached to the -OH group of serine or threonine side chain by an acyl bond, whereas the *N*-linked glycans are high-weight and highly branched oligosaccharides attached to asparagine residues within the sequon Asn-X-Ser/Thr (Mora-Montes *et al.*, 2009). The biosynthesis of both *N*-linked and *O*-linked glycans is a multi-step process that takes place in the ER and Golgi complex and involves glycosyltransferases, glycosidases, and other carbohydrate modifying enzymes (Mora-Montes *et al.*, 2009; Martinez-Duncker *et al.*, 2014).

The *N*-linked glycan synthesis begins with the assembly of a dolichol-linked oligosaccharide precursor, $Glc_3Man_9GlcNAc_2$, in the ER and its subsequent transfer in a co-translational mechanism by an oligosaccharyltransferase complex to the amide side chain of the asparagine of a nascent polypeptide (Martinez-Duncker *et al.*, 2014). Then, this precursor is processed to $Glc_1Man_9GlcNAc_2$, which is key for protein folding in the glycoprotein quality control mediated by the calnexin/calreticulin chaperones (Helenius and Aebi, 2001). Once the polypeptide is properly folded, the oligosaccharide is further processed to $Man_8GlcNAc_2$, which is directed to the Golgi complex for additional processing by mannosyltransferases, generating high mannose content *N*-linked glycans (Martinez-Duncker *et al.*, 2014). In *C. albicans*, the synthesis of an outer chain, and attachment to $Man_8GlcNAc_2$ takes place in the Golgi complex. This process begins with the α-1,6-mannosyltransferase Och1, which is responsible for adding one α-1,6-mannose unit to $Man_8GlcNAc_2$ (Martinez-Duncker *et al.*, 2014). Then, a chain containing 50 or more α-1,6-mannose residues is built on the initial mannose added by Och1, generating the outer chain backbone (Bates *et al.*, 2006). The enzymes involved in the backbone elongation are Van1, Anp1, Mnn9, Mnn10, and Mnn11 (Martinez-Duncker *et al.*, 2014). Then, lateral oligosaccharides containing α-1,2-mannose units branched the backbone and may be decorated with α-1,3-mannose or β-1,2-mannose units (Mora-Montes *et al.*, 2009). The enzymes required for the synthesis of this outer chain are listed in **Table 3**. In *C. neoformans*, the *N*-linked glycans contain xylomannan, having a long nucleus of α-1,6-mannoses with side chains consisting of α-1,2-mannoses ending in a xylose residue (Park *et al.*, 2013). In *Aspergillus fumigatus*, the outer chain has a different structure,

Table 3 Enzymes involved in the biosynthesis of fungal *N*-linked and *O*-linked glycans

Enzyme	*C. albicans*	*A. fumigatus*	*Function*
Och1	✓	✓	α-1,6-Mannosyltransferase; initiates *N*-glycan outer chain branch addition
Van1	✓	✓	Member of Mnn9 family of mannosyltransferases
Anp1	✓	✓	Putative Golgi-resident mannosyltransferase; member of Mnn9p family
Mnn9	✓	✓	Mannosyltransferase involved in the *N*-linked outer-chain mannan biosynthesis
Mnn10	✓	✓	α-1,6-Mannosyltransferase involved in biosynthesis and organization of cell wall polysaccharides
Mnn11	✓	✓	Role in protein *N*-linked glycosylation, protein glycosylation, and α-1,6-mannosyltransferase complex localization
Mnn2	✓	✓	α-1,2-Mannosyltransferase, required for normal cell wall mannan content
Mnn6	✓	✓	Role in protein glycosylation and Golgi apparatus localization
Mnn1	✓	✓	Putative α-1,3-mannosyltransferase; of the mannosyltransferase complex
Pmt1-Pmt6	✓	✓	Protein mannosyltransferase, member of the PMT family which includes Pmt1p, Pmt2p, Pmt4p, Pmt5p, and Pmt6p
Mnt1/Ktr1	✓	✓	α-1,2-Mannosyl transferase; predicted type II Golgi membrane protein
Mnt2	✓	✓	α-1,2-Mannosyl transferase, partially redundant with Mnt1
Mnt3	✓	✓	Mannosyltransferase

Notes: The presence or absence of the enzymes involved in the synthesis of *N*-linked and *O*-linked glycans in *Candida albicans* and *Aspergillus fumigatus* was determined. The presence of these enzymes was investigated in their respective genome databases (http://www.candidagenome.org/, http://www.aspgd.org/).

instead of a linear nucleus of α-1,6-mannose units there is a repeated tetrameric mannan composed of α-1,2-mannose-α-1,6-mannose-α-1,2-mannose-α-1,2-mannose with β-1,5-galactofuranose side chains (Henry *et al.*, 2016; Engel *et al.*, 2012) (see **Table 3**).

The *O*-linked glycosylation pathway also begins in the ER, where the Pmt1-Pmt6, a family of protein *O*-mannosyltransferases (PMT) takes a mannose unit from dolichol-phosphate-mannose and transfer it to an acceptor protein (Martinez-Duncker *et al.*, 2014; Mora-Montes *et al.*, 2009). Then, additional mannoses are added to the oligosaccharide by Golgi resident α-1,2-mannosyltransferases, generating a short chain of α-1,2-mannose residues that may contain up to seven mannose units (see **Table 3**) (Diaz-Jimenez *et al.*, 2012). *A. fumigatus* has *O*-linked mannogalactans, and there is evidence that the mannose chain has α-1,6- rather than α-1,2- bonds, although this fungus has Mnt1, the enzyme responsible for the production of α-1,2-mannan (Leitão *et al.*, 2003). In *C. albicans* and *C. neoformans*, Pmt2 is essential for cell viability (Willger *et al.*, 2009).

The *Candida albicans* Cell Wall Proteome

There are quantitative and qualitative differences in the *C. albicans* CW protein composition in both yeast and hyphae. The different proteins can be attached to the wall by a GPI anchor (Klis *et al.*, 2001; Satala *et al.*, 2020), these account for about 88% of all covalently linked proteins, and aspartyl proteinases, chitinases, glucanases, phospholipases, adhesins, and proteins for β-1,6-glucan biosynthesis are among the best GPI-containing wall proteins (Richard and Plaine, 2007). Moreover, it has been predicted that other 115 proteins with unknown functions are attached to the wall *via* a GPI anchor (Richard and Plaine, 2007).

Another type of wall proteins is released by extraction from intact cells with reducing agents, suggesting they are linked through disulfide bridges to other wall proteins (Klis *et al.*, 2001). Among these proteins, Eng1 (endo-β-1,3-glucanase), Gca1, Bgl2 (β-1,3-glucosyltransferase), Pdi, Pir1, MP65 (glucan metabolism), Sim1 (cell wall maintenance), Tos1, Pra1, and Iff2/Hyr3 have been identified (Caminero *et al.*, 2014). The last type of wall proteins are those linked directly to β-1,3-glucan through an alkali-sensitive linkage (without an interconnecting β-1,6-glucan moiety), the proteins have internal repeats and have been designated as Pir (Klis *et al.*, 2001).

The proteins mentioned above contain a signal sequence at the N-terminus that allows transportation to the cell surface, *via* the conventional secretory pathway, but other proteins lacking that signal and non-covalently associated with the wall are also found (Satala *et al.*, 2020). In *C. albicans*, the presence of moonlight proteins in the CW has been described, and include Cdc25 (CDC25 cell division cycle), Aco1 (aconitate hydratase), Adh1 (alcohol dehydrogenase), Fba1 (fructose bisphosphate), Gap (glyceraldehyde-3-phosphate dehydrogenase), Icl1 (isocitrate lyase), Pdc11 (pyruvate decarboxylase), Act1 (actin), various Rpl (ribosomal proteins), Hsp90 and Hsp70 (heat shock protein), Tef1 (translation elongation factor eEF1 α-A chain) Atp1 (ATP synthase alpha subunit) Cit1 (citrate synthase), among others (Satala *et al.*, 2020). Some of these proteins have been described to have adhesion properties but others an unknown function in the CW (Satala *et al.*, 2020; Castillo *et al.*, 2008).

The Cell Wall Composition in Other *Candida* Species

Although the structure and composition of the *C. albicans* CW are well known, this is not the case for other *Candida* species, such as *Candida guilliermondii*, *Candida tropicalis*, *Candida krusei*, *Candida auris*, *Candida dubliniensis*, *Candida parapsilosis*, *Candida glabrata*, *Candida lusitaniae*, and *Candida orthopsilosis*, mainly because it was thought that the *C. albicans* wall model could be applied to all these species (Navarro-Arias *et al.*, 2019).

Our group has determined the basic cell wall composition of *C. tropicalis*, *C. krusei*, *C. guilliermondii*, and *C. auris* (Navarro-Arias *et al.*, 2019). The walls from the analyzed species had differences in the levels of chitin, glucan, mannan, and wall proteins, when compared to *C. albicans*: (1) *C. auris* has higher chitin levels; (2) *C. krusei* contains higher chitin levels and low mannan, glucans, and wall proteins contents; (3) *C. guilliermondii* possesses higher mannan levels; (4) and *C. tropicalis* and *C. albicans* have similar amounts of all the mentioned components. When the content of cell wall *O*-linked and *N*-linked mannans were also analyzed, it was observed that *C. guilliermondii* had increased levels of both mannans, while *C. krusei* showed lower levels when compared to *C. albicans* (Navarro-Arias *et al.*, 2019). Two parameters associated with the mannan length, named phosphomannan content and wall porosity, were also evaluated: *C. albicans* and *C. auris* had similar phosphomannan levels; *C. tropicalis* and *C. guilliermondii* had a higher phosphomannan content, and *C. krusei* had a lower phosphomannan abundance (Navarro-Arias *et al.*, 2019). The CW porosity was similar for *C. tropicalis*, *C. guilliermondii*, and *C. krusei*, but it was lower in *C. albicans* and *C. auris* (Navarro-Arias *et al.*, 2019). Additionally, the experimental evidence suggested that both β-1,3-glucan and chitin are underneath the external mannan layer in all the analyzed species (Navarro-Arias *et al.*, 2019).

Another study also reported the CW composition of *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, and *C. guilliermondii* (Walker and Munro, 2020). They found that all of the species have similar glucan levels, except for *C. tropicalis* that had reduced glucan content, and *C. glabrata*, which had a higher content when compared to *C. albicans* (Walker and Munro, 2020). Mannan content was reduced in *C. glabrata*, *C. parapsilosis*, and *C. guilliermondii*; while chitin was in higher amounts in *C. tropicalis* but in lower levels in *C. glabrata* and *C. guilliermondii* (Walker and Munro, 2020).

The CW mannan structure from different *Candida* species has also been reported. In *C. krusei*, it was found that *N*-linked and *O*-linked mannans have a similar composition than that reported for *C. albicans* (Kuraoka *et al.*, 2019). *C. tropicalis* and *C. albicans* were found to share certain mannan moieties; while *C. glabrata* mannans contain small branches with low α-mannan content and one or two α-1,2-linked mannose residues (Nguyen *et al.*, 2018). In *C. parapsilosis*, the *N*-linked mannans are shorter and less complex than in *C. albicans*; and the *O*-linked mannans play a more important role during the interaction with the host, representing about half of the total wall mannan content (Perez-Garcia *et al.*, 2016).

The *Aspergillus* Cell Wall

Aspergillus spp. encompass several environmental filamentous fungi considered as opportunistic, being *A. fumigatus* the main etiological agent of invasive aspergillosis, a disease linked with high mortality rates in immunocompromised patients (Garcia-Rubio *et al.*, 2020). They possess two different morphotypes, conidia, and hyphae, and although the same polysaccharides are found in both, the CW organization is different (Beauvais *et al.*, 2014). In general, the wall can be divided into an alkali-soluble fraction, which represents the wall outer layer, and an alkali-insoluble fraction, which is the inner layer. In the alkali-soluble fraction, linear α-1,3-glucans (92%), galactosaminogalactan (GAG) (7%), and galactomannan (3%) are found, while the insoluble fraction, which is thought to provide rigidity, is composed of galactomannan (8%), chitin (22%), chitosan (7%), β-1,3-glucans (51%) that can extend up to 1500 residues long, and a linear β-1,3/1,4-glucan (6%), component with an unknown role that has not been described in any other fungal species (Garcia-Rubio *et al.*, 2020). The wall core is the β-1,3-glucan polymer, highly branched with β-1,6-linkages, to which chitin, chitosan, galactomannan, and β-1,3/1,4-glucan are covalently linked (Beauvais *et al.*, 2014).

The β-1,3-glucan synthesis is essential for *A. fumigatus* viability, while defects in the chitin or galactomannan synthesis generate only sick cells with virulence attenuation (Bernard and Latgé, 2001; Schmalhorst *et al.*, 2008).

In *A. fumigatus*, two types of wall-associated proteins can be found: (1) not glycosylated or glycosylated proteins without galactofuranose residues, which have been found also in the culture medium; (2) and *N*-linked and *O*-linked glycosylated GPI proteins containing galactofuranose, such as Gel2 and Ecm33, which are thought to be indispensable for the wall synthesis and vital for fungal growth (Jin, 2012). Gel2 belongs to a family of β-1,3-glucanosyltransferases, and its absence leads to slower growth, abnormal conidiogenesis, altered wall composition, and reduced virulence (Jin, 2012); while Ecm33 has an unknown function but participates in maintaining the correct wall architecture, and its disruption results in conidial separation defects, chitin accumulation, rapid conidia germination, and increased virulence (Jin, 2012; Romano *et al.*, 2006). No proteins covalently bound to the *A. fumigatus* wall polysaccharides have been found, however, a proteomic analysis of wall-associated proteins described that an acid phosphatase is the major GPI-anchored protein strongly associated with β-1,3-glucan, although the presence of a covalent link was not established (Bernard *et al.*, 2002).

The conidial CW is a two-layered structure that can be observed as a dense pigmented outer layer and as a translucent inner layer (Bernard and Latgé, 2001). It is known that this morphotype is covered by a superficial rodlet layer composed of the hydrophobic proteins RodA and RodB, known as hydrophobins (Beauvais *et al.*, 2014; Bernard and Latgé, 2001), organized in an amyloid configuration making the wall waterproof (Latge *et al.*, 2017). There is a dihydroxy naphthalene (DHN) melanin layer underneath the hydrophobins, important for the CW structuration and stiffness (Latge *et al.*, 2017). These structures are required for conidia survival and dispersion into the air, and both overlap with the α-1,3-glucans (Latge *et al.*, 2017).

The rodlet layer also confers immunological inertness to the conidia by functioning as a masking mechanism to avoid the recognition of β-1,3-glucans by dectin-1 (Beauvais *et al.*, 2014), and RodA was reported as the only essential protein for rodlet structure formation (Latge *et al.*, 2017). When RodA is missing from the surface, these morphotype is easily recognized by immune cells (Aimanianda *et al.*, 2009). However, conidia without hydrophobins can still bind to the host cells *in vitro* and *in vivo*, with no changes in the virulence (Girardin *et al.*, 1999).

Melanin presence in the wall helps conidia to counteract the effect of reactive oxygen species since albino conidia are more susceptible to these radicals and show a lower survival rate within phagocytic cells (Pihet *et al.*, 2009). It was demonstrated that melanin also plays an indirect role in the fungal pathogenesis, allowing the correct assembly of the wall layers of resting conidia (Pihet *et al.*, 2009). Analysis of albino mutants showed considerable changes in the conidial wall organization, with the loss of the outermost electron-dense layer, the absence of the rodlet layer, and a decrease in the cell electronegative charge (Pihet *et al.*, 2009).

The presence of sialic acid has also been reported in the conidial wall, being recognized by the *Sambucus nigra* agglutinin, a specific lectin that binds N-acetyl-neuraminic acid (Warwas *et al.*, 2007). High-performance liquid chromatography and mass spectrometry analyses confirmed the presence of this component in the conidia wall, and digestion with neuraminidase demonstrated that it helps the fungus to bind to the host extracellular matrix (Warwas *et al.*, 2007).

When conidia germinate due to water entrance and glycerol accumulation, the isodiametric growth gets replaced by polarized growth, causing the conidial wall rupture to form a germ tube with a wall that extends from the inner layer of the conidia. In consequence, several structures change in the cell, including the loss of the rodlet and melanin layers, which turn the hydrophobic surface of the conidium into hydrophilic; and the exposure of the β-glucans in the surface of the swollen conidia, which increases the surface adhesive properties (Latge *et al.*, 2017). The α-glucans exposed interact with each other, causing the swollen conidia aggregation (Latge *et al.*, 2017; Beauvais *et al.*, 2014).

In mycelia, around 90% of the CW is composed of polysaccharides, some of which are structurally and covalently bound to the β-1,3-glucan core; while others, such as α-1,3-glucan and GAG, fill up the pores between fibrillar polysaccharides (Latge *et al.*, 2017). After swelling, conidia germinate and develop into hyphae that can grow differently depending on the conditions, (1) as a network of agglutinated hydrophobic hyphae, under static and aerated conditions; (2) or as separate hyphae, under shaken liquid conditions. Electron microscopy analysis has shown that in the first case the wall looks like a single electron-translucent layer; while in the second case, it looks like a thin electron-dense layer with extracellular material covering the CW (Latge *et al.*, 2017).

In addition to the differences in the CW polysaccharides composition and organization in the two morphologies, these may also vary depending on the growth and nutritional conditions. For example, when the fungus is growing as a biofilm, the α-1,3-glucans are found on the surface of the mycelial wall, but when the growth is under shaken and submerged conditions, they are localized on the CW inner layer (Beauvais *et al.*, 2014). Also, *A. fumigatus* growth under limiting glucose concentrations results in a reduction of the β-1,3-glucan synthase activity, with a consequent reduction of CW β-1,3-glucan levels (Clavaud *et al.*, 2012). On the other hand, hypoxia is associated with an increment in β-1,3-glucans and chitin, and with the reduction of α-1,3-glucans (Shepardson *et al.*, 2013).

GAG is a heterogeneous linear polymer composed of α-1,4-linked galactose, N-acetyl-galactosamine, and galactopyranose, and is secreted by growing hyphae (Speth *et al.*, 2019). It is an important component during biofilm formation since it binds to the hyphal surface, creating a polysaccharide envelope that covers and protects the growing fungus, forming an extracellular matrix between hyphae (Speth *et al.*, 2019). When GAG deficient strains were evaluated in mouse models of invasive aspergillosis, they were incapable of forming biofilms and showed an attenuated virulence (Gravelat *et al.*, 2013; Speth *et al.*, 2019). Also, GAG is a major hypha adhesin, because mutants incapable of synthesizing this compound do not form biofilms, and exhibit a reduced adherence to pulmonary epithelial cells (Speth *et al.*, 2019; Gravelat *et al.*, 2013). Moreover, strains that overexpress this wall component exhibit an increased binding to the host cells and other surfaces (Neves *et al.*, 2017).

It is also known that GAG needs to be deacetylated by the enzyme Agd3, not only to bind to the hyphae but also to bind to other surfaces (Lee *et al.*, 2016). Agd3 mutants showed adhesion and virulence defects both *in vitro* and *in vivo* (Lee *et al.*, 2016). This wall component has been described in several *Aspergillus* species, including *A. fumigatus*, *Aspergillus nidulans*, *Aspergillus niger*, and *Aspergillus parasiticus* (Lee and Sheppard, 2016).

The Fungal Cell Wall and the Development of Antifungal Drugs

Since the CW has an essential role in maintaining cell integrity, this structure represents an attractive target for the development of antifungal drugs. Additionally, many wall components are not synthesized by the mammalian host, potentially reducing the collateral effects when developing a compound with antifungal properties. Besides the selection of the target, antifungals should ideally meet some criteria: (1) the activity of the antifungal must have a broad spectrum against yeast and filamentous fungi; (2) it should be fungicidal rather than fungistatic; (3) to be directed at a specific fungal target and to have no interference with host targets; (4) to have multiple delivery methods, particularly oral availability; and (5) to have minimal side effects or toxicities (Mazu *et al.*, 2016).

The chitin synthesis inhibitors act as analogs of the UDP-GlcNAc substrate for Chs. Among these inhibitors, we found polyoxins, which are closely related to nucleotide antibiotics that are produced by *Streptomyces cacaoi* var *asoensis* (Endo *et al.*, 1970). The polyoxin D action mechanism is the competitive inhibition of Chs for UDP-GlcNAc (Endo *et al.*, 1970). The polyoxins are dipeptidyl or tripeptidyl nucleosides that are transported into the cell *via* a peptide transport system (Naider *et al.*, 1983). Nikkomycins X and Z are also competitive inhibitors of fungal Chs that are structurally similar to polyoxins. These compounds showed *in vivo* and *in vitro* antifungal activity against *Blastomyces dermatitidis* and *Coccidioides immitis* (Hector *et al.*, 1990). However, some fungi like *Candida* spp., *C. neoformans*, *Aspergillus* spp. have been reported to be resistant to nikkomycins (Hasim and Coleman, 2019).

Other Chs inhibitors are HWY-289, arthrichitin (activity against *Candida* spp., *Trichophyton* spp.), and FR-9000403 (Mazu *et al.*, 2016). A recent study found two benzothiazole compounds, described as IMB-D10 and IMB-F4, which inhibited Chs *in vitro*, reduced chitin levels in yeast cells, and inhibited the *S. cerevisiae* and *C. albicans* growth (Li *et al.*, 2019). Nikkomycin Z has shown to have a synergistic interaction with fluconazole and itraconazole against *C. albicans, C. parapsilosis, C. neoformans,* and *C. immitis;* while combined with itraconazole, Nikkomycin Z showed synergism against *A. fumigatus* and *Aspergillus flavus* (Li and Rinaldi, 1999).

Echinocandins are semisynthetic lipopeptides produced from fungal precursors: caspofungin from *Glarea lozoyonensis* pneumocandin B_0, anidulafungin from *A. nidulans* echinocandin B_0, and micafungin from *Coleophoma empedra* hexapeptide FR901370 (Eschenauer *et al.*, 2007). Echinocandins are hexapeptide with *N*-linked acyl fatty acid chains that intercalate in the cell membrane phospholipids (Lima *et al.*, 2019). The echinocandin's action mechanism is the noncompetitive inhibition of β-1,3-glucan synthase, which causes the decrease in the β-1,3- and β-1,6-glucan network, leading to a disordered and osmotically unstable CW and fungal death (Lima *et al.*, 2019). Echinocandins inhibit the Fsk subunit of the glucan synthase complex and mutations in this subunit lead to the resistance showed in some *Candida* species (Perlin, 2011).

Ibrexafungerp (SCY-078 formerly MK-3118) is a semisynthetic orally bioavailable enfumafungin derivative, structurally distinct from echinocandins. This compound is a potent inhibitor of glucan synthase, with activity even in echinocandin-resistant *Candida* strains (Pfaller *et al.*, 2017). Moreover, Ibrexafungerp exhibited antifungal activity against *C. auris* strains resistant to echinocandin and fluconazole (Arendrup *et al.*, 2020). In *C. glabrata*, spontaneous mutations in the β-1,3-glucan synthase *FKS* subunit showed partially overlapping but an independent binding sites for Ibrexafungerp and echinocandins (Jiménez-Ortigosa *et al.*, 2017).

The combination of nikkomycin Z and echinocandin (anidulafungin or micafungin) showed synergistic effects against *C. albicans* isolates and laboratory-derived echinocandin-resistant *fks* mutants (Cheung and Hui, 2017); while a similar observation was reported with echinocandin FK463 in *A. fumigatus* (Chiou *et al.*, 2001). The combination of different compounds with distinct targets may be a good solution for the arising problem of fungal resistance against antifungal drugs.

One of the proteins involved in GPI synthesis is Gwt1 (GPI-anchored wall protein transfer 1), an inositol acyltransferase that catalyzes inositol acylation (Lima *et al.*, 2019). The compound 1-[4-butylbenzyl]isoquinoline (BIQ) inhibits the expression of GPI-mannoproteins in both *S. cerevisiae* and *C. albicans*, resulting in growth inhibition (Tsukahara *et al.*, 2003). A missense mutation in *GWT1* suppressed the drug-induced growth-inhibitory phenotype, suggesting that the encoded protein is the target of this compound (Tsukahara *et al.*, 2003). A derivative compound from BIQ, E1210, has a broad-spectrum antifungal activity that also inhibits the fungal GPI biosynthesis and showed potent antifungal activity against *C. albicans, C. tropicalis, A. flavus, A. fumigatus,* and *Fusarium solani* (Hata *et al.*, 2011).

Another class of compounds are members of the pradimicin family. Pramidicins appear to act by calcium-dependent complexing with the saccharide portion of cell surface mannoproteins, which leads to the perturbation of the cell membrane, leakage of intracellular contents and cell death (Gonzalez *et al.*, 1998). A derivative of paramedicine, BMS-181184, was proven to have *in vitro* antifungal activity against *Candida* spp., *C. neoformans, A. fumigatus,* dermatophytes, and *Sporothrix schenckii* but it had no activity against *A. niger, A. flavus, Malassezia furfur, Fusarium* spp, and *Pseudallescheria boydii* (Fung-Tomc *et al.*, 1995).

Thus, the fungal CW components and their biosynthetic pathways still are novel and important targets for the development of new antifungal drugs.

Immune Sensing and the Fungal Cell Wall

The immune system has developed very efficient strategies to control fungal pathogens, generally ensuring a result in our favor (Hernandez-Chavez *et al.*, 2017). Once the pathogen has entered the host, it is likely eliminated by phagocytic cells, such as macrophages and neutrophils of the innate immune system that belong to the first defense line (Hernandez-Chavez *et al.*, 2017; Erwig and Gow, 2016). Both kinds of phagocytic cells are efficient in fungal recognition, mainly through interaction with the CW, which contains molecules that cannot be synthesized by the host, and therefore are detected as pathogen-associated molecular patterns by various patterns recognition receptors (Díaz-Jiménez *et al.*, 2012; Becker *et al.*, 2015). It is known that polysaccharides are the most abundant CW components, and almost all of them can be recognized by specific receptors and stimulate immune responses (Erwig and Gow, 2016). However, to avoid host defense mechanisms, fungi have developed strategies to escape immune sensing (Hernandez-Chavez *et al.*, 2017).

The CW changes both composition and architecture when environmental conditions are adverse, and when the cell is under high-stress levels, contributing to the strategies required for adaptation and survival (Díaz-Jiménez *et al.*, 2012; Gow *et al.*, 2017). This is considered an advantage for some fungi since those changes can provide advantages to resist or disguise the action of immune effectors (Hernandez-Chavez *et al.*, 2017).

The recognition of CW components is essential to trigger a host protective immune response, and the outcome of this will depend on the ligands-receptors engaged and the downstream signaling pathways activated (Hernandez-Chavez *et al.*, 2017). The pattern recognition receptors are classified into four families: Toll-like receptors (TLR), NOD-like receptors (NLR), retinoic acid-inducible gene I (RIG-I), and C-type lectins (CLR), most of which are expressed in dendritic and other myeloid cells (Netea *et al.*, 2008). When interacting with *C. albicans*, TLR2 recognizes phospholipomannans, TLR4 recognizes *O*-linked mannan, TLR6 is involved in zymosan recognition, and TLR9 detects fungal DNA (Netea *et al.*, 2008). C-type lectin receptors are primarily membrane-bound receptors that recognize polysaccharide structures. Dectin-1 recognizes β-1,3-glucans, mannose receptor and DC-SIGN recognize *N*-linked mannans, the macrophage-inducible C-type lectin (Mincle) recognizes α-mannans, and galectin-3

Table 4 Pathogen-associated molecular patterns (PAMPs) present in some pathogenic fungi and recognition of fungal components by pattern recognition receptors (PRRs)

Pathogens	*PAMPs*	*PRRs that recognize PAMPs*
C. albicans	β-1,3-Glucans, β-1,6-glucans, *N*-linked mannans, *O*-linked mannans, α-mannans, α-mannose oligosaccharides, β-1,2- oligomannans, chitin.	TLR4, TLR2, TLR9, Dectin-1, Dectin-2, Dectin-3, DC-SIGN, MR, Galectin-3, Mincle
A. fumigatus	Galactomannan, α-1,3-glucans, β-1,3-glucans, β-1,6-glucans, β-1,4-glucans	DC-SIGN, Dectin-1, Dectin-2, TLR2, TLR4

detects mannans and β-1,2-oligomannans (Drummond *et al.*, 2011) (see **Table 4**). Several studies have shown that Dectin-1 mediates a wide variety of antifungal cellular responses, such as phagocytosis, cytokine, and chemokine production, which contribute to a strong inflammatory immune response. Some of these activities are stimulated when both dectin-1 and TLR2 interact with β-1,3-glucan and establish a co-stimulatory effect that positively boosts those immunological elicitors (Dennehy *et al.*, 2008; Netea *et al.*, 2008).

As mentioned, CW polysaccharides are organized in different layers, and to avoid its sensing by immune cells, fungi manage to "hide" and cover structural polysaccharides, in particular the β-1,3-glucans and chitin, with different molecules such as mannans, working as a protective shield (Heinsbroek *et al.*, 2005; Hernandez-Chavez *et al.*, 2017). This mechanism is not exclusive of *C. albicans* though. In *A. fumigatus* conidia, the hydrophobins and melanin mask the β-glucans, evading the host defense system (Steele *et al.*, 2005). The *C. neoformans* capsule masks the recognition of cell wall mannan and β-1,3-glucan; however, the cell does not go completely unnoticed by the innate immune system, because the capsule can be recognized by TLRs that trigger an inflammatory response (Marcos *et al.*, 2016). Dectin-2 and Dectin-3 receptors are also transmembrane proteins of the C-type lectin family that recognize α-mannans (Zhu *et al.*, 2013).

Chitin interacts with different receptors, in a size- and concentration-dependent manner (Da Silva *et al.*, 2009; Wagener *et al.*, 2014). At low concentrations, small particles of 1–10 μm can induce an anti-inflammatory response through the mannose receptor, in conjunction with TLR9 and NOD2, leading to secretion IL-10 (Wagener *et al.*, 2014). Particles of intermediate size (40–70 μm) induce a pro-inflammatory response, triggering the production of TNFα and IL-17 (Marcos *et al.*, 2016). Very large chitin particles (70–100 μm) and very small particles < 2 μm are inert and do not trigger an immune reaction (Marcos *et al.*, 2016). It is likely that during the infection process, and assuming that the chitin particles that remain are of intermediate size, an inflammatory process is induced and disrupts fungal cells, and from these, small particles arise to trigger an anti-inflammatory response, limiting the immunological reaction (Da Silva *et al.*, 2009).

Melanins, which are complex amorphous polymerized phenolic compounds, are also found in the CW of some dimorphic fungi. These compounds prevent complement activation, neutralize antimicrobial peptides, and protect cells from oxidative damage (Nosanchuk and Casadevall, 2006). Because of its importance in fungal virulence, the host immune system has evolved antifungal strategies that allow melanin detection (Smith and Casadevall, 2019). One of the strategies is the use of C-type lectins such as the melanin receptor MelLec, responsible for recognizing the naphthalene-diol unit of DHN-melanin. MelLec recognizes melanin from cell surface such as that found on *A. fumigatus* conidia and in other DHN-melanized fungi (Stappers *et al.*, 2018).

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The Fungal Chitinases

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Introduction

Chitin derives from the Greek word chiton (*χιτών*) meaning garment, and constitutes one of the most abundant biopolymers in nature. It consists of β-1–4 linked N-acetyl-D-glucosamine (GlcNAc) residues, forming a linear long chain and it is a structural component in many organisms. Chitin is synthesized by chitin synthases, which are plasma membrane-associated enzymes transferred by small vesicles called chitosomes (Bartnicki-Garcia, 1987). These enzymes are responsible for catalyzing the transfer of GlcNAc from uridine (UDP)-N-acetylglucosamine to the growing chitin chain. The chitin chain then inserts into the cell wall adjacent to plasma membrane, aided by hydrogen bonds (Bowman and Free, 2006).

The fungal cell wall is a dynamic compartment, contributing to hyphal ability to cope with different environmental stresses. Its composition is mostly β-1.3 and β-1.6 glucans, chitin and glycoproteins and differs from those in oomycetes and plants, where chitin is absent or exists in very small amounts (Melida *et al.*, 2013; Hinkel and Ospina-Giraldo, 2017). Chitin plays an important role in fungal cell wall rigidity and plasticity, although the percentage of chitin in filamentous fungi does not exceed 10%–20% of the total cell wall dry biomass (Specht *et al.*, 1996; de Nobel *et al.*, 2000), while in yeast-like fungi the proportion is even less (1%–2%) (Klis *et al.*, 2002). Chitin plays also a key role in fungal-plant interactions since it is recognized by plant receptors and triggers basal plant defense mechanisms resulting in pattern-triggered immunity (PTI) (Boller and Felix, 2009). Except fungal cell walls, chitin can be found in insects and nematodes (Hill *et al.*, 1991; Merzendorfer and Zimoch, 2003), in crustacean and mollusks shells (Peters, 1972; Kurita, 2006), in protozoa and algae (Mulisch, 1993; Kapaun and Reisser, 1995). However, it is absent from vertebrates and plants, meaning that it can be an appropriate target for anti-fungal drugs (Chaudhary *et al.*, 2013).

Chitinases (EC 3.2.1.52) are hydrolytic enzymes, responsible for chitin degradation. They cleave the β-1.4-bond releasing oligomeric, dimeric (chitobiose), and polymeric GlcNAc products. Yeast-like fungi contain fewer chitinase genes than the filamentous ones, and the number highly varies between species; from only one in the yeast *Schizosaccharomyces pombe* to 36 in *Trichoderma virens*. Chitinases are categorized into two glycoside hydrolase (GH) families; 18 and 19. Chitinases from filamentous fungi belong exclusively to GH18, while GH19 chitinases have been identified in the microsporidia phylum (Henrissat, 1991; Rönnebäumer *et al.*, 2006). Chitinases in GH18 and GH19 families display limited sequence similarity, differences in their three-dimensional structures and in their catalytic mechanisms (Perrakis *et al.*, 1994; Brameld *et al.*, 1998). Fungal species also contain hydrolytic enzymes that degrade chitobiose to monomers. These enzymes are called *N*-acetylhexosaminidases (NAGases) and belong to the GH20 family (Cantarel *et al.*, 2009).

Chitinases can be classified as endochitinases or exochitinases depending on their cleavage patterns (**Fig. 1**). Exochitinases can cleave the chitin chain from the ends producing chitobiose, while endochitinases cleave the chitin chain at random positions (Horn *et al.*, 2006; **Fig. 1**). Endo- and exochitinases also show differences in their catalytic clefts; exochitinases have tunnel-shaped clefts while the substrate cleft in endochitinases are open and shallow (van Aalten *et al.*, 2001; Hurtado-Guerrero and van Aalten, 2007). Chitinase binding sites are long and contain at least five sugar units. All GH18 proteins contain a $(\alpha/\beta)_8$ barrel (TIM barrel) fold and the substrate-binding amino acids are located in loops extending from the $(\alpha/\beta)_8$ barrel (Lienemann *et al.*, 2009). Finally, GH18 have been reported to be involved in transglycosylation process as well (Boer *et al.*, 2004).

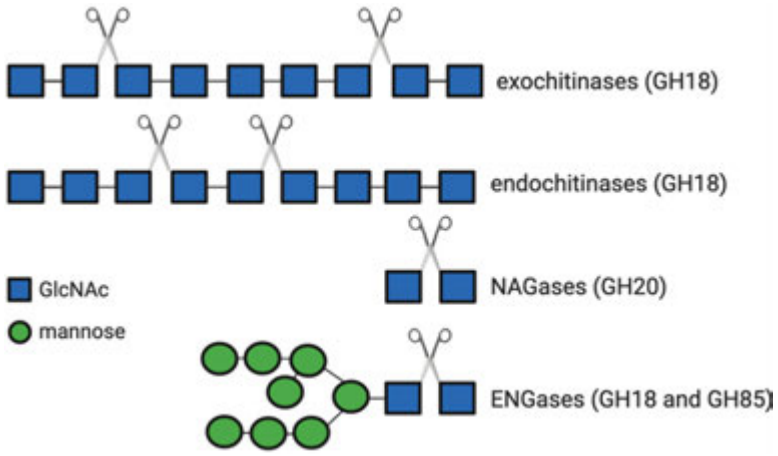

Fig. 1 The enzymatic activity of GH18s and GH20s in fungi. Endochitinases cleave the chitin chain randomly, exochitinases cleave from the open ends producing chitobiose (dimers) further cleaved by GH20 enzymes. ENGases are responsible for the deglycosylation of *N*-glycoproteins, cleaving the *N*, *N*-diacetylchiotobiose moiety from high mannose N-linked glycans. Figure was created with BioRender.

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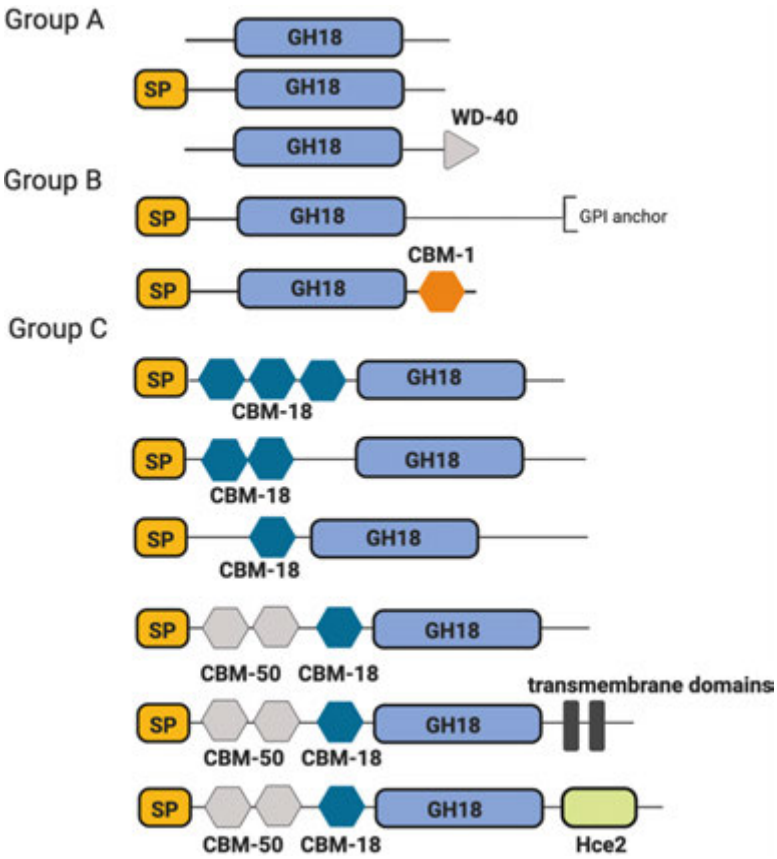

Fig. 2 The domain structure of fungal chitinases. Abbreviations: SP: signal peptide, GH18: glycoside hydrolases family 18, CBM-1: carbohydrate binding module family 1; cellulose-binding, CBM-18: carbohydrate binding module family 18; chitin-binding, CBM-50: carbohydrate binding module family 50; LysM, GPI: glycosylphosphatidylinositol structure. Figure was created with BioRender.

Phylogenetic analyses of fungal GH18 enzymes show that they cluster in three distinct groups; A, B and C (Seidl *et al.*, 2005; Karlsson and Stenlid, 2008; Karlsson *et al.*, 2016). These groups are further subdivided in several subgroups: Group A contains four subgroups (A-II, A-III, A-IV, and A-V), group B contains six subgroups (B-I, B-II, B-III, B-IV, B-V, and B-VI) while group C is divided into two subgroups (C-I and C-II). In certain fungal species, a chitinase of bacterial origin has been identified, probably as a result from horizontal gene transfer (Ubhayasekera and Karlsson, 2012).

Chitinases in all three groups typically contain a GH18 module with the catalytic DxxDxDxE motif, necessary for chitinolytic activity (van Aalten *et al.*, 2001; Gruber *et al.*, 2011a; Tzelepis *et al.*, 2012). In addition to their catalytic module, many GH18 enzymes contain additional domains such as the carbohydrate binding module family 1 (CBM1) or CBM50 (peptidoglycan binding LysM domain) (Seidl *et al.*, 2005; Tzelepis *et al.*, 2012; **Fig. 2**). These modules are not involved in the catalytic activity but enhance the enzymes ability for binding to crystalline chitin (Suzuki *et al.*, 1999).

Group A Chitinases

Most chitinases in this group are predicted to have exochitinase activity since they typically have a narrow substrate catalytic cleft. Many members contain a signal peptide at their N-termini, indicating that they are targeted to the endoplasmic reticulum (ER) and are possibly secreted (**Fig. 2**). In addition to the GH18 catalytic module, a WD40 domain has been found in chitinases from the entomopathogenic fungus *Beauveria bassiana* (Agrawal *et al.*, 2015) (**Fig. 2**). This domain is involved in a variety of cellular processes, such as protein-protein or protein-DNA interactions (Xu and Min, 2011). Fungal genomes contain variable numbers of genes encoding group A chitinases. For instance, the basidiomycete *Ustilago maydis*, which causes smut disease in maize, contains only two genes (Kamper *et al.*, 2006), while the soil-borne pathogen *Fusarium oxysporum* contains 12 genes. The well-studied mycoparasitic *Trichoderma* species contain an average number of seven group A chitinase genes (Kubicek *et al.*, 2011), while the model species *Neurospora crassa* has five (Tzelepis *et al.*, 2012).

One of the best studied group A chitinases is Ech42 from *Trichoderma*. Its enzymatic function has been characterized and is atypical for group A as it is shown to possess endo-activity (Carsolio *et al.*, 1994; Hayes *et al.*, 1994). It contains at least seven subsites and cleave the chitin chain preferably between the second and the third sugar from the reducing end and it can degrade chitohexose

$(GlcNAc)_6$ into three $(GlcNAc)_2$ units (Hartl *et al.*, 2012). When it comes to regulation and function, the *ech42* gene was highly induced under different conditions such as fungal-fungal interactions, starvation, autolysis and during cell wall degradation (Gruber and Seidl-Seiboth, 2012). Deletion of this gene did not affect *Trichoderma* mycoparasitic ability against *Rhizoctonia solani* or *Sclerotium rolfsi* (Carsolio *et al.*, 1999), while reduced anti-fungal activity against *Botrytis cinerea* was observed (Woo *et al.*, 1999). In *T. reesei*, three additional group A chitinase genes were expressed during similar conditions as *ech42* (Seidl *et al.*, 2005).

In the mycoparasitic species *Clonostachys rosea*, the homolog to the *ech42* gene was also induced during interactions with *B. cinerea* and on media where chitin was the sole carbon source (Mamarabadi *et al.*, 2008a; Tzelepis *et al.*, 2015). Deletion of this gene led to lower *in vitro* antagonistic activity but did not reduce *C. rosea* biocontrol ability (Mamarabadi *et al.*, 2008b). In addition to *ech42*, *C. rosea* contains seven other genes in group A (Karlsson *et al.*, 2015; Tzelepis *et al.*, 2015). None of these genes were induced during interactions between *C. rosea* and *B. cinerea*, *R. solani* or *F. graminearum*, while some of them were up-regulated when *C. rosea* was exposed to carbon starvation or during growth on chitin-medium, as compared to carbon rich medium (Tzelepis *et al.*, 2015). In the entomopathogenic species *Metarhizium anisopliae*, nine group A chitinases have been identified (Junges *et al.*, 2014). The *chimaA1* gene was up-regulated on media containing chitin or GlcNAc, while high transcript levels of *chimaA7* were measured during growth on high glucose medium (Junges *et al.*, 2014). Furthermore, three and two genes were induced at the conidial stage or during appressoria formation, respectively (Junges *et al.*, 2014).

In *N. crassa*, deletion of group A chitinases did not have any impact in fungal phenotypes, such as growth rate, conidiation or colony morphology, while variable transcription patterns were observed (Tzelepis *et al.*, 2012). For instance, two genes (*gh18–3* and *gh18–4*) were induced during interaction between *N. crassa* and *F. sporotrichoides*, (Tzelepis *et al.*, 2012). In the opportunistic human pathogen *Aspergillus fumigatus*, deletion of the group A *chiB1* chitinase gene did not cause any changes in colony morphology but resulted in lower chitinolytic activity during the autolytic phase in batch cultures (Jaques *et al.*, 2003). However, a functional analysis of the group A ChiB chitinase in *A. nidulans* revealed a crucial role in autolysis (Yamazaki *et al.*, 2007; Pocsi *et al.*, 2009).

In the ectomycorrhizal species (ECM) *Laccaria bicolor* there are three putatively secreted group A chitinases, which are induced at different time points of ECM development, indicating a continuous degradation of chitin during the establishment of the mutualistic relationship (Veneault-Fourrey *et al.*, 2014).

In yeast-like fungi, the number of genes encoding chitinases is lower compared to filamentous species. In the smut fungus *U. maydis*, the group A chitinase Cts1 is involved in cytokinesis (Langner *et al.*, 2015). Deletion of the *cts1* gene led to changes in colony morphology and sedimentation rate. Cts1 was also shown to be essential during separation of mother and daughter cells and during filamentous growth (Langner *et al.*, 2015), possibly by degrading chitin in the cell wall. The model yeast species *Saccharomyces cerevisiae* contains only one chitinase gene in group A (*ScCTS2*) that is involved in asci formation (Giaever *et al.*, 2002). Deletion of the group A *AgCTS2* gene from the plant pathogenic fungus *Ashbya gossypii*, homologous to *ScCTS2*, impacted the phenotype of spores (Dünkler *et al.*, 2008). The wild-type spores have a hyaline appearance, while spores from the *AgCTS2* mutants were speckled with dot-like, vesicular bodies. (Dünkler *et al.*, 2008). However, deletion of the *AgCTS2* gene did not affect spore germination or polarity (Dünkler *et al.*, 2008). Interestingly, complementation of *AgCTS2* with the homologs *ScCTS2* and *CaCTS4* from *S. cerevisiae* and *Candida albicans* respectively, restored the phenotype of spores indicating a conserved function in sporulation over species boundaries (Dünkler *et al.*, 2008). In *C. albicans*, *CaCTS4* is induced during the yeast growth phase but down-regulated during the hyphal growth phase (McCreath *et al.*, 1996). However, deletion of this gene did not cause any obvious phenotype (Dünkler *et al.*, 2005). Finally, the opportunistic human pathogen *Cryptococcus neoformans* contains three homologs to the *ScCTS2* gene, which are involved in sexual reproduction of this species (Baker *et al.*, 2009).

Certain predicted group A GH18 proteins contain mutations in the catalytic DxxDxDxE motif, indicating loss of enzymatic activity. For instance, *T. reesei* contains four genes in subgroup A-II and A-IV with these type of amino acid substitutions (Karlsson and Stenlid, 2009). The A-IV subgroup CHI18–3 protein is predicted to be localized in the mitochondria (Seidl *et al.*, 2005) and contains two S-globulin domains, often associated with GH18 modules (Shewry and Halford, 2002). Genome analyses revealed that there are orthologs in other fungal species as well (Seidl *et al.*, 2005; Tzelepis *et al.*, 2012, 2015), indicating that the presence of a putative mitochondrial non-chitinolytic GH18 protein is widespread among the Sordariomycetes. It is possible that these enzymatically inactive GH18 proteins retain the ability for chitin binding, i.e., evolve into lectins, as was recently shown for the group A protein MpChi from the basidiomycete cacao pathogen *Moniliophthora perniciosa*. The enzymatically inactive MpChi binds to chitin and thereby suppresses chitin-triggered plant immunity and facilitates infection (Fiorin *et al.*, 2018). A paralog to MpChi in the closely related cacao pathogen *M. roreri* carries a single substitution in its catalytic motif that results in reduced chitinolytic activity and was shown to suppress plant immunity in a similar fashion as MpChi (Fiorin *et al.*, 2018). Genome mining revealed that many plant pathogens contain predicted GH18 proteins with degenerate catalytic motifs, suggesting that recognition of chitin by plant pattern recognition receptors drives evolution of chitin-scavenging lectins in plant pathogenic fungi (Fiorin *et al.*, 2018).

Group B Chitinases

GH18 enzymes in this group are divided in six subgroups (B-I through B-VI) (Seidl *et al.*, 2005; Karlsson *et al.*, 2016). They are predicted to have a more shallow and wider catalytic cleft as compared to chitinases in group A, indicating endochitinase activity (**Fig. 2**). However, certain enzymes from the B-V subgroup have a mannosyl glycoprotein endo-N-acetyl-β-D-glucosaminidase (ENGase) type activity (Stals *et al.*, 2010; **Fig. 2**). The number of group B chitinase genes varies between one in *S. cerevisiae* to 11 in mycoparasitic *Trichoderma* species (Kuranda and Robbins, 1991; Seidl *et al.*, 2005). They are also predicted to vary in size, from small

(30–40 kDa) to large (80–90 kDa) proteins. They typically contain a signal peptide at the N-terminus, indicating that they are targeted to the ER. Cellulose-binding domains (CBM1), which also bind chitin (Boraston *et al.*, 2004), are often found in group B chitinases. *Trichoderma* species contain high numbers of chitinases with CBM1 domains (Seidl *et al.*, 2005). Experimental addition of CBM1 domains to group B chitinases in *Trichoderma* resulted in an increase of its antifungal activity (Limon *et al.*, 2001, 2004), suggesting a role in fungal-fungal interactions. Certain large group B chitinases contain a glycosylphosphatidylinositol structure (GPI anchor) in the C-terminus indicating that they are localized in the cell wall (Yamazaki *et al.*, 2008; Tzelepis *et al.*, 2012). The group B chitinase Chit33 from *Trichoderma* has been enzymatically characterized. It is a typical endochitinase having a shallow and open substrate-binding site with at least six subsites, producing $(GlcNAc)_2$ and $(GlcNAc)_4$ (Hartl *et al.*, 2012).

In *T. reesei*, the *chi18–13* gene was expressed in presence of *R. solani* cell wall components and during confrontation with *B. cinerea* (Seidl *et al.*, 2005). Similarly, its homolog in *T. atroviride* was highly induced before and during interactions with *R. solani*, indicating that group B chitinases are involved in mycoparasitic interactions (Seidl *et al.*, 2005). The group B chitinase Chi18–15/Chit36/Ech37 is an interesting example of a horizontal gene transfer event, from a bacterial donor (likely related to *Streptomyces*) to a hypocrealean acceptor, as it is found in both *Trichoderma* and *Clonostachys* (Karlsson and Stenlid, 2009; Ubhayasekera and Karlsson, 2012). Gene expression analysis showed that *chi18–15* was induced during growth on chitin and fungal cell walls, perhaps suggesting a function in nutrient acquisition (Viterbo *et al.*, 2002). Besides the *ech37* gene, the *C. rosea* genome contains two additional group B chitinases, *chiB1* and *chiB2* (Tzelepis *et al.*, 2015). The *chiB1* gene was highly induced during interactions with *R. solani*, while both genes were up-regulated on media where chitin was the sole carbon source (Tzelepis *et al.*, 2015). The saprotrophic species *N. crassa* also contains two genes in group B and one of them, *chit-1*, was induced on chitin media as well (Tzelepis *et al.*, 2012). Analysis of a *chit-1* deletion strain showed that this mutant grew slower as compared to the wild type strain, indicating a role in hyphal growth (Tzelepis *et al.*, 2012).

The fungus *M. anisopliae* contains seven genes in this group (Junges *et al.*, 2014). Among those, *chiMaB7* showed significantly higher transcript levels during infection-related conditions (blastospores, tick cuticles and appressoria), while the *chimaB4* gene was up-regulated during growth on media contained chitin or GlcNAc as sole carbon sources (Junges *et al.*, 2014). Furthermore, the *M. anisopliae* B group endochitinase CHI2 seems to be involved in virulence, since overexpression of this chitinase led to an increased efficacy of the fungus to kill the insect *Dysdercus peruvianus* while gene deletion resulted in a reduced fungal infection efficiency (Boldo *et al.*, 2009). Similarly, the CHI30 chitinase is also shown to be involved in virulence and heat shock adaptation in *M. anisopliae* (Staats *et al.*, 2013).

In the ectomycorrhizal species *Tuber melanosporum* two B group chitinases, *TmelCHT2.1* and *TmelCHT2.2*, are highly induced in the symbiotic stage. This indicates the importance of cell wall modification during the switch from the free-living to symbiotic stage (Balestrini *et al.*, 2012). Moreover, in *L. bicolor* there is a chitinase gene in this group that was up-regulated at different time points of ECM development, similar to the group A genes mentioned previously (Veneault-Fourrey *et al.*, 2014).

Regarding yeast-like species, the group B *S. cerevisiae CTS1* chitinase gene was expressed in the mother and daughter cells during the early stages of separation (Langner and Gohre, 2016). Deletion of the *CTS1* gene led to a defect in cell separation and formation of multicellular aggregates (Kuranda and Robbins, 1991). The *C. albicans* genome contains three group B chitinase homologs to *CTS1*, where deletion of *CaCHIT1* and *CaCHIT2* resulted in increased hyphal growth on solid media (Dünkler *et al.*, 2005). CaCHIT2 is a typical large group B chitinase with a GPI anchor at the C-terminus (Dünkler *et al.*, 2005). Deletion of the *CaCHIT3* resulted in formation of chains of non-separated cells similar to the *S. cerevisiae CTS1* deletion phenotype, indicating a role of these genes in cytokinesis (Dünkler *et al.*, 2005). The group B endochitinase KlCTS1 from the yeast *Kluyveromyces lactis* appears to provide the same function in cytokinesis, since deletion of this gene led to inefficient cell separation (Colussi *et al.*, 2005).

As mentioned above, certain members of the B-V subgroup are shown to exhibit ENGase (EC 3.2.1.96) activity (Stals *et al.*, 2010) (**Fig. 2**). They contain a GH18 module with a conserved catalytic motif similar to active chitinases (DxxDxDxE) and likely evolved from an ancestral chitinolytic enzyme through neofunctionalization (Karlsson and Stenlid, 2008, 2009). Some of these enzymes are involved in deglycosylation of misfolded *N*-glycoproteins in the ER-Associated Degradation (ERAD) process, cleaving the glycoside bond in the *N*,*N*-diacetylchiotobiose moiety as shown in **Fig. 1** (Suzuki *et al.*, 2002). Filamentous ascomycetes typically contain a cytosolic ENGase, while a second, putatively secreted protein has been identified in certain species (Tzelepis *et al.*, 2017). In general, GH18 ENGases are absent from yeast-like species, such as *Saccharomyces*, *Candida*, and *Cryptococcus* (Tzelepis *et al.*, 2017). In Basidiomycetes, the situation is mixed with ENGases being absent from *Heterobasidium*, *Ustilago*, and *Puccinia* genera, while *Schizophyllum* and *Stereum* species have one putative ENGase in their genomes (Tzelepis *et al.*, 2017). Deletion of cytosolic ENGases led to severe phenotypic changes in filamentous ascomycetes (Dubey *et al.*, 2012; Tzelepis *et al.*, 2012). In *N. crassa*, deletion of the cytosolic *gh18–10* ENGase resulted in slower mycelial growth, increased conidiation and tolerance to abiotic cell wall stress agents, and reduced amount of secreted proteins (Tzelepis *et al.*, 2012). Similar results were observed in *T. atroviride* Δ*Eng18B* strains, which is homolog to the *gh18–10* gene (Dubey *et al.*, 2012). Interestingly, this mutant lost its antagonistic ability against *B. cinerea* as well, possibly as an effect from the reduced secretion phenotype (Dubey *et al.*, 2012). Further analysis on Eng18B enzymatic function revealed that it is an active de-glycosylating enzyme, involved in the ERAD process (Tzelepis *et al.*, 2014a).

Group C Chitinases

Chitinases in this group are predicted to exhibit exochitinase activity although no member from this group has yet been biochemically characterized. They are present only in filamentous ascomycetes and are subdivided to two subgroups (C-I and C-II)

(Karlsson and Stenlid, 2008; Gruber *et al.*, 2011a). They show sequence similarities to the α/β subunits of the killer toxin zymocin produced from the diary yeast *K. lactis* and are therefore often referred to as "killer toxin-like" chitinases (Magliani *et al.*, 1997). In *K. lactis*, the function of the α subunit chitinase is to degrade the antagonist cell wall in order to facilitate penetration of the γ subunit, which is the active toxin (Butler *et al.*, 1991). A similar function has been hypothesized for the group C chitinases (Tzelepis and Karlsson, 2019). Genome analysis in *Trichoderma* species revealed that aggressive mycoparasitic species, such as *T. virens* and *T. atroviride*, contain higher number of C group chitinase genes, as compared to the weak mycoparasite *T. reesei* (Ihrmark *et al.*, 2010).

Most C group chitinases are predicted to have an intact catalytic domain, while few of them contain mutations that would render them devoid of chitinolytic activity (Karlsson and Stenlid, 2009; Gruber *et al.*, 2011b). As it was shown in **Fig. 2**, group C chitinases contain a signal peptide at the N-terminal and multiple domains (Tzelepis and Karlsson, 2019). In the C-I subgroup, chitinases usually contain one to three carbohydrate-binding family 18 (CBM18) together with the catalytic GH18 module, both located at the N-terminus (**Fig. 2**). Members in the C-II subgroup typically contain one CBM18 and two LysM CBM50 domains (Gruber *et al.*, 2011a,b; Tzelepis *et al.*, 2012, 2014b). The CBM18 domains bind to chitin and it is suggested to increase adherence of chitinases to the substrate (Boraston *et al.*, 2004). The CBM50 modules have peptidoglycan and chitin binding affinity (Buist *et al.*, 2008) and their role in fungal-plant interactions is well established, since they bind to chitin and stealth fungal hyphae from plant recognition (de Jonge *et al.*, 2010). This mechanism seems to be universal in the fungal kingdom, since it has been described in both ascomycete and basidiomycete phytopathogenic species (Mentlak *et al.*, 2012; Takahara *et al.*, 2016; Dölfors *et al.*, 2019). Furthermore, some members in the C-II subgroup contain transmembrane domains at the C-terminus, indicating a plasma membrane localization (Tzelepis *et al.*, 2012; 2014b; **Fig. 2**). Finally, it has been reported that a small protein domain (Hce2) is fused to the C-termini of certain C-II chitinases (Stergiopoulos *et al.*, 2012). This protein domain is homologous to the necrosis-inducing Ecp2 effector, which has previously been found in phytopathogenic species such as *Mycosphaerella graminicola* and *M. fijiensis* (Stergiopoulos *et al.*, 2010). A chitinase from *T. reesei* is predicted to contain an epidermal growth factor-1-like module at the C-terminus, potentially involved in protein-protein interactions (Wouters *et al.*, 2005). Although the exact roles of Hce2 and EFG-1 remain to be elucidated, it is speculated that they serve a role similar to the zymocin γ-subunit (Tzelepis and Karlsson, 2019).

When it comes to regulation of C-group chitinases, it was initially speculated that the primary function of these enzymes was in fungal-fungal interactions. However, gene expression data later suggested their involvement in other aspects of fungal biology as well. In the *N. crassa* genome, there are three members in the C group. The C-I member *chi18–9* was induced during carbon starvation conditions and not during interactions with fungal species, in contrast to the other C-II members, *chi18–6* and *chi18–8*, which were induced during interspecific interactions and during self-interactions (Tzelepis *et al.*, 2012). However, expression patterns differed during interactions with different fungal species, ascomycetes (*B. cinerea*) verses basidiomycetes (*R. solani*), suggesting that differences in cell wall composition may control regulation (Tzelepis *et al.*, 2012). The *A. nidulans* genome contains four genes in C-II subgroup and gene expression analysis showed that all of them were highly induced during interactions either with ascomycetes (*B. cinerea*) or basidiomycetes (*R. solani*), while there were not induced during interactions with *Phytophthora* species, which lacks chitin in the cell wall, or when dead cell wall material was the only carbon source (Tzelepis *et al.*, 2014b). Expression studies of C-group chitinases have been described in other fungal species as well, such as in the thermophile *Myceliophthora thermophile* where up-regulation was observed during growth on straw (Kolbusz *et al.*, 2014), and in the mycoparasite *Tolypocladium ophioglossoides* during parasitism of truffle tissue (Quandt *et al.*, 2016).

Analysis of expression patterns in *Trichoderma* species support the concept that C-group chitinases are involved in different aspects of fungal life and not only in interspecific interactions (Seidl *et al.*, 2005; Gruber *et al.*, 2011a,b). For instance, in *T. atroviride*, all eight C-group chitinase genes in *T. atroviride* were up-regulated during interactions with *B. cinerea* but not with *R. solani*, and no induction was observed on dead *R. solani* cell wall material (Gruber *et al.*, 2011a). Furthermore, four genes were induced on media where chitin was the sole carbon source, indicating their involvement in nutrient acquisition, while two of them were induced in the central part of the fungal colony, with older hyphae, compared to younger hyphae in periphery, suggesting an additional role in hyphal branching and autolysis (Gruber *et al.*, 2011b). In contrast, gene expression analyses in *T. virens* showed that only four of totally 14 C-group chitinase genes were induced during interactions with either *B. cinerea* or *R. solani*, while 11 of them were induced on fungal cell wall material (Gruber *et al.*, 2011b).

The structure of chitin also seems to influence the induction of these genes, since different genes were up-regulated by different forms of chitin (crude or colloidal) (Gruber *et al.*, 2011b). Finally, *Trichoderma* genome analyses revealed that *tal* genes, predicted to contain only CBM50 modules without any catalytic domain, are often clustered and regulated together with C-group chitinase genes (Gruber *et al.*, 2011a). Functional analysis showed that they are involved in hyphal growth (Seidl-Seiboth *et al.*, 2014). Furthermore, Tal6 protects hyphae from chitinases, increases *Trichoderma* virulence and modulates the activation of plant immunity (Romero-Contreras *et al.*, 2019). In contrast, the mycoparasite *C. rosea* contains a reduced number of genes encoding C-group chitinases as compared to *Trichoderma* mycoparasitic species. There is one member in the C-I and one in the C-II subgroup (Tzelepis *et al.*, 2015). Transcriptional analysis in this species showed that none of these genes were induced in interaction with *Fusarium* species, while the C-I member was only induced on chitin media (Tzelepis *et al.*, 2015). *Metarhizium anisopliae* contains four genes in this group that appeared to be constitutively expressed (Junges *et al.*, 2014).

Functional analyses of C-group chitinases have been conducted in model filamentous species, such as *N. crassa* and *A. nidulans*. In *N. crassa*, deletion of either C-II subgroup genes did not affect its growth or tolerance to abiotic stress conditions (Tzelepis *et al.*, 2012). In *A. nidulans*, deletion of either of the four C-II subgroup genes resulted in increased biomass production in liquid cultures. Deletion of *chiC2–2*, predicted to contain the Hce2 domain, resulted in mutants with reduced *in vitro* antagonistic ability against *B. cinerea* (Tzelepis *et al.*, 2014b). Moreover, three deletion strains showed to be slightly more tolerant in abiotic stress conditions (Tzelepis *et al.*, 2014b).

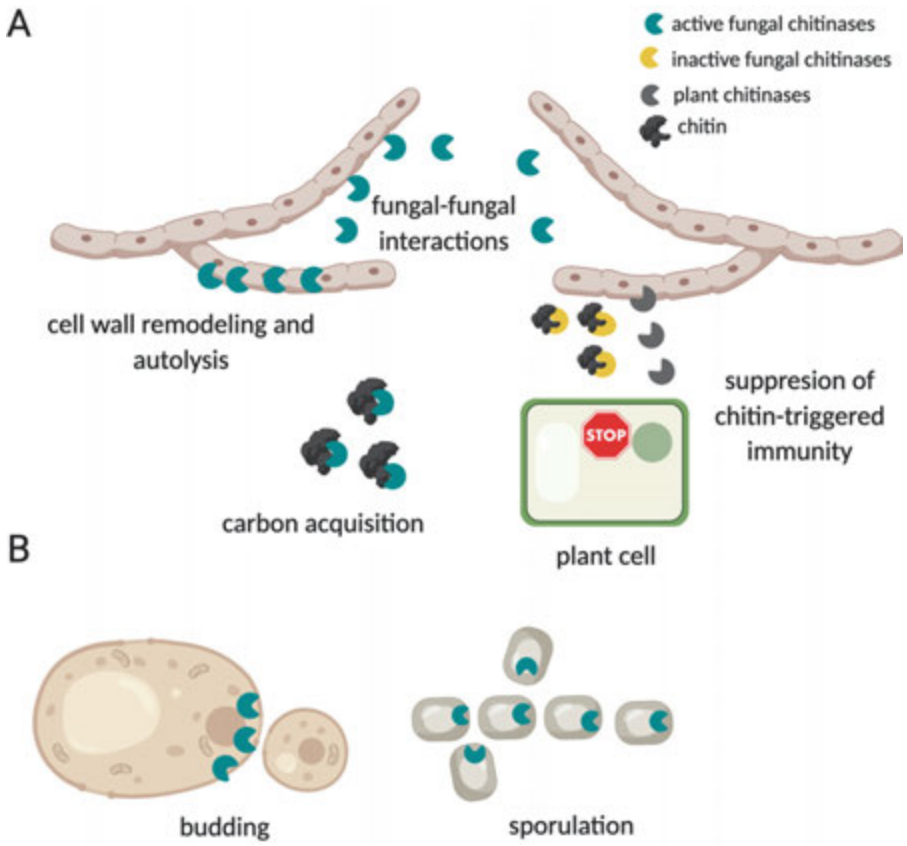

Fig. 3 Proposed functions of fungal chitinases. (A) chitinases in filamentous species have a function in fungal-fungal interactions, degradation of exogenous chitin for nutrient acquisition, autolysis and hyphal branching. Inactive fungal chitinases are involved in perturbation of chitin-triggered immunity during fungal-plant interactions. (B) chitinases in yeast-like fungal species are involved in cytokinesis, such as separation of mother and daughter cells during budding and separation and formation of spores. Figure was created with BioRender.

In *Trichoderma* mycoparasitic species, functional studies are limited. It is possible that gene redundancy in these species prevents observable phenotypes. The only study comes from deletion of a C group gene in *T. atroviride*, where deletion led to reduced conidiation (Seidl-Seiboth *et al.*, 2014). However, this gene carries mutations in the catalytic motif indicating that its chitinolytic activity has been abolished (Gruber *et al.*, 2011a). In contrast, deletion of the *C. rosea* C-II chitinase *chiC2* gene resulted in mutants with reduced *in vitro* antagonistic activity against *B. cinerea* and *R. solani*, but not against *Fusarium graminearum* (Tzelepis *et al.*, 2015). However, deletion of *chiC2* did not affect the biocontrol ability of *C. rosea* against *B. cinerea* (Tzelepis *et al.*, 2015).

Concluding Remarks

Chitinolytic enzymes are present in all fungal species and they are involved in degradation of chitin, an important structural component of fungal cell walls. Fungal chitinases from filamentous fungi belong to GH18, while a single GH19 chitinase has been described in the microsporidial species *Encephalitozoon cuniculi* (Rönnebäumer *et al.*, 2006), a very basal fungal group. Based on their catalytic activities, they can be categorized as exochitinases, which cleave the chitin chain from the ends, or as endochitinases, which cleave the chitin at random positions. Exochitinases release chitobiose, which is further degraded by GH20 NAGases. Phylogenetically, fungal GH18 genes are divided into three groups A, B, and C, further sub-divided in many subgroups. Since gene redundancy is one of the main problems in chitinase functional studies, their precise roles in fungal biology still remain unclear. From the available studies, data show that chitinases play roles in different aspects of fungal physiology, such as degradation of exogenous chitin for nutrient release, in mycoparasitic interactions, during carbon starvation and autolysis, in cell wall remodeling and hyphal growth (**Fig. 3(A)**). In yeast-like species, the number of chitinase genes is lower, typically between one and four, and hence more functional data are available. In these species, chitinases are involved in cytokinesis, such as mother and daughter cell separation during budding (**Fig. 3(B)**), and in sporulation, such as spore formation and separation (**Fig. 3(B)**). The killer toxin-like chitinases in group C is an interesting case. They are present only in filamentous ascomycetes and contain multiple domains such as CBM50 and CBM18, and all of them are predicted to be secreted (**Fig. 2**). In some of them, the Hce2 domain, homologous to the Ecp2 effector, is present. Many aspects of these chitinases remain to be elucidated. Since transcriptional data show that

induction of these genes is often triggered in fungal-fungal interactions, important questions concerns host specificity and cell wall composition or elicitors produced by the living cells that may trigger their expression. Furthermore, the role of the CBM50 and Hce2 modules should be clarified. It is speculated that they may be released as individual peptides after secretion through proteolytic activity, and may function in self-protection and as a toxin, respectively.

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GTPases in Hyphal Growth

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Hyphal Growth in Filamentous Fungi

Filamentous fungi are an evolutionarily flourishing group of microorganisms of significant ecological importance (Evans and Hedger, 2001). They have a considerable impact on our economy and ecosystem because important enzymes derive from fungi with applications in food, textile, recycling and other industries (Cairns *et al.*, 2018). In addition, many fungi are human and plant pathogens that pose a threat to public health and agriculture (Fisher *et al.*, 2012). Among the filamentous fungi there are also the mycorrhizal fungi, which bring countless benefits to the host plant (Smith and David, 2008), and edible fungi, such as truffle, one of the most valuable foods (Hall *et al.*, 2003). Last but not least, fungi are extremely important for understanding many functions of eukaryotic cells in vastly researches and their secondary metabolism is of significant pharmaceutical relevance (Keller *et al.*, 2005).

A better understanding of cytoplasmic organization and behavior can lead to higher and more diverse levels of insight to biology of cells and their evolutionary histories.

The dominant cell type of filamentous fungi are hyphae, which are filamentous of elongated cells that expand at the apex of the tip cell (Steele and Trinci, 1975). The processes involved in the growth of hyphae have affected many scientists for many years. Thanks to the progress in efficient technological DNA sequencing and gene deletion methods, today there is a drastic increase in the number of data. Despite these new resources, filamentous growth is a critical aspect of fungal biology that is not yet comprehensively understood (Meyer *et al.*, 2016). Hyphae exhibit a highly polarized form of cell growth that requires the regulation of numerous processes including cell wall synthesis, polarized vesicle transport, exocytosis, endocytosis, cytoskeletal function, turgor pressure, organelle positioning and bulk cytoplasmic flow. These actions result in apically growing tube-shaped hyphae.

Cell Wall Synthesis

Hyphal growth is accompanied by the secretion of exoenzymes that participate in lysis of the substrate or are involved in the synthesis of the fungal cell wall (Archer and Wood, 1995). The fungal cell wall plays a significant role in development and integrity of the fundamental architecture required for survival and proliferation of the filamentous fungus. The structure of the cell wall is composed typically of polysaccharides and glycoproteins, that can be considered as an extracellular gel-like matrix (Ruiz-Herrera and Ortiz-Castellanos, 2019). The cell wall of most filamentous fungi containsβ (1,3)- β (1,4)- β (1,6)- β (1,7)- glucans, α (1,3)-glucans, chitin, galactomannoproteins, and other less well-characterized glucans. It has been seen that in the alkaline-insoluble fraction are present fibrils of β (1,3)- and β (1,7)-glucans and chitin. Glycoproteins together with alkaline-soluble polysaccharides form an amorphous matrix (Klis *et al.*, 2006). These matrix glycoproteins are presynthesized and packaged into vesicles at the Golgi and delivered along cytoskeleton to the specific points of wall growth. After exocytosis glycosylphosphatidylinositol (GPI) anchors glycoproteins to the plasma membrane (Steinberg *et al.*, 2017).

The polysaccharides are synthesized by enzymes chitin synthases (CHS) and glucan synthases (GS), which are transported on the cell membrane in an inactive form within vesicles, where the enzymes are inserted to synthesize in situ the chitin and β (1,3)-glucans (Sanchez-Leon *et al.*, 2011). Chitin is thought to be the major polysaccharide found in the *Neurospora crassa* septae (Potapova, 2014). Filamentous fungi generally contain several genes encoding CHSs and they are organized into seven different groups or classes, as opposed to three classes of CHSs in yeast or dimorphic species, which correlates with the lowest chitin content in their cell wall (Riquelme and Bartnicki-Garcìa, 2008). The 1–3-glucan synthase complex (GSC) synthesizes β (1,3)-glucans: it contains a catalytic subunit (Fks) and a regulatory subunit (Rho1). Only one essential *Fks* gene has been identified in filamentous fungi. While in *Saccharomyces cerevisiae* there are two *Fsk* genes and they haven't distinct functions. Catalysis products operated by Fsk transmembrane protein poured out of the plasma membrane. Rho1 is a GTPase which is synthesized in the endoplasmic reticulum and inserted into plasma membrane by geranylgeranylation (Inoue *et al.*, 1999).

Today the most accepted view proposes that cell wall-loosening enzymes, such as chitinases and glucanases, participate in the breakage of polysaccharide chains, such as chitin and β (1,3)-glucans, allowing the addition of newly arrived material and generating free ends, substrate for cross-linking enzymes, that rigidify the cell wall (Riquelme *et al.*, 2018).

The Spitzenkörper and Vesicles Pathway

The apex of filamentous fungi is a highly dynamic region where cell growth and morphogenesis occur through the coordinated events of exocytosis, cytoskeletal dynamics and cell wall synthesis. Spitzenkörper (Spk) is where secretory vesicles self-assemble. Using phase-contrast light microscopy, the Spk is observed as a phase-dark body, in most taxa, is partially or completely surrounded a phase-bright central core (Roberson *et al.*, 2010). The Spk is important for hyphal growth and its position in the growing hypha determines the directionality of growth (Riquelme *et al.*, 2000). At the ultrastructural levels the Spk contains vesicles of different sizes,

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actin, microtubules and above all ribosomes, suggesting that translation of mRNA and protein synthesis happen in the hyphal apex (Howard and Aist, 1979). It is thought that the Spk functions as a vesicles supply center. It receives Golgi derived vesicles that releases exocytic vesicles in a controlled manner: in fact, the Golgi cisternae are distributed along the hyphae (Riquelme *et al.*, 2018). It determines an exocytosis gradient that fixes the shape of the fungal apex (Bartnicki-Garcìa *et al.*, 1989). In *N. crassa* the enzymes responsible for the synthesis of the cell wall, CHS and GCS, were located in the Spk. Specifically, CHS was identified at the core of Spk, where microvesicles concentrate and GCS at the Spk outer layer (Riquelme *et al.*, 2007). A biochemical stratification of Spk was detected in *Aspergillus nidulans*, but not the precise location of CHS synthetases (Takeshita *et al.*, 2015).

The biogenesis and release of vesicles take place through different passages, as in other eukaryotes: scission from the donor membrane-vesicle formation, vesicle transport, vesicle docking and vesicle fusion. There are involved coat complexes, which promote vesicle formation and recognize cargo-sorting signals; tethers which interact with coat proteins and mediate docking; and SNAREs (soluble NSF [N-ethylmaleimide-sensitive factor] attachment protein [SNAP]receptors) which facilitate the fusion between the donor and target membrane (Bonifacino and Glick, 2004). Rab GTPases are molecular switches to regulate these steps of vesicular transport along cytoskeletal elements. In *N. crassa* homologous genes have been identified that encode for Rab GTPases that occupy two distinct parts of Spk (Gould and Lippincott-Schwartz, 2009): YPT-1Rab1 was found in the core, while SEC-4Rab8 and YPT-31Rab11 were located in the outer layer. This suggests that distinct Rabs regulate the traffic of the different vesicle populations to the Spitzenkörper (Sánchez-León *et al.*, 2015). FRAP (fluorescence recovery after photobleaching) experiments allowed the identification of the time of the vesicular turnover at the Spk, which is rates from 20 to 40s (Sánchez-León *et al.*, 2015). Scientists speculate that the vesicles of Rab that reach the Spk derive from the apparatus of Golgi (Pantazopoulou *et al.*, 2014).

Once the vesicles have reached the membrane, they are fused through exocysts. In *A. nidulans*, *Candida. albicans* and other filamentous fungi, fluorescently imprinted exocysts components localized at growth sites, but if there is a tethering mechanism of exocytosis it is little understood (Riquelme *et al.*, 2014). After exocytosis, the last stage of secretory pathway, vesicles fuse with their target membrane by SNAREs interactions. The synaptobrevin vesicular SNARE protein SynA was observed at the Spk of *A. nidulans* and at the apical plasma membrane (Taheri-Talesh *et al.*, 2008).

It seems that exocytosis and endocytosis work in tandem for hyphal morphogenesis (Delgado-Alvarez *et al.*, 2010; Upadhyay and Shaw, 2008). Endocytosis is the result of the extra plasma membrane released by exocytosis (Riquelme *et al.*, 2018).

Structure and Action Mechanism of Small GTPases

Structure

The small GTPases proteins have been studied by crystallographic analysis, and it has emerged that they consist of five fairly conserved domains responsible for binding with GTP, from G1 to G5. G1 motif (I) is a purine nucleotide binding signal; G2 motif (E) is in one of two segments that redirects with GDP or GTP binding function and provides major component of the effector binding surface; G3 motif (II) is involved in Mg^{2+} binding; G4 (III) motif brings the hydrogen bond into contact with the guanine ring; the G5 (IV) motif creates indirect associations with the guanine nucleotide (Wennerberg *et al.*, 2005; Colicelli, 2004; Goitre *et al.*, 2014).

Action Mechanism

Small GTPases perform their physiological activity switching the molecular structure in two forms which support mutual transformation, GTP-binding activated state and GDP-binding non-activated state, which can also be called as "ON" state and "OFF" state, respectively (Jhonson and Chen, 2012).

The multiple biological functions of the small GTPases proteins are mediated through a highly regulated GTP/GDP binding cycle. Three different classes of proteins are required for the regulation: (1) guanine nucleotide exchange factors (GEFs), which stimulate the GTP–GDP exchange reaction; (2) GTPase-activating proteins (GAPs), which stimulate the GTP-hydrolyzing reaction; and (3) guanine nucleotide dissociation inhibitors (GDIs), which antagonize the actions of GEFs and GAPs and regulate the subcellular localization and the cycling of GTPases between membrane and cytosol (Hoffman *et al.*, 2000; Rivero *et al.*, 2002). The active GTP-bound GTPases interact with a myriad of effectors that relay upstream signals, inducing a number of downstream events, including rearrangements of the actin cytoskeleton network and protein kinase-dependent induction of transcription (Hall, 1998; Jaffe and Hall, 2005).

GEFs and GAPs coexist in most cells, increasing the diversity of signals that regulate small GTPases activity (Goitre *et al.*, 2014). Guanine nucleotide dissociation inhibitors (GDIs) are contrary to exchange factors (Menotta *et al.*, 2008). GDI specifically binds GDP-bound GTPase and inhibits GDP release (Rak *et al.*, 2003; Malagnac *et al.*, 2013).

Classification

Ras proteins were the first to be discovered, as their mutation led to various forms of cancer. Subsequently, the researches brought to light several GTPase enzymes with a 3D structure similar to Ras. To date, over 150 members of this family are known, therefore it is counted among the superfamilies.

Ras-like proteins have been named small G proteins. They are divided into five subfamilies, according to their amino acid sequence, their structure and function (Kahn *et al.*, 1992).

In the various organisms these proteins belonging to each subfamily have been extensively studied, and it has been found that they can have overlapping functions. Ras are involved in cell proliferation, Rho in cell morphology, Ran plays a role in nuclear transport and Rab and Arf in vesicular traffic (Goitre *et al.*, 2014).

Ras

Ras superfamily is the largest and most diverse superfamily. In mammals it mainly plays a role in regulating the mechanisms of immunity and inflammation (Colicelli, 2004; Goitre *et al.*, 2014). The GTPases of this group are a general component of the eukaryotic signaling pathway and contribute to the processes of development, proliferation, differentiation and survival of the eukaryotic cell.

It has also been shown that the different isoforms (the highly conserved H-, K- and N-Ras) perform specific biological functions (Haigis *et al.*, 2008; Karnoub and Weinberg, 2008).

Rho

Small GTPases of the Rho family function as molecular switches in separate or correlative signaling pathways that tightly regulate diverse cellular functions (Karnoub *et al.*, 2004). These signaling pathways link the cytoplasmic receptors to activate cytoskeleton reorganization and the subsequent biological effect. They are ubiquitously expressed in eukaryotes and are grouped in three major subfamilies: Rho, Rac, and Cdc42.

The Rho GTPases were thought to be primarily involved in the regulation of cytoskeleton organization (Hall, 1998) required for vesicle trafficking, motility, adhesion and morphogenesis (Kaibuchi *et al.*, 1999; Rivero and Somesh, 2002). Moreover, they play the same significant role in modulating cell polarity, genetic transcription, cell cycle progression, extracellular matrix (ECM) remodeling apoptosis, tumorigenesis (Johnson, 1999; Jaffe and Hall, 2005) and various enzymatic activities (using NADPH oxidase activity to generate the reactive oxygen, ROS) (Li *et al.*, 2014).

Rab

Rab subfamily is the largest subfamily in small GTPase Protein family (Pereira-Leal and Seabra, 2001). In *Arabidopsis* 93 small GTPases family members were detected, and among them 57 belong to Rab subfamily. Rab GTPases modulate membrane traffic processes (vesicle formation, vesicle movement along actin and tubulin networks, membrane fusion). Through these processes the surface proteins are transported from the Golgi apparatus to the plasma membrane and are then recycled.

Over 60 types of Rab GTPases have been found, with the function of stimulating and regulating the docking and fusion of the trafficking vesicles to the membranes; at least one is found for each cytoplasmic organelle and the different membranes have different Rab proteins.

Arf

The Arf protein (ADP ribosylation factor) is the main regulator of the biosynthesis of trafficking vesicle in eukaryotic cells (Donaldson and Jackson, 2011; Ma *et al.*, 2020). It has strong homology with Rab GTPase, but unlike this, which acts in a single step in the transport process to the membrane, Arf acts in several steps. For example, Arf1 protein functions in retrograde transport from Golgi to the ER through recruitment of COPI coated vesicle proteins and in the Trans-Golgi-Network (TNG, ie the formation of vesicles) (Beck *et al.*, 2009); Arf1 also regulates recruitment of clathrin through AP-1, AP-3 and AP-4 complexes (Wennerberg *et al.*, 2005).

Ran

Small GTPase Ran is one of the most expressed in eukaryotes (Moore, 1998). It is involved in nucleocytoplasmic transport, participating both to the import and the export from the nucleus of proteins and RNAs, modulate the formation of cell spindle apparatus, cell cycle progression, structure and function of nucleoplasm, cell redox reaction, RNA synthesis and processing, etc. (Sazer and Dasso, 2000). Nuclear import receptors such as importin beta bind their substrates only in the absence of GTP-bound RAN, forming the tripolymer, and release them upon direct interaction with GTP-bound RAN, while export receptors behave in the opposite way. Thereby, Ran controls cargo loading and release by transport receptors in the proper compartment and ensures the directionality of the transport. Similarly Ran regulates the export of the core (Kim *et al.*, 2001).

Function of Small GTPase in Hyphal Growth

GTPases are molecular switches or timers in many cellular processes (Gilman, 1987). They are involved in signal transduction in response to activation of cell surface receptors, including transmembrane receptors; protein biosynthesis at the ribosome; regulation of cell differentiation, proliferation, division and movement; translocation of proteins through membranes; transport of vesicles within the cell, and vesicle-mediated secretion and uptake, through GTPase control of vesicle coat assembly (Threadgill *et al.*, 1997; Parri and Chiarugi, 2010).

In general, we can summarize from numerous scientific researches, that the different GTPses have the following prevalent roles: Ras sub- family modulation of the gene expression; Rho subfamily regulation of cytoskeleton reorganization, cell wall synthesis,

cell cytokinesis, and MAP kinase cascade pathway; Rab sub-family and Sar/Arf subfamily modulate the trafficking and formation of coated vesicles; Ran subfamily acts in transport into and out of the cell nucleus during interphase and also in mitosis.

Numerous studies have concerned their involvement in hyphal growth in filamentous fungi, in the acquisition of the fundamental polarity for growth directionality and in the underlying cytoskeletal morphological changes.

Ras

Ras GTPases are fundamental for detecting and responding to environmental signals in all eukaryotes (Goitre *et al.*, 2014; Arkowitz and Bassilana, 2015). Their action is expressed through the activation of numerous effectors belonging to kinases pathways (such as the conserved Ras/cAMP/protein kinase A, PKA) (D'Souza and Heitman, 2001; Fimia and Sassone-Corsi, 2001; Gerits *et al.*, 2008). Many studies, using mutant deletion, have shown that Ras plays a key role in hyphal growth and morphogenesis.

S. cerevisiae has two Ras (Ras1 and Ras2), one Rap (Rsr1/Bud1) and one Rheb (Rhb1) ortholog, whereas *Schizosaccharomyces pombe* has only one Ras, one Rheb, but no apparent Rap ortholog.

In particular Ras 2 in *S. cerevisiae* is involved in hyphal growth consequent to starvation (Mösch *et al.*, 1996). Its connection to nutrient availability in fission yeast has been shown also by Chen *et al.* (2019) in 2019. Since the conserved NDR/LATS kinase Orb6 responds to nutritional cues, Orb6 increases the protein levels of a Ras1 GTPase activator, the guanine nucleotide exchange factor Efc25 and regulates Ras1 GTPase activity. These evidences remark the involvement of Ras in promoting cell adaptation, balancing the opposing demands of promoting cell growth and extending chronological lifespan.

In Daniels (2012) it was shown that the formation of the peculiar finger structure, induced in *C. albicans* by the presence of CO_2, is coordinated by Ras 1, and also in *Cryptococcus neoformans* Ras 1 is involved in hypoxia-induced growth (Chang *et al.*, 2014).

In filamentous fungi the role of Ras is crucial for hyphal growth, as reported in literature (Arkowitz and Bassilana, 2015; Kanauchi *et al.*, 1997; Feng *et al.*, 1999; Leberer *et al.*, 2001; Zhu *et al.*, 2009; Huang *et al.*, 2009; Som and Kolaparthi, 1994; Fortwendel *et al.*, 2004; Truesdell *et al.*, 1999; Alspaugh *et al.*, 2000; Waugh *et al.*, 2002; Lee and Kronstad, 2002; Boyce *et al.*, 2005; Bluhm *et al.*, 2007; Zhang *et al.*, 2012a,b; Knabe *et al.*, 2013; Minz Dub *et al.*, 2013).

In filamentous fungi the activation mechanism of Ras, dependent on the GTP/GDP bound cycle, has been studied by means of mutants and the corresponding GEFs (Guanine nucleotide Exchange Factors) and GAPs (GTPase Activating Proteins) have been shed light on.

Numerous studies on the Cdc25 homolog in *Ustilago maydis*, *Yarrowia lipolytica*, *C. albicans*, *A. nidulans* and *Colletotrichum orbiculare* have highlighted its importance in the onset of pathogenesis (in *U. maydis*, Müller *et al.*, 2003), in filamentous growth (*C. albicans*, Shapiro *et al.*, 2009), in the maintenance of polarity (in *A. nidulans* Harispe *et al.*, 2008), in hyphal morphology (*C. orbiculare*, Schubert *et al.*, 2006; Harata and KuboRas, 2014). Therefore Ras, like all GTPases, depends on the GTP/GDP cycling.

GTPase Ras is localized at the level of plasma membrane through the farnesylation of a cysteine in the conserved CAAX tail, in combination with a palmitoylation (Eisenberg *et al.*, 2013; Wright and Philips, 2006). In yeasts and *Candida*, the palmitoylation occurs on a single cysteine residue that allows the membrane anchoring. In filamentous fungi, two conserved palmitoylation cysteines have been identified (Fortwendel *et al.*, 2012; Nichols *et al.*, 2009). Ras localization is spread evenly across the membrane in *C. albicans* and in *Aspergillus fumigatus* (Fortwendel *et al.*, 2012; Piispanen *et al.*, 2011).

The palmitoylation is crucial for several cellular events, e.g. mating in *S. pombe* (Onken *et al.*, 2006), hyphal growth at extreme temperature in *C. neoformans* (Nichols *et al.*, 2009), while in *A. fumigatus* is necessary for hyphal growth, cell wall development, and virulence (Fortwendel *et al.*, 2012). Ras, depending on its location, has specific effectors in the different membrane compartments.

A Ras GTPase associated protein, MadC a GTPase activating protein (GAP), affects the circadian clock output in *N. crassa*, as reported in Polaino *et al.*, 2017, thus it is a target for photoreponses.

Moreover in pathogen filamentous fungi such as *Magnaporthe oryzae* Ras is involved in appressorium formation and in fungal infection. In fact, as shown in Hendy *et al.* 2019, the action of farnesyltransferase β-subunit gene, RAM1, that regulate post-translational farnesylation process can affect the proper localization of many proteins in signal transduction, including Ras, and consequently can modulate hyphal growth and sporulation.

In Martin-Vicente *et al.* 2019, another Ras regulator, a Ras-subfamily-specific guanine nucleotide exchange factors (RasGEFs) in the human pathogen *A. fumigatus*, has shown to affect the properly timed polarity establishment during early growth and branch emergence as well as for cell wall stability, in that it is essential for the integration of multiple signaling networks performed by Ras.

Rho subfamily

Rho

Cdc42 and Rho1 were shown to be required for viability and for cell polarization in *S. cerevisiae* and *S. pombe* (Bi and Park, 2012; Perez and Rincón, 2010; Arkowitz and Bassilana, 2015; Brauns *et al.*, 2020). Moreover Rho1 is involved in cell wall synthesis, in that it modulates glucan synthesis and the specific MAP-kinase pathway.

Among filamentous fungi, Rho 1 is essential for viability and hyphal growth in *C. albicans* and *U. maydis* (Arkowitz and Bassilana, 2019; Wakade *et al.*, 2020; Lu *et al.*, 2014; Corvest *et al.*, 2013; Pham *et al.*, 2012; Smith *et al.*, 2002; Dünkler and Wendland, 2007), while not in *Y. lipolytica* and *Fusarium oxysporum* where it is nonessential (León *et al.*, 2003; Martínez-Rocha *et al.*, 2008). Cell wall

integrity (Arkowitz and Bassilana, 2019; Corvest *et al.*, 2013; Edlind *et al.*, 2005) and virulence (Roemer *et al.*, 2003) of *C. albicans* depends on Rho1; it is the regulatory component of a complex (β-1,3-glucan synthase complex, GSC) responsible of β-1,3-Glucans synthesis (Arkowitz and Bassilana, 2019).

In *U. maydis* the expression alteration of Rho1 determinates growth defects. In Paul *et al.*, 2014 an interaction between Rho1 and an ammonium transporter crucial for hyphal growth regulation has been reported. Studies on *C. neoformans* revealed the presence of three Rho1 homologs, Rho1, Rho10 and Rho11 (Lam *et al.*, 2013), with different roles, such as viability, temperature reactivity and cell wall integrity. Dominant active forms of Rho1 are somewhat defective in capsule formation, suggesting this GTPase may also be important for virulence. Mutations in Rho1 GEF, rom2, exhibited alterations in temperature sensitive growth, actin organization, cell morphology and infectious capacity (Fuchs *et al.*, 2007; Tang *et al.*, 2005).

Conditional rho1 mutants provoke swell at the hyphal tip, apical lyses at the non-permissive temperature, hypersensitivity to cell wall damage and limitation in cell wall integrity MAP kinase signaling in *N. crassa*, (Richthammer *et al.*, 2012). The mutation of GAP and GEF Rho regulators causes similar morphological effects, in addition, a defect in sub-apical branching and sensitivity is observed in GAP mutants if exposed to an actin depolymerization substance (Richthammer *et al.*, 2012; Vogt and Seiler, 2008).

In *A. nidulans* the hydrolysis blockage and the alteration of the GAP domain induces a serious defect in germ tube emergence. In *A. fumigatus*, Rho1 is crucial (Dichtl *et al.*, 2012) and its GEF regulator (Rom2) is fundamental in cell wall integrity (Samantaray *et al.*, 2013). In *Ashbya gossypii*, there are two Rho1 homologs, Rho1a (RhoH) and Rho1b (Rho1): the presence of only one required for viability, but they are both critical for cell wall integrity and remodeling in dimorphic and filamentous fungi (Köhli *et al.*, 2008; Walther and Wendland, 2005; Wendland and Philippsen, 2001). Similar effects are shown in the Tus1 GEF mutant (Lengeler *et al.*, 2013).

In *C. albicans*, *U. maydis*, *A. fumigatus* and *N. crassa*, Rho1 and/or its GEF Rom2 are localized to the hyphal apex (Caballero-Lima *et al.*, 2013; Dichtl *et al.*, 2010; Pham *et al.*, 2012; Richthammer *et al.*, 2012; Samantaray *et al.*, 2013). In *N. crassa* the presence of Rho1 GAP Lrg1 to the hyphal tip is dependent on the growth rate (Vogt and Seiler, 2008).

In *C. albicans* active Rho1 was broadly associated with the hyphal tip, consistent with the Rom2 distribution, and a ~10-fold increase in the levels of active Rho1 was observed at the septum upon cell division (Corvest *et al.*, 2013). In *A. gossypii*, Rho1b was localized predominantly to the hyphal tip, whereas Rho1a was localized to the septum at 25°C (Köhli *et al.*, 2008), while after heat shock conditions both Rho GTPases were localized to the entire hyphal cortex, demonstrating their different roles.

Cdc42

Cdc42 is the main regulator of cell polarity and has been studied in numerous filamentous fungi.

Cdc42 and Rac have similar roles, as shown in *A. nidulans* (ModA and RacA), *N. crassa* (CDC-42 and RAC-1), and *U. maydis* (Cdc42 and Rac1) (Virag *et al.*, 2007; Araujo-Palomares *et al.*, 2011; Lichius *et al.*, 2014; Harris and Momany, 2004; Riquelme *et al.*, 2018) in particular as concern cell polarity machinery. Both anchor the membrane via a typical C-terminal CAAX motif. In *N. crassa*, the spatial distribution of these Rho GTPases changes during development. Cdc42 and Rac regulate the chemotropism exhibited during germ tube development and together with Cdc24, Cdc42, are localized at the apical dome in mature hyphae.

In *Penicillium marneffei* and *C. albicans* Cdc42 is necessary for viability, (Bassilana *et al.*, 2003; Boyce *et al.*, 2003, Boyce *et al.*, 2001; Mahlert *et al.*, 2006; Michel *et al.*, 2002; Ushinsky *et al.*, 2002), as well as cell polarization. The GEF Don1 is critical for cell separation and localizes to endosomal vesicles (Hlubek *et al.*, 2008; Schink and Bölker, 2009; Weinzierl *et al.*, 2002).

In *U. maydis*, Cdc42 is required for virulence and cell separation during budding, yet this protein does not appear to be essential for cell polarity (Mahlert *et al.*, 2006).

Cdc42 has a role in polarized growth in *C. albicans* and strains with reduced levels of Cdc42 arrested growth (Bassilana *et al.*, 2003; Arkowitz and Bassilana, 2015; Ushinsky *et al.*, 2002). Furthermore, Cdc42 and its activator Cdc24 are required for the yeast to hyphal transition, tropic responses and virulence (Bassilana *et al.*, 2003; Brand *et al.*, 2008; VandenBerg *et al.*, 2004), and the level of active Cdc42 is critical for the initiation and maintenance of hyphal growth (Bassilana *et al.*, 2005), as shown in Corvest *et al.* 2013. Also the localization of its GAP is important for maintaining active Cdc42 at apical level (Zheng *et al.*, 2007a,b).

In *A. nidulans* functional analysis of the homologs of the yeast GEF Cdc24 and the yeast GAP Rga1 shown that Cdc24 is important for the establishment of hyphal polarity and localizes to hyphal tips, and that Rga1 is necessary for the suppression of branching in developing conidiophores.

Results showed in Menotta *et al.*, 2007 suggest a fundamental role of Cdc42 in cell polarity development in *Tuber borchii* Vittad. Immunolocalization experiments revealed an accumulation of Cdc42 in the apical tips of the growing hyphae, and very interestingly, the expression of the constitutively active TbCdc42 Q63L transformed in yeast cells switched on a series of evident morphological modifications, since elongated shape, and giant cells and cell aggregations were present.

Moreover, in a few fungi, this highly conserved small GTPase also plays roles in cell separation and/or cytokinesis, which may reflect its importance in septin-dependent processes. In fact, in yeast the ring diameter is set through the dynamic interplay of septin recruitment and Cdc42 polarization, establishing it as a model for size homeostasis of self-assembling organelles (Kukhtevich *et al.*, 2020).

Also in *C. neoformans* Cdc42 is necessary for septin localization (Ballou *et al.*, 2010).

In *A. gossypii*, Cdc42 is essential for spore germination (Wendland and Philippsen, 2001).

In contrast, Cdc42 is not required for viability in other filamentous fungi, e.g. *N. crassa*, *A. nidulans*, *A. niger*, *Magnaporthe grisea* and *Claviceps purpurea*. In conditional cdc42 mutants *N. crassa* hyphae grew in an uncoordinated, zig-zag fashion (Seiler and Plamann, 2003), were distorted (loss of polarity) and periodic reinitiation of growth at the swollen tips were visible

(Araujo-Palomares *et al.*, 2011). Moreover cdc24 mutants exhibited increased tip branching and bem1 mutants resulted in chains of spherical cells (Seiler and Plamann, 2003), even if subsequent studies concluded that bem1 is not critical for hyphal polarity establishment in *N. crassa* (Schürg *et al.*, 2012).

Cdc42 was found to be important also for hyphal branching in *A. nidulans*, *Curvularia trifolii* and *Schizophyllum commune* (Chen *et al.*, 2006; Virag *et al.*, 2007; Weber *et al.*, 2005; Si *et al.*, 2016). In *A. niger* a reduced and delayed germ tube formation was observed in the cdc42Δ mutant, while no significant effects were observed in hyphal morphology (Kwon *et al.*, 2011). It has been reported a role in virulence mechanisms in *M. grisea*, where cdc42 is probably involved in appressorium formation (Zheng *et al.*, 2009; Dagdas *et al.*, 2012). In the plant pathogen *C. purpurea*, Cdc42 was found to be important for pathogenicity, branching and conidation (Scheffer *et al.*, 2005).

In some filamentous fungi Cdc42 is important for mycorrhiza development, as shown in Menotta *et al.*, 2007, where RT-real-time PCR analyses revealed an increased expression of Tb*cdc42* during the phase preparative to the instauration of symbiosis, in particular after stimulation with root exudate extracts.

From research carried out on filamentous fungi, it is shown that in most cases cdc42 has a fundamental role in hyphal growth and hyphal branching.

As concern Cdc42 localization, in *C. albicans* this GTPase is located at apical tip level (Bassilana and Arkowitz, 2006; Crampin *et al.*, 2005; Hazan and Liu, 2002) in F-actin dependent way (Hazan and Liu, 2002), as well as its coordinators Cdc24 and Bem1 (Bassilana *et al.*, 2005; Pulver *et al.*, 2013). The GAP Bem3 localized to a diffuse patch at the hyphal tip, which sometimes appeared as a ring, whereas the GAP Rga2 was largely cytoplasmic (Court and Sudbery, 2007; Zheng *et al.*, 2007a,b). Also in *A. gossypii*, *N. crassa*, *C. purpurea* and *S. commune* Cdc42 is visualized a cortical cap or crescent at the hyphal tip (Araujo-Palomares *et al.*, 2011, Herrmann *et al.*, 2014; Köhli *et al.*, 2008; Weber *et al.*, 2005). Cdc24 and Bem1 are located at the hyphal tip in *A. gossypii* and in *Epichloë festucae*; while in *N. crassa* Cdc24 and Bem1 localized similarly to the hyphal tip (Köhli *et al.*, 2008; Takemoto *et al.*, 2011) and they appear more broadly distributed (Araujo-Palomares *et al.*, 2011; Schürg *et al.*, 2012). While Cdc42 was observed at the hyphal septa, active Cdc42 was not detected at this location (Corvest *et al.*, 2013; Hazan and Liu, 2002).

Rac

The Rac small GTPase is highly homologous to Cdc42, has similar functions but also specific roles. In *P. marneffei* and *C. albicans*, Rac1 is not essential for viability but is important for filamentous growth (Bassilana and Arkowitz, 2006; Boyce *et al.*, 2003; Hurtado *et al.*, 2000). In *P. marneffei*, a rac1 deletion strain produced aerial hyphae, extensive apical branching and altered actin cytoskeleton (Boyce *et al.*, 2003). In *C. albicans*, it seems that the lacking of active rac1 does not cause an alteration in the actin cytoskeleton, but can compromise the invasive growth (Bassilana and Arkowitz, 2006). Mahlert *et al.* (2006) reported that Rac1 is not essential in *U. maydis*, but has a role in cell morphology and hyphal growth.

Two Rac paralogs (Rac1 and Rac2) were found in *C. neoformans* (Ballou *et al.*, 2013). Defective Rac1 impaired completely the hyphal growth (Vallim *et al.*, 2005), but Rac1 nor Rac2 were necessary for virulence in an inhalation model of cryptococcosis (Ballou *et al.*, 2013; Vallim *et al.*, 2005). Rac has a less important role in hyphal branching while it is fundamental in polarized growth during response to specific inducers.

Rac mutants generated hyperbranching at apical tip in *N. crassa*. Rac and Cdc42 together were found to be necessary for polarity establishment and maintenance (Araujo-Palomares *et al.*, 2011).

Rac alone is not essential for viability in the three *Aspergillus* species, *A. fumigatus*, *A. nidulans* and *A. niger*, (Kwon *et al.*, 2011; Virag *et al.*, 2007; Si *et al.*, 2016), but double rac, cdc42 mutants were inviable in *A. nidulans* and *A. niger* (Kwon *et al.*, 2011; Virag *et al.*, 2007). In *A. fumigatus* and *Aspergillus niger* rac deletion mutants exhibited multiple axes of polarity, with increased apical branching (Kwon *et al.*, 2011).

In *M. grisea*, rac1 defect was compatible with life, while conidial and appressorium development was repressed (Chen *et al.*, 2008). In *C. purpurea*, hyphae of both rac and cla4 mutants were shorter and wider than wild-type cells and exhibited hyper-branching (Rolke and Tudzynski, 2008).

The p21-activated kinase Cla4 is an important effector of Rac in fungi (Cotteret and Chernoff, 2002; Boyce and Andrianopoulos, 2011; Bustelo *et al.*, 2007). BcRac, the Rac homolog of the gray mold fungus *Botrytis cinerea*, was found to have Cla4 as effector; BcCla4 protein was found to mediate all the Rac driven processes, including hyphal growth and morphogenesis, conidia production and pathogenicity (Minz-Dub and Sharon, 2017).

In *Trichoderma reesei* Rac1 defect induces hyperbranching, apolar growth and impacts on cellulase activity (Fitz *et al.*, 2019).

Therefore, in filamentous fungi, Rac is central for filament branching.

As regards the localization in *P. marneffei* Rac1 it is found at the level of the hyphal apices and in the division sites, while in *C. albicans* it is widespread in the cell cytoplasm. Instead Rac1 was observed in the nucleus in *C. albicans* (Bassilana and Arkowitz, 2006; Boyce *et al.*, 2003; Vauchelles *et al.*, 2010). The two forms of rac present in *C. neoformans*, on the other hand, are localized at the membrane level, both cellular and organellar (Ballou *et al.*, 2013). The two forms of Rac in *N. crassa* are located in a differentiated way under the apical region, reflecting the different functions attributed to them (Araujo-Palomares *et al.*, 2011).

Rab

Rab GTPases are the largest group of the small GTPases family, plays a pivotal role in the secretion of proteins and serve as regulators of the intracellular membrane trafficking system (Mizuno-Yamasaki *et al.*, 2012; Li and Marlin, 2015; Pfeffer, 2017).

Fungal hyphae extend by apical growth and this process requires continuous polarized trafficking of secretory vesicles to a special structure called Spitzenkörper (SPK).

Rab family orchestrates the vesicle secretion in all eukaryotic cells, in coordination with protein coats, molecular motors, tethering factors and SNAREs (Grosshans *et al.*, 2006). Rab proteins are as signaling molecules, safeguarding the arrival of vesicles to the specific domains (Jin *et al.*, 2011; Hervé and Bourmeyster, 2018).

In addition, in *N.crassa* YPT-1 has been shown to be involved in the CHS traffic activity through tests of immunoprecipitation followed by mass spectrometry that supposed YPT-1 is one of the CHS-1, CHS-4 and CHS-5 interacting proteins (Fajardo-Somera *et al.*, 2015) and through characterization by density gradient centrifugation (Verdín *et al.*, 2015). Benli *et al.* (1996) indicated Ypt31 and Ypt32 (Rab11 orthologs) in *S. cerevisiae* as helpers of vesicular carriers.

RabE, orthologue of Rab11, in *A. nidulans* has been seen to play a role in the transformation of the Golgi's cisternae into post-Golgi (Pantazopoulou *et al.*, 2014). Sánchez-León *et al.* (2015) have found the different localization of the homologs of *S. cerevisiae* YPT-1 (Rab1) and YPT-31 (Rab11) in *N. crassa*: laser scanning confocal microscopy showed that YPT-1 occupied the Spk microvesicular core and YPT-31 was at the macrovesicular layer of the Spk. This research confirmed that distinct Rabs participate in micro and macro vesicles pathway.

Other studies suggested that YPT-31 is not significant for hyphal growth, because through FRAP analysis, the YPT-31 levels in some fungal systems are similar in *N. crassa* where the growth was lower (Jones and Sudbery, 2010; Pantazopoulou *et al.*, 2014).

With quantitative superresolution localization microscopy of live *A. nidulans* cells it has discovered that chitin synthase ChsB, located in Spk, is transported with two different speeds for hyphal growth (Zhou *et al.*, 2018). These findings provide the characteristic intermittent cell growth and shed light on how downstream regulators coordinate the vesicles release. In *S. cerevisiae*, Sec4p is the Rab8 homolog that is involved in the last anterograde vesicles transport (Walworth *et al.*, 1992).

Sec4p, as YPT-31, GS-1 and FKS-1, in *N. crassa* is allocated at the marginal layer of the Spk (Sánchez-León *et al.*, 2015). Sec4p associates with Sec15p and this interaction can control the exocyst assembling (Guo *et al.*, 1999). But the location seen by Sánchez-León *et al* in 2015, suggests that Sec4p can also takes part in the transport of GSC (Sánchez-León and Riquelme, 2015).

Nevertheless, further investigation is necessary to explain the role of Sec4p for GSC and especially how the different Rabs come into play in the pre-exocitary phase.

Arf

In all eukaryotes, membrane/protein trafficking to the plasma membrane is mediated by vesicular transport between different cellular compartments (Donaldson and Jackson, 2011). The small GTPases of the Arf (ADP-ribosylation factor) family guide membrane/protein trafficking. In *S. cerevisiae* has been describing the processes controlled by the ADP ribosylation factors of the Arf/Sar family, in particular vesicle formation and trafficking, cytoskeletal rearrangements, cell polarity and budding (Roth, 1999; Lambert *et al.*, 2007; Suda *et al.*, 2018). *S. cerevisiae* Arf family includes seven members: Arf1, Arf2, Arf3, Arl1, Arl3, Cin4, and Sar1. Ar1 and Arf2 together with Ar11 have regulating role in the secretory pathway. Specifically Arf1/2 are involved in the formation of COPI vesicles and clathrin coated vesicles at cis and trans Golgi cisternae, respectively (Roth, 1999; Suda *et al.*, 2018).

In contrast to *S. cerevisiae*, there are still unknown aspects of Arf/Arl proteins in filamentous fungi. Labbaoui *et al.* (2017) investigated Arf/Arl proteins in *C. albicans* and they identified Arf2 and Arl1 as key regulators of membrane traffic, critical for this fungal hyphal growth and virulence. However ArfB in *A. nidulans* is the *S. cerevisiae* Arf3 homolog, and it is involved in polarized growth and endocytosis (Lee *et al.*, 2008), also as its homolog in *M. oryzae*, Arf6, during asexual development (Labbaoui *et al.*, 2017). Also within the Nematode-trapping (NT) fungus *Arthrobotrys oligospora* was found an ortholog of Arf-GAP of *S. cerevisiae*, Aoglo3, to be involved within the regulation of multiple cellular processes such as mycelial growth, conidiation, environmental adaption, endocytosis and pathogenicity. Moreover, the deletion of Aoglo3 gene, in particular, shown growth defects, a rise in hyphal septation and the Aoglo3 mutant sporulation capacity decreased (Ma *et al.*, 2020).

Fiedler *et al.* (2018) characterized ArfA in *A. niger* and demonstrated that it impacts fungal growth rates, hyphal tip morphology and protein secretion. Subcellular localization experiments of fluorescently labeled proteins associated with cytoskeletal elements provided evidence that the position of the endocytic actin ring is impacted by both lowered and elevated levels of ArfA expression, and ArfA secretion at septa level was hypothesized. Fluorescent microscopy shown that the position of the actin ring at the hyphal tip is affected by the ArfA expression in *A. niger*.

Moreover it was reported in yeast, that Arf3 regulates the Bud1 GTPase activating protein Bud2p and the GTP/GDP exchange factor Bud5; since Bud1 affects Cdc24, and subsequently Cdc42, Arf3 is upstream an important hierarchical GTPase cascade that regulate actin dynamics necessary for polar growth (Hsu and Lee, 2013).

The actin ring is the site of endocytosis, and the location of this cytoskeletal apparatus at the hyphal tip is vital for polar growth (Taheri-Talesh *et al.*, 2008). In *A. nidulans*, the actin ring is maintained precisely 1–2 μm behind the hyphal apex, even in rapidly growing hyphae (Taheri-Talesh *et al.*, 2008). It is seen that the *A. niger* glucoamylase might not only be secreted at the hyphal tip, but also at hyphal septa as previously suspected (Gordon *et al.*, 2000). This is consistent with studies using *A. oryzae*, where the major extracellular protein alpha-amylase was observed to localize in the space between the plasma membrane and cell wall at septa [i.e., the septal periplasm (Hayakawa *et al.*, 2011)]. Septal exocytosis is required for secondary cell wall thickening, intercalary hyphal growth, and branch initiation in filamentous fungi (Hayakawa *et al.*, 2011; Read, 2011). Therefore, ArfA is also required for normal exocytic and/or endocytic processes at the hyphal septum.

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Membrane Transporters, an Overview of the Arbuscular Mycorrhizal Fungal Transportome

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Introduction

Membrane transport in fungi contributes to key aspects of their growth and development, and to their adaptation to multiple ever-changing environments. These transport systems, consisting of one or more proteins embedded in the cell membranes, enable the cell to ensure the uptake of essential nutrients and the efflux of toxic compounds, and play key roles in ion homeostasis and cell signaling. Transport proteins can be simple channels or pores created in the membrane, that facilitate diffusion of compounds down their concentration gradient, or active transporters that require metabolic energy to drive the transport of solutes against their concentration gradient (Busch and Saier, 2004).

One of the main changing extracellular conditions is nutrient availability. Adaptations to these conditions require nutrient-sensing mechanisms in order to supply specific nutrients and to adapt cellular metabolism, growth and development accordingly. One fundamental mechanism for this adaptation is regulated transport across cell membranes through the coordinated uptake or efflux of nutrients across the plasma membrane and intracellular membranes. Recently, the identification of transceptors, membrane proteins that act both as transporters and receptors, establishes a link between transport and signaling (Steyfkens *et al.*, 2018).

Membrane transport has been a main topic of research during the last century and remains an active field of study. Among fungi, most research has been performed in *Saccharomyces cerevisiae*. Even before the emergence of high-throughput genome sequencing techniques, genes encoding transport proteins of this model yeast were cloned and characterized. Progression into the genomics era led to rapid advances in the catalog of available fungal genomes for mining and *in silico* predictions of all membrane transporters of a fungal species (De Hertogh *et al.*, 2002; Grigoriev *et al.*, 2014). It is now known that approximately 10% of a yeast genome corresponds to membrane transporters (De Hertogh *et al.*, 2006) and it is firmly established that many of these transport proteins belong to protein families conserved in organisms ranging from bacteria to humans (André, 1995). The important transport processes operating in several fungal species have been described (Benito *et al.*, 2011; Hora'k, 2013; Dutta and Fliegel, 2018) and excellent comprehensive reviews have summarized the yeast transport processes (Conrad *et al.*, 2014; Ramos *et al.*, 2016). For a detailed description of the membrane transport proteins readers are referred to the Transporter Classification Database (TCDB) webpage (See Relevant Websites Section). This chapter summarizes the general types of transport processes that enable translocation of substrates across fungal membranes and provides an overview of the transport systems characterized so far in arbuscular mycorrhizal (AM) fungi, the most ancient and widespread fungal plant symbionts (Smith and Read, 2008). AM fungi are obligate biotrophs that enhance host plant nutrient acquisition in exchange for carbon compounds. This requires an efficient nutrient transport system in which the transporters are key actors.

Types of Transport Processes

Transport of solutes across biological membranes may occur via simple diffusion, facilitated diffusion or active transport (**Fig. 1**).

Simple Diffusion

Simple diffusion is a passive mechanism that does not involve the input of energy by the fungus to accomplish the movement of gases or uncharged small molecules, such as ethanol, across the membrane lipids. Simple diffusion can be affected the plasma membrane composition and proton motive force, but specific transporters are not usually involved.

Facilitated Diffusion

In facilitated diffusion solutes are transported across the membrane along a concentration with the assistance from membrane proteins, such as channels or transporters (also named facilitators, carriers, or permeases). No metabolic energy is expended since this transport process is governed by the concentrations of the molecule of interest on either side of the membrane. If the concentration on one side of the membrane barrier is higher than on the other side, the movement of molecules through the connecting channel or facilitator will naturally occur, in order to balance the concentrations on both sides of the membrane (Eddy and Barnett, 2007).

Channels

Membrane channels are complexes of membrane proteins or peptides that mediate passive transport of solutes by forming an aqueous diffusion pore. They can operate by different mechanisms, being the most common the gated channel, which requires a

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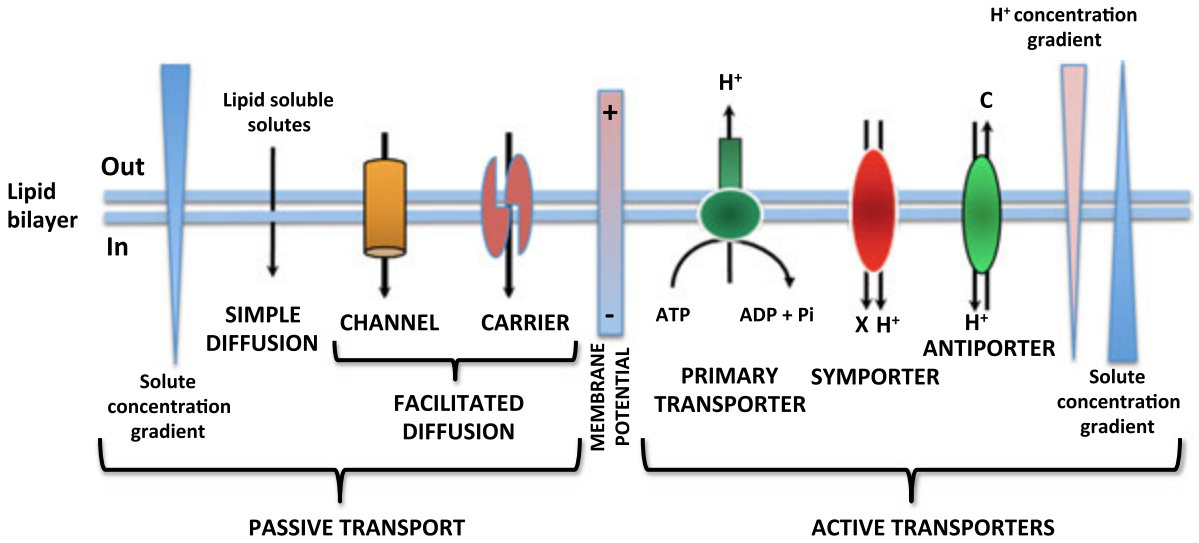

Fig. 1 Types of membrane transport processes. Passive transport processes do not require energy input. Lipid soluble solutes enter the cells via simple diffusion. In facilitated diffusion solutes are transported along a concentration gradient through channels or carriers. Solutes moving against a concentration gradient are transported at the expense of metabolic energy. Primary transporters couple ATP hydrolysis to the transport of a solute against its concentration. The plasma membrane H^+-ATPase generates the proton motive force required for the activity of the secondary transporters. Symporters transport two solutes in the same direction and antiporters in the opposite direction. X (anion or a solute moved against its concentration), C (cation).

trigger, such as a change in membrane potential in voltage-gated channels, to unlock or lock the pore opening. Ion channels play a key role in cation homeostasis, being the best characterized the *S. cerevisiae* efflux voltage-gated K^+ channel Tok1, which is activated by membrane depolarization (Ketchum *et al.*, 1995).

Aquaporins, belonging to the family of major intrinsic proteins, also form pores in the membrane facilitating mainly the transport of water into and out of the cell. Aquaporins form tetramers in the cell membrane, with each of the four monomers acting as a water channel. The driving force for water movement is the gradient of chemical potential of water (osmotic and/or hydrostatic pressure) between both sides of the membrane and predicted to occur in either direction (Finkelstein, 1984). In the fungal kingdom, there are five groups of fungal aquaporins, with two groups of classical aquaporins and three groups of aquaglyceroporins. Although water is the main substrate transported by aquaporin, they can transport other substrates, such as glycerol, H_2O_2, NH_4^+, boron, urea, and CO_2 (Sabir *et al.*, 2016).

Another important family of channels is the ammonium channel transporter (AMT) family. AMT proteins are homotrimers, in which each subunit contains a narrow pore through which substrate transport occurs. As expected for a channel, NH_3 uniport appears to occur by energy-independent, non-concentrative, bidirectional diffusion (Loque *et al.*, 2007), but NH_4^+ may be the true substrate requiring in this case metabolic energy (Fong *et al.*, 2007). AMT proteins appear to function as channel/carrier hybrids. *S. cerevisiae* has three AMT homologs named Mep1, Mep2, and Mep 3, that also transport methilammonium and present different affinities for ammonium. Mep2 has been shown to function both as a transporter and as a sensor, generating a signal that regulates filamentous growth in response to ammonium starvation (Lorenz and Heitman, 1998).

Copper (Cu^+) transporters of the Ctr family also function by a channel mechanism that mediate Cu^+ uptake by a passive, membrane potential-dependent mechanism (Dumay *et al.*, 2006). They function as trimmers forming a channel in the membrane. Three homologs have been described in *S. cerevisiae*, two located in the plasma membrane (Ctr1 and Ctr3) and one in the vacuolar membrane (Ctr2) (Puig and Thiele, 2002). However, multiple homologs have been identified in *Schizosaccharomyces pombe* (Beaudoin *et al.*, 2013).

Transporters or carriers

In contrast to a channel, a transporter is assumed to transfer the solute across the membrane by undergoing reversible conformational changes that expose its solute-binding site alternately on each side of the membrane. For example, in *S. cerevisiae* hexoses are taken up by facilitated diffusion through hexose transporters. The high rates of phosphorylation of hexoses together with its subsequent metabolism provide the driving force for continued translocation into the cell. The *S. cerevisiae* hexose transporter family is comprised of 17 members, with different substrate affinities for glucose (Leandro *et al.*, 2009). Given that facilitated diffusion systems operate optimally around their substrate affinity value or Km, it is believed that the wide range of substrate affinities of the *S. cerevisiae* hexose transporters (ranging from 1 to 100 mM) will enable uptake of glucose and other hexoses across the wide range of hexose concentrations present in the yeast environment. However, in other organisms uptake of hexoses requires metabolic energy, as it is presented below for the AM fungal hexose RiMST2 (Helber *et al.*, 2011).

Active Transport

Active transport is the process of moving solutes across a membrane against a concentration gradient and requires metabolic energy. There are two types of active transporters, primary transporters that use adenosine triphosphate (ATP), as a source of energy and secondary transporters that use an electrochemical gradient.

Primary active transporters

Primary transporters are integral membrane proteins that couple ATP hydrolysis to the transport of a solute against its concentration. These transporters play a key role in the generation of gradients of protons and cations across the membranes and in the detoxification of toxic compounds. There are four types of primary transporters: P-type ATPases, V-ATPases, F-ATPases, and ABC transporters.

P-type ATPases

P-type ATPases are transmembrane proteins that couple ATP hydrolysis to the efflux of a cation out of the cytosol. They function as pumps for various cations (H^+, Ca^{2+}, $Cu^{+/2+}$, Zn^{2+}) across the plasma or intracellular membranes. The plasma membrane H^+-ATPase is by far the most extensively studied ATP-driven transport system in yeast (Serrano *et al.*, 1986) and in many other fungi (Ghislain and Goffeau, 1991; Kühlbrandt *et al.*, 2002). Its primary function is to provide an energy source for the transport of nutrients into the cell. The yeast PMA1 H^+-ATPase is an electrogenic enzyme since it extrudes positive charges forming a membrane potential (negative on the inside) that serves as a source of energy for the activity of secondary active transporters (Barnett, 2008).

A sub-family of P-type ATPases comprises the Ca^{2+}-transporting ATPases, which in *S. cerevisiae* play a key role in calcium storage in the vacuoles and in the secretory compartments of the endoplasmic reticulum and Golgi through the activity of the Pmc1 and Pmr1 Ca^{2+}-ATPases, respectively (Cunningham and Fink, 1994). A second sub-family of P-type ATPases is the P_{1B}-type ATPases also known as Heavy Metal ATPases that pump metals across membranes against their electrochemical gradient. The best-characterized member of this sub-family is the product of the *CCC2* gene that is required for exporting Cu^{2+} from the cytosol to the secretory pathway (Yuan *et al.*, 1995).

V-ATPases

V-ATPases consist of peripheral and integral membrane subunits. They are proton pumps that acidify organelles, such as vacuole, lysosomes, endosomes, and Golgi (Kane, 2006; Forgac, 2007). Together with the plasma membrane H^+-ATPase plays a key role in fungal pH and in the generation of the proton gradients that serve as a source of energy for secondary transporters.

F-type ATPases

F-type ATPases are located in the mitochondria and function mainly as an ATP synthase utilizing ADP, inorganic phosphate and an electrochemical gradient of protons.

ABC (ATP binding cassette) transporters

Members of the ATP-binding cassette (ABC) superfamily catalyze the ATP-dependent transport of chemically diverse compounds across the plasma membrane or intracellular membranes. They play key roles in the efflux of xenobiotic compounds, physiological substrates, and toxic intracellular metabolites. The yeast genome contains 30 ABC proteins that are classified in different sub-families (Paumi *et al.*, 2009). The best-characterized yeast ABC transporter is the vacuolar yeast cadmium factor that transports glutathione-complexes to the vacuole and plays a role in detoxifying metals (Li *et al.*, 1996).

Secondary transporters

Antiporters

An antiporter is a membrane protein that transports two molecules at the same time in the opposite direction. Usually the transport of one ion or molecule is against its electrochemical gradient and the movement is powered by the free energy stored in the proton gradient generated by the plasma membrane H^+-ATPase or the vacuolar ATPase. A primary example of this type of transporters is the family of Na^+/H^+ antiporters or Na^+/H^+ exchangers (NHEs), proteins essential to keep the Na concentrations low in the cytosol (Dutta and Fliegel, 2018). For example, the genome of *S. cerevisiae* encodes three Na^+/H^+ antiporters: the plasma membrane Nha1 that has similar affinity for Na^+ and K^+ (Bañuelos *et al.*, 1998), Nhx1 that is the main proton-coupled antiporter mediating potassium or sodium transport across the vacuolar membrane (Cagnac *et al.*, 2007) and ScKha1p that is localized in the Golgi (Maresova and Sychrova, 2005).

Two other examples of antiporters are the family of calcium proton exchangers that play a key role in calcium homeostasis (Cunningham and Fink, 1996) and the cation diffusion facilitator (CDF) family that function by H^+ antiport for metal efflux (Montanini *et al.*, 2007). Members of the CDF family transport heavy metals including cobalt, cadmium, iron, zinc and possibly nickel, copper and mercuric ions and are involved in metal tolerance/resistance by efflux (Paulsen and Saier, 1997).

Symporters

Symporters are proteins that simultaneously transport two molecules across a membrane in the same direction. The most widely held model for this process has the molecules binding to the transport protein that is exposed on the external surface of the

membrane. In an energy-dependent process, these molecules are driven through a central region of the protein to emerge on the opposite side of the membrane. The protein molecule remains stationary. Examples of symporters are the family of sulfate transporters that mediate transport of SO_4^{-2} by using the electrochemical gradient of protons (Cherest *et al.*, 1997) and the Pho family of phosphate transporters that also use the proton gradient as a driven force for translocation of phosphate ions in a symport manner (Bun-ya *et al.*, 1991). The potassium transporters of the Trk family normally function also as H^+/K^+ or Na^+/K^+ symporters and are thought to be driven by the membrane potential created by the plasma membrane H^+-ATPase. Fungi usually have two Trk systems (Corratgé-Faillie *et al.*, 2010). In *S. cerevisiae*, Trk1 and Trk2 strongly differ in their affinity for potassium, being Trk1 and high-affinity K transporter and Trk2 a low-affinity one (Ko and Gaber, 1991).

Amino acid transport is also a primary example of a symport transport process since amino acids are generally present in the natural environment at concentrations far lower than those found in the cytoplasm. Amino acids are, therefore, taken up against its concentration gradient by members of the amino acid transporter (AAT) family (Gournas *et al.*, 2018). Members of the proton-dependent oligopeptide transporter (POT/PRT) family also function by a proton symport process (Hauser *et al.*, 2001).

The mechanism of transport of several secondary transporters has not been determined yet. This is the case of the members of the Zinc (Zn^{2+})-Iron (Fe^{2+}) permease (ZIP) family (Gaither and Eide, 2001). The energy source for metal translocation is not ATP and several driving forces, including bicarbonate, pH dependence or phosphorylation, have been proposed.

Why Arbuscular Mycorrhizal Fungi?

Arbuscular Mycorrhizal (AM) fungi, belonging to the subphylum Glomeromycotina within the Mucoromycota (Spatafora *et al.*, 2016), are complex but extremely successful soil-borne microorganisms. They are obligate biotrophs that establish a compatible interaction with plants, called arbuscular mycorrhiza, by either avoiding or suppressing plant defense reactions while redirecting host metabolic flow to their benefit without being detrimental to their host (Gianinazzi-Pearson, 1996). AM fungi have accompanied land plants through evolution and survived across periods of important environmental change to become ecologically and agriculturally important symbionts that improve overall fitness of most land plants (Smith and Read, 2008). Substantial evidence supports their use as natural biofertilizers and bioprotectors in agriculture (Gianinazzi *et al.*, 2010; Berruti *et al.*, 2016).

The main benefit for the plant is an improved mineral nutrition, especially of phosphorus, nitrogen, zinc and copper. The fungus develops in the soil an extensive network of extraradical hyphae that overgrows the soil surrounding the plant roots and functions as an additional absorptive surface area for the plant increasing its capacity to forage nutrients beyond their depletion zone (**Fig. 2(A)** and **(B)**). In return, the plant provides to the fungus the carbon compounds, either in the form of carbohydrates or lipids, required to complete its life cycle. This nutrient exchange between partners occurs in the arbuscules, specialized and highly branched structures formed by the fungus in the root cortical cells (**Fig. 2(C)**). AM fungal nutrient uptake from the soil and coordinated exchanges between the plant and the fungus at the symbiotic interface formed in the cortical cells colonized by arbuscules is controlled by the coordinated regulation of specialized plant and fungal membrane transport systems. Despite nutrient exchange between partners and nutrients themselves seem to be at the heart of the evolutionary success of this symbiosis, the mechanisms underlying these processes are not yet fully understood. So far, molecular players controlling nutrient transport processes in the symbiosis have been mainly identified and characterized on the plant side (Wipf *et al.*, 2019). However, much less is known on the fungal side.

AM fungi are obligate biotrophs incalcitrant to pure culture in the absence of a host plant, possess coenocytic hyphae and multinucleated spores and so far no sexual reproduction has been described yet (Gianinazzi-Pearson *et al.*, 2012). These characteristics introduce inherent limitations in the application of standard techniques, such as genetic transformation and mutant generation/characterization, for their study, which greatly hinders advances in the knowledge about gene function in these crucial group of fungi. Nevertheless, significant progress has been made recently in our understanding of their biology through the release and subsequent mining of genome sequences. *Rhizophagus irregularis* (formerly named *Glomus intraradices*)

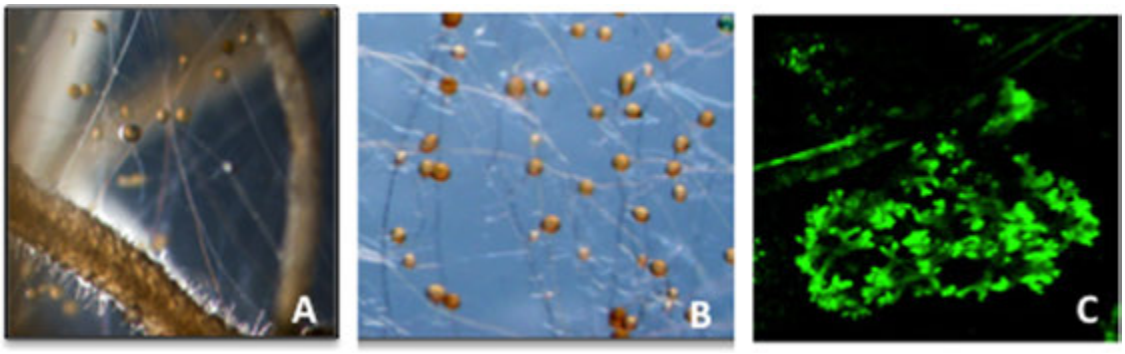

Fig. 2 (A) *In vitro* culture of *R. irregularis* in association with carrot roots. (B) Extraradical mycelium of *R. irregularis* showing hyphae and spores. (C) Arbuscule of *R. irregularis* detected by wheat germ agglutinin-FITC labeling on a root section.

DAOM197198 was the species chosen for the first genome-sequencing project on AM fungi (Tisserant *et al.*, 2012). Nowadays, the genomes or transcriptomes of several AM fungi, such as *Rhizophagus clarum* (Kobayashi *et al.*, 2018), *Diversispora epigaea* (formerly *Glomus versiforme*; Sun *et al.*, 2019), *Gigaspora margarita* (Salvioli *et al.*, 2016), and *Gigaspora rosea* (Tang *et al.*, 2016) have been sequenced and annotated. These gene repertoires have enabled the identification of genes potentially encoding for transport proteins in different AM fungal species.

AM Fungal Transportome

Current knowledge on the transportome in AM fungi, that is, the complete repertoire of fungal genes encoding membrane transporters is still low. Much of the information generated on the Glomeromycotina transportome focuses on the model species *R. irregularis*.

Primary Active Transporters

P-type ATPases

As mentioned above, P-ATPases are enzymes that couple ATP hydrolysis to the transport of a cation out of the cytosol, being the plasma membrane H^+-ATPase an essential protein for the functioning of the secondary transporters. In the Glomeromycotina, two H^+-ATPases genes have been characterized in the AM fungus *Funneliformis mosseae* (formerly, *Glomus mosseae*) (Ferrol *et al.*, 2000; Requena *et al.*, 2003). Both genes are differentially expressed in the different developmental stages of the fungus and are more highly expressed in the intraradical than in the extraradical mycelium. Expression of *GmHA5* is up-regulated by phosphate, the main nutrient transferred by the fungus to the plant, suggesting that this isoenzyme provides the proton motive force required for phosphate uptake by a symport transport process. Interestingly, *GmHA5* was found to be highly expressed in the arbuscules (Balestrini *et al.*, 2007). As found for *F. mosseae*, *R. irregularis* also expresses two plasma membrane H^+-ATPase genes (Tisserant *et al.*, 2012). A total of 35 genes putatively encoding P-type ATPases were identified in the *R. irregularis* transcriptome, ten potentially encoding Ca-ATPases likely involved in Ca homeostasis and four encoding heavy metal ATPases likely involved in copper/metal homeostasis (Tamayo *et al.*, 2014).

ABC transporters

The first ABC transporter described in the Glomeromycotina was GintABC1, an ortholog of the yeast cadmium factor (ycf1) that transports metal-glutathione complexes into the vacuoles and that was suggested to play a role Cu and Cd tolerance in *R. irregularis* (González-Guerrero *et al.*, 2010). Thirty-one more genes putatively encoding ABC transporters were identified in the *R. irregularis* transcriptome. They include ABC multidrug transporters, multidrug resistance-associated proteins, MRP-like ABC transporters and oligomycin resistance ATP-dependent permeases.

Transporters of Macronutrient Ions

Phosphate transporters

Improved phosphorus uptake is the main benefit of the AM symbiosis for the host plant. The first step of symbiotic phosphorus uptake is phosphate uptake from the soil by the extraradical mycelium, which is believed to occur through by a phosphate/proton symporter of the *S. cerevisiae* Pho84 family. Phosphate transporters have been described in *D. epigaea* (Harrison and van Buuren, 1995), *F. mosseae* (Benedetto *et al.*, 2005), *R. irregularis* (Maldonado-Mendoza *et al.*, 2001) and *G. margarita* (Xie *et al.*, 2016). These transporters are expressed in the extraradical mycelium, suggesting a role in phosphate uptake from the soil. However, this hypothesis has not been proven due to the lack of stable transformation systems for AM fungi. By using yeast as a heterologous expression system, it was shown that the *D. epigaea* and G. *margarita* phosphate transporters *GvPT* and *GigmPT* encode high-affinity transporters that are dependent on the metabolic energy generated by the H^+-ATPase. These transporters are also expressed in the intraradical mycelium suggesting a second role in phosphate reabsorption from the apoplast of the symbiotic interface created in the arbuscule-colonized cortical cells (Benedetto *et al.*, 2005). Inactivation of *GigmPT* by host-induced gene silencing impaired arbuscule development, supporting the view that phosphate itself acts as a signal in the establishment of the symbiosis and that GigmPT is a transceptor that is involved both in Pi transport and Pi sensing (Xie *et al.*, 2016).

The *R. irregularis* genome also has homologs of the *S. cerevisiae* Na^+/phosphate symporter Pho89p and of the low-affinity vacuolar phosphate transporter Pho91 (Tisserant *et al.*, 2012). However, these transporters have not been characterized yet.

Ammonium transporters

Two high-affinity ammonium transporters, GintAMT1 (López-Pedrosa *et al.*, 2006) and GintAMT2 (Pérez-Tienda *et al.*, 2011), and one low-affinity GintAMT3 (Calabrese *et al.*, 2016) have been characterized in *R. irregularis*. GintAMT1 is highly expressed in the extraradical mycelium and is believed to mediate soil NH_4^+ uptake when NH_4^+ is present in low concentrations, for example, in acidic conditions. However, GintAMT2 might be involved in the recovery of NH_4^+ leakage during fungal metabolism, as it is highly expressed in the arbuscules. Finally, the low-affinity ammonium transporter GinAMT3, which is also expressed in the

arbuscules, could be also involved in NH_4^+ retrieval form the symbiotic interface but when the NH_4^+ concentrations are high. Both the high-affinity and low-affinity NH_4^+ transport processes require metabolic energy provided by the plasma membrane H^+-ATPase, as both transport activities where inhibited when the extraradical mycelium was supplied with the ionophore carbonylcyanide m-chlorophenylhydrazone and the ATP-synthesis inhibitor 2,4-dinitrophenol (Pérez-Tienda *et al.*, 2012). Orthologs of the *R. irregularis* AMT genes have been identified in the genomes of *G. rosea*, *G. margarita*, and *D. epigea*, but they have not been characterized yet.

Nitrate transporters

Although AM fungi show a clear preference for NH_4^+ they are also able to take up nitrate (Villegas *et al.*, 1996). Only a high-affinity nitrate transporter (GiNT) has so far been partially characterized in *R. irregularis* (Tian *et al.*, 2010). A role for GiNT in transporting nitrate into the extraradical mycelium was suggested, as its expression is regulated by nitrate availability.

Other transporters of inorganic ions

Despite several studies have shown the ability of AM fungi to improve potassium or sulfur nutrition of their host plants (Smith and Read, 2008), the fungal transporters involved in the symbiotic transport process of these nutrients have not been described yet. Genes encoding putative potassium transporters of the Trk (acronym of transport of K) and HAK (high-affinity K uptake) families and sulfate transporters have been identified in the genomes of the sequenced AM fungi. However, further research is needed to elucidate their role in the mycorrhizal transport pathway of these macronutrients.

Metal Transporters

Metal ions are essential micronutrients for fungal metabolism, as they are essential components of a wide variety of metalloproteins, transcription factors, and other proteins. Fungi possess a repertoire of metal transport proteins mediating active uptake of metals when present at low concentrations (Radisky and Kaplan, 1999) and intracellular transport systems delivering metals to the different organelles (Luk *et al.*, 2003). However, high metal ion concentrations are toxic. Under these conditions, transport systems pumping metals out of the cytosol and compartmentalizing excess metal in the vacuoles contribute to metal detoxification (Ruotolo *et al.*, 2008). The importance of metal transporters in AM fungi relies not only on their role on AM fungal homeostasis but also on the importance of these fungi on host plant homeostasis (Ferrol *et al.*, 2016). In fact, AM fungi increase the uptake of low mobility metal micronutrients when plants grow in soils deficient in these elements and, on the other hand, to alleviate metal toxicity in contaminated soils. Several genes putatively encoding proteins implicated have been identified in the genomes of the sequenced AM fungi and mined in the genome of *R. irregularis* (Tamayo *et al.*, 2014).

Zinc transporters

Two families of Zn transporters have been described in *R. irregularis*: the ZIP and the CDF families. The *R. irregularis* ZIP family is composed of five members that, although have not been characterized yet, are likely involved in transport of Zn and/or other metal ion substrates from the extracellular space or organellar lumen into the cytoplasm. In contrast, CDF proteins transport Zn and/or other metal ions from the cytoplasm into the lumen of intracellular organelles or to the outside of the cell (Eide, 2006). Out of the six genes potentially encoding CDF transporters in *R. irregularis* only *GintZnT*1 has been functionally characterized (González-Guerrero *et al.*, 2005). GintZnT1 was shown to be involved in Zn detoxification, as its expression in yeast decreased Zn cytosolic levels and its expression pattern in response to Zn correlated with the accumulation pattern of Zn in the fungal vacuoles.

Copper transporters

The *R. irregularis* genome has two genes encoding Cu transporters of the Ctr family and a CTR-like protein (Gómez-Gallego *et al.*, 2019). Members of this family mediate Cu^+ uptake by a passive, membrane potential-dependent mechanism. Functional analyses in yeast and gene expression patterns in response to copper revealed that *RiCTR1* encodes a plasma membrane Cu transporter that is involved in Cu uptake by the extraradical mycelium and *RiCTR2* a vacuolar transporter involved in Cu mobilization of vacuolar stores. The third gene RiCTR3 produces, as a consequence of an alternatively spliced event, two transcripts, RiCTR3A and RiCTR3B. RiCTR3B was suggested to be a receptor involved in Cu tolerance, as it confers copper tolerance to the metal sensitive Δ*yap-1* mutant yeast and its expression is highly induced by Cu toxicity in the extraradical mycelium (Gómez-Gallego *et al.*, 2019).

Iron transporters

Three members of the reductive pathway of Fe assimilation, the ferric reductase (RiFRE1) and the high-affinity Fe permeases (RiFTR1–2), have been characterized in the model fungus *R. irregularis* (Tamayo *et al.*, 2018). In this high-affinity uptake process, the metal is reduced from Fe^{3+} to Fe^{2+} by membrane-bound ferrireductases, and then it is rapidly internalized by the concerted action of a ferroxidase and a permease that form a plasma membrane protein complex (Kwok *et al.*, 2006). Functional analyses in yeast mutants and gene expression patterns in response to Fe indicate a role for the plasma membrane RiFTR1 in Fe acquisition by both the extraradical and intraradical mycelium and for FiFTR2 in Fe homeostasis under Fe-deficient conditions.

Arsenite efflux pump

Although specific transporters for non-essential metals, such as arsenate, unlikely exist in AM fungi, uptake of these non-essential and toxic metals is mediated by transporters of essential metals or even through other type of transporters. For example, in *R. irregularis* uptake of the metalloid arsenate occurs via the high-affinity phosphate transporter GiPT (González-Chávez *et al.*, 2011). However, arsenite detoxification is partially mediated via the specific efflux pump of arsenite GiArsA (González-Chávez *et al.*, 2014).

Transporters of Organic Compounds

Organic nitrogen transporters

Besides inorganic nitrogen, AM fungi can take up from the soil organic nitrogen in the form of amino acids, such as glycine, glutamic acid and arginine, and in the form of small peptides (Hawkins *et al.*, 2000). So far, an amino acid permease (GmosAPP1) and a dipeptide transporter (RiPT2) have been characterized in *F. mosseae* and *R. irregularis*, respectively. GmosAPP1 is expressed in the extraradical mycelium and transports non-polar and hydrophobic amino acids, such as proline, serine, glycine, and glutamine, by a proton-coupled, pH and energy-dependent process (Cappellazzo *et al.*, 2008). The *R. irregularis* dipeptide transporter RiPTR2 is expressed both in the extraradical and intraradical mycelium and has been suggested to play a role in the uptake of small peptides from the soil and the reuptake of peptides from the interfacial apoplast (Belmondo *et al.*, 2014). As yet uncharacterized oligopeptide transporter was also found to be overexpressed in the intraradical mycelium of *G. rosea* (Tang *et al.*, 2016).

Sugar transporters

As obiligate biotrophs, AM fungi need the supply of carbon compounds from the host plant for their growth and metabolism. Based on stable isotope labeling experiments, it has long been considered that AM fungi receive carbohydrates and specifically glucose from the plant (Pfeffer *et al.*, 1999; Trepanier *et al.*, 2005). However, it has been recently shown that despite lipids comprise up to 95% of spore dry weight (Bécard *et al.*, 1991) and up to 47% of hyphal volume in some regions of the extraradical mycelium (Bago *et al.*, 2002), AM fungi are auxotrophs for fatty acids and they also receive lipids from the plant host (Bravo *et al.*, 2017; Jiang *et al.*, 2017); however, the fungal transporter mediating lipid uptake remains uncharacterized.

Sugar uptake from the apoplast of the symbiotic interface created in the arbuscule-colonized cortical cell is mediated *in R. irregularis* by the monosaccharide transporter RiMST2 (Helber *et al.*, 2011). RiMST2 is a high-affinity monosaccharide transporter that operates by a H^+ cotransport process with a broad substrate spectrum. Besides glucose, it is able to transport xylose, mannose, and fructose with decreasing affinity in that order. RiMST2 is primarily expressed in intraradical fungal structures, and its silencing results in impaired formation, malformed arbuscules. Additional monosaccharide transporters (RiMST3, RiMST4, RiMST5 and RiMST6) and a putative sucrose transporter (RiSUC1) have been identified in *R. irregularis* (Helber *et al.*, 2011). However, their contribution to sugar uptake has not been clarified yet.

Water Channels

Aquaporins have been functionally characterized in *R. irregularis* and *R. clarus*. In *R. irregularis*, the two identified aquaporins RiAQP1 and RiAQP2 were found to be expressed both in the extraradical mycelium and in the arbuscules (Li *et al.*, 2013). Three genes potentially encoding aquaporins have been identified in *R. clarus*. *RAQP3 aquaporin 3* was most highly expressed in intraradical mycelia and encodes an aquaglyceroporin responsible for water transport across the plasma membrane. Knockdown of *RcAQP3* by virus-induced gene silencing revealed a role for this water channel in phosphate translocation from the outer to the inner hyphae (Kikuchi *et al.*, 2016).

Concluding Remarks

During the last decades research on membrane transport has made continuous progress and still remains an active field of study. A tremendous number of integral membrane proteins have been characterized in different fungi, especially in the model yeast *S. cerevisiae*, and the mechanisms by which their function is regulated have been elucidated.

Despite nutrient transport has been one of the more extensively studied aspects of the AM symbiosis; current knowledge on AM fungal transporters is still in its infancy. Significant progress has been made recently through the release and subsequent mining of genome sequences. The repertoire of annotated genes potentially encoding membrane transport proteins represents valuable sequence data for further functional validation. *S. cerevisiae* has been shown to be a useful tool to determine the function of the AM fungal transporters identified so far. Although AM fungi can not be genetically modified, development of host-induced and virus-induced gene silencing techniques of AM fungal genes has allowed functional analysis of a few genes expressed in the intraradical mycelium. It is expected that developments and advances in technologies, such as – omics, live cell imaging, stable isotope tracking and genetic manipulation, will provide a holistic view of the transport systems operating in AM fungi. This knowledge will be crucial to understand their biology and adaptation to environmental stresses.

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