Understanding Germination and Pathogenicity in Zygomycota Species through Genomic and Transcriptomic Approaches

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Abstract

Mucorales spores are the causative agents of the emerging disease mucormycosis. Mucorales species are also responsible for high quantities of food spoilage annually. The mechanism by which Mucorales spores cause disease and rot relies upon spore germination, however the mechanism underlying germination in these species remains poorly understood. Presented here are results which characterise Mucorales spore germination, through phenotypic and transcriptional studies (RNA-Seq), which followed the defined germination phenotype throughout. Hallmark pathways are identified through analysis of differentially expressed genes and co-transcriptional networks, providing targets for germination inhibition. With the resulting transcriptional data, the genome of *Rhizopus delemar* was enriched and analysed, thus providing better information on the Mucoralean genome. Comparative genomics was also employed to better understand genotypic variation between Mucorales species. To examine the differences in pathogenicity between species, and assess the impact of germination stage on pathogenicity, the transcriptional profile (RNA-Seq) of selected Mucorales species was examined upon phagocytosis by innate immune cells. To better understand the corresponding host response, the transcriptional response (single cell RNA-Seq) of innate immune cells to Mucorales infection was also examined. Finally, germination targets identified through the described analyses were targeted with suspected inhibitors to confirm function in germination regulation. This work has furthered our basic understanding of germination in these ancient fungi, indicated pathways essential to the germination programme of Mucorales species, and demonstrated a crucial role played by many of these pathways in host-fungal interactions of the Mucorales.

Acknowledgements

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I would like to say a special thank you to Daniel, for all of his support, curiosity and encouragement. For celebrating triumphs with me, sharing frustrations with me and always encouraging me to go after my goals. It is truly appreciated!

Last but not least, I would like to say thank you to my parents, for encouraging me for as long as I can remember! For encouraging curiosity and always showing an interest in whatever I was most fascinated with, thank you! Finally, I would like to say thank you to my Gran, for all of her encouragement along the way!

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Chapter 1: Introduction

Introduction: Mucorales

The following work has been adapted from the book chapter "Spore Germination of Pathogenic Filamentous Fungi" (Sephton-Clark and Voelz 2017), for which I performed the literature search, wrote the manuscript, completed revisions, and prepared the figures.

Mucorales species (Figure 1), belonging to the Mucorales order of the Zygomycota division (Mucoromycotina subdivision), are ancient diverging pathogenic fungi, capable of causing mucormycosis. These species are also known as food spoiling agents, predominantly spoiling soft fruits, vegetables and baked goods. Mucorales species are disseminated in their spore form, which swell and produce aseptate hyphae upon germination (Hoffmann et al. 2013). They reproduce sexually, via the combination of two hyphae (of opposite mating types) producing zygospores, or asexually (Mendoza et al. 2014). Asexual reproduction is quicker and leads to the formation of sporangiospores. These structures contain many spores which are dispersed via water, air, or animal disruption (Moore-Landecker 2011). Sexual reproduction introduces genetic variation into the population, allowing for the adaptation to changing environments (Mendoza et al. 2014), whereas asexual reproduction and sporulation provides an advantage in terms of dissemination, dispersal and colonisation of new territories. Propagated spores are found ubiquitously throughout the environment and remain dormant until favourable conditions prompt germination. Upon germination spores swell and grow to produce aseptate hyphae (Hoffmann et al. 2013). Once hyphal growth is

initiated, Mucorales species are characterised by rapid growth which allows them to cause infection and food spoilage.

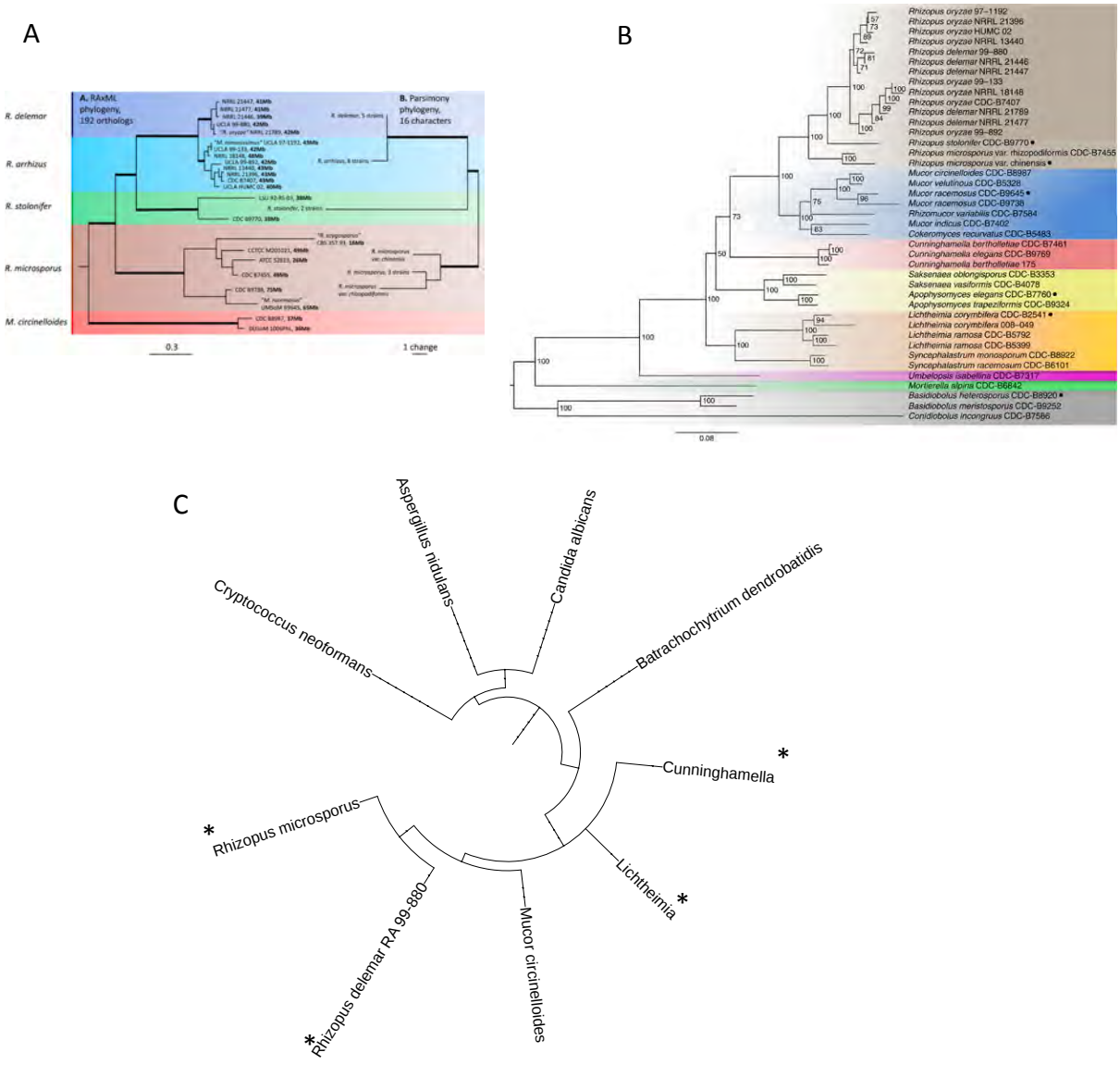


Figure 1. Phylogeny of the Mucorales A) Figure adapted from "*Phylogenetic and Phylogenomic Definition of Rhizopus Species"*, Gryganskyi et al, G3: GENES, GENOMES, GENETICS, 2018. This figure shows the phylogeny of 5 *Rhizopus* species, including multiple *R. delemar* and *R. microsporus* species, as well as *Mucor circinelloides*. This analysis has been performed based on 192 orthologues genes, and compares this phylogeny to parsimony phylogeny. Genome sizes have also been included in bold. B) Figure adapted from "*An integrated genomic and transcriptomic survey of mucormycosis-causing fungi"*. Chibucos et al, Nature Communications, 2016. This analysis shows the broader phylogenetic relationship of 38 Mucorales species, including multiple *R. oryzae* species, based on the relationship between 76 orthologous proteins. C) Phylogenetic tree based on NCBI taxonomy, generated with phylot. The asterisk denotes species worked with throughout chapter 2-6.

Food Spoilage

Worldwide one in eight people are malnourished, whilst it is predicted that a third of all food produced annually is wasted. Reducing this waste is crucial to improving food security. Food spoilage is a significant contributor toward food waste; it has been estimated that 20% of harvested fruit and vegetables are spoiled by microbes (Jay 1992; Barth et al. 2009), whilst in east Asian countries, rice losses due to bruising, moulds and pest spoilage can be as high as 80% (Fox and Fimeche 2013). It has also been estimated that up to 5% of baked goods are spoiled by fungi every year, with fungal spoilage estimated to cost $10,000,000 a year in Australia alone (Dao and Dantigny 2011). Food spoilage due to fungal contamination occurs at pre-harvest, storage, processing and packaging stages of food production (Bond et al. 2013). The ability to grow in acidic conditions, as well as at temperature extremes, has led to the spoilage of fruit juices, pasteurized and refrigerated foods, predominantly by fungi (Dao and Dantigny 2011).

Fungi of the Mucorales order are capable of invading plant tissue due to their rapid growth, with fruits and vegetables providing an optimal pH, high water content and nutrient source for growth (Turgeman et al. 2016). Sweet potatoes, cherries, peaches and tomatoes in particular are commonly affected by *Rhizopus spp.* spoilage (J W Eckert and Sommer 1967). *Rhizopus* may even spoil unbroken fruits, as it is able to penetrate the skin by secreting esterase enzymes (Baggio et al. 2016). Control of spoilage agents can be achieved through storage of produce below 5°C, however it is not viable to store all fruit and vegetables at this temperature (Joseph W Eckert and Ogawa 1989). Biological agents such as *Candida guilliermondii* and *Acremonium cephalosporium* have been used effectively to decrease *Rhizopus* spoilage of grapes (Zahavi et al. 2000), whilst *Pichia membranefaciens* effectively

inhibits spoilage within peach wounds through a proposed mechanism of competitive wound colonisation (Qing and Shiping 2000; Bonaterra et al. 2003). Although storage at low temperatures works well as a preventative measure, this is not feasible in all countries. With few alternative options, it is necessary to develop new measures to reduce food spoilage and increase food security.

Mucormycosis

Human mucormycosis, an emerging fungal infection caused by members of the Mucorales order, has become a growing concern due to difficulty treating, resulting in mortality rates of up to 90% (Trzaska et al. 2015; Brown et al. 2012; Kontoyiannis et al. 2012). *Rhizopus, Mucor* and *Lichthemia* species are thought to account for 70-80% of all mucormycosis infections (Gomes, Lewis, and Kontoyiannis 2011), whilst *Cunninghamella* has been reported as one of the most aggressive and pathogenic species (Petraitis et al. 2013a). Mucormycosis mainly affects immunocompromised patients, such as those having undergone transplants, or in many cases individuals suffering from ketoacidic phases due to diabetes (Lanternier and Lortholary 2009). Mucormycosis is especially prevalent in countries with high counts of uncontrolled diabetes, as is the case in India (Chakrabarti and Singh 2014). Mucormycosis diagnosis due to traumatic injuries is common, whilst nosocomial acquisition in susceptible patients is also on the rise (Skiada et al. 2012). Mucorales species manage to cause invasive infection due to their ability to germinate, grow and proliferate within the host. They avoid killing by the host in immunocompromised individuals, causing angioinvasion and tissue necrosis (Ibrahim et al. 2012). Current treatment consists of lipid forms of Amphotericin B and surgical debridement (Spellberg and Ibrahim 2010). Statins also effectively decrease the growth of *Rhizopus delemar*, by attenuating germination and increasing the pathogens

susceptibility to oxidative stress (Bellanger et al. 2016). Although alternative treatment options are being explored, mortality rates remain high, available treatment options for mucormycosis are severely limited and the outcome often leads to patients having affected areas amputated.

Mucorales spores and germination regulation

Mucorales spores have been detected in a range of environments, from the sands of Saudi Arabia to the forests of China (Murgia et al. 2019; Walther et al. 2013). They appear dark due to the melanin within the cell wall, a feature common to many fungi which is thought to protect against UV damage (Moore-Landecker 2011). These hardy spores can survive temperatures of 60-70°C (maintaining viability), however once germination is initiated, these spores become increasingly prone to damage (Turgeman et al. 2013). The composition of the Mucorales cell wall changes over germination; under aerobic conditions chitin increases over germination, with chitosomes acting as a reservoir for chitin synthase (Kamada, Bracker, and Bartnicki-Garcia 1991). At rest, Mucorales spore cell walls contain large quantities of lipids, accounting for 10-40% of the cell wall (Feofilova et al. 2015). The cell wall also contains considerable chitin/chitinosan (11.6%), sugars (49.3%), protein (16.1%), phosphate (2.6%) and melanin (10.3%) (Bartnicki-Garcia 1968).

Germination may be initiated upon cell wall breakage, removal of unfavourable conditions or the introduction of specific cues and nutrients - such as water, carbon and nitrogen (Feofilova et al. 2012; Mendoza et al. 2014). Essential nutrients for triggering germination of *Rhizopus oligosporus* includes glucose, phosphates and a mixture of amino acids, with leucine displaying a strong inductive effect on germination (Thanh, Rombouts, and Nout 2005). Blue

and green light have also been proposed as germination regulators for the light-sensitive protein possessing Mucorales species *Mucor circinelloides* (Herrera-Estrella and Horwitz 2007). Germination of *Rhizopus delemar* can be achieved with acidified glucose alone, as pH regulates germination via the recruitment of aquaporins (RdAQP1 and RdAQP2) that enable swelling (Turgeman et al. 2016). In Mucorales spores, the amount of RNA and protein within the spore appears to increase exponentially as soon as germination is induced, however DNA synthesis has not been reported to occur until 30 minutes before the production of germ tubes (Cano and Ruiz-Herrera 1988).

Germination as a mechanism of pathogenicity

Spores of *Cryptococcus neoformans* and yeast cells of *Candida albicans* have been shown to be key to dissemination throughout the host (Walsh et al. 2019; Seman et al. 2018). *C. neoformans* spores are the infectious propagules which lead to greater dissemination and mortality, when compared to infection with the yeast form (Walsh et al. 2019). Similarly, *Aspergillus* species are disseminated throughout the host via their conidial (spore) forms, however hyphae are often required for tissue damage and invasion (Bertuzzi, Schrettl, Alcazar-Fuoli, Cairns, Munoz, et al. 2014; Seman et al. 2018; Ben-ami et al. 2009; Ben-Ami et al. 2009). Spores are the infectious particles of *Aspergillus* species and germination is central to pathogenicity (Zhao et al. 2006; Fortwendel et al. 2005). *Aspergillus* has long been used as a model for understanding the lesser studied Mucorales species, and though research in this field provides a framework, a full understanding of Mucormycete pathogenicity requires comprehensive investigation into mucorales species. The transition from Mucorales spore to hyphae appears to be a key pathogenicity factor (Inglesfield et al. 2018; Mendoza et al. 2014), however the underlying mechanisms which control this event in Mucorales species is poorly understood.

As filamentous growth leads to tissue damage, the rate of germination can also be a contributing factor to virulence. In *Rhizopus* species, iron availability is known to regulate virulence (Andrianaki et al. 2018) as iron limitation leads to inhibition of germination (Kousser et al. 2019), and excess iron induces expression of the invasion mediating CotH (Gebremariam et al. 2016). *Cunninghamella* spp. are also known as one of the more aggressively invasive Mucorales species sets, the rate of germination of *Cunninghamella* spp. is increased, when compared to that of other Mucorales species. *Mucor circinelloides* shows increased virulence in its hyphal form, when compared to the yeast form

(Herrera-Estrella and Horwitz 2007; Walsh et al. 2019; Seman et al. 2018; Ben-ami et al. 2009; Zhao et al. 2006; Fortwendel et al. 2005; Inglesfield et al. 2018; Andrianaki et al. 2018; Lee et al. 2013).

In immunocompetent individuals phagocytes inhibit spore germination (Inglesfield et al. 2018), a mechanism key to infection control in immunocompetent patients. Phagocytes are rapidly recruited to the site of infection, and form innate granuloma-like structures around spores, leading to a latent infection. Phagocytes of immunocompromised patients fail to inhibit germination and subsequently life threatening infections develop. Despite the vital role played by the innate immune system in controlling mucormycosis, the interaction between Mucor species and innate immune cells is poorly understood.

There are several challenges when working with species of the Mucorales order. These include: a complete absence of chromosomal level genome assemblies (the highest resolution *Rhizopus* assembly consists of 83 contigs) (Ma et al. 2009; Horn et al. 2015; Mondo et al. 2017); the repetitive nature of Mucorales genomes which makes for difficult assembly; unclear species phylogeny (Gryganskyi et al. 2018; Hoffmann et al. 2013); minimal or absent genome annotation; limited genetic tools for manipulation (until recently) (Garcia, Vellanki, and Lee 2018); and large phenotypic variation and genotypic variation between species within the order. Further to this, understanding of the pathogenicity mechanisms employed by Mucorales species is limited (Gebremariam et al. 2014), compared to better studied species such as *Cryptococcus*, *Candida* and *Aspergillus spp.* Aside from the work presented here, there have been few comparative genomic and phenotypic studies

of Mucorales species. To date, there have been no transcriptional (high-resolution) studies of Mucorales germination, few studies which explore the transcriptional basis of Mucorales-host interactions and only one which explores germination inhibition as a means to inhibit pathogenicity (Trzaska et al. 2015).

Project Aims

This project aims to further understanding of the mechanisms of germination in the Mucorales species, and determine how this programme of morphological change and rapid growth contributes to pathogenicity. Once a basic phenotypic and transcriptional understanding of germination has been established, this project aims to detect mechanisms key to pathogenicity and identify pathways targetable to inhibit germination and reduce pathogenicity.

Literature Review

The following literature review will give an overview of knowledge about fungal morphology and germination regulation in multiple fungal species. Subsequent chapters will include literature reviews on the current knowledge of: phenotypes of fungal germination (Chapter 2), hallmarks of the fungal genome (Chapter 3), transcriptional regulation of fungal germination (Chapter 4), fungal-immune cell interactions (Chapter 5) and inhibition and modulation of fungal germination (Chapter 6), relevant to the work presented in these sections.

Introduction to fungal morphotypes: Spores and Hyphae

Spores may be formed either through sexual or asexual reproduction. Asexual reproduction is thought to provide an advantage due to the speed at which the spores can be produced and disseminated. Sexual reproduction, though often a longer process, presents an advantage through introduction of genetic variation into the population (Moore-Landecker 2011; Mendoza et al. 2014). The method of reproduction may be determined by the environment encountered by the fungi. For example, It has been suggested that the decision to reproduce sexually is regulated by trehalose homeostasis in *Cryptococcus neoformans* (Botts et al. 2014), whilst *Aspergillus* species conidiate when grown in nutritionally sparse conditions (Adams, Wieser, and Yu 1998). Although the nutritional triggers of sexual reproduction in the Mucorales order have not been well studied, trisporic acid is capable of triggering this process (Schimek and Wostemeyer 2012). Mucorales species produce this pheromone prior to sexual reproduction: both mating types must co-operate to complete production, as they rely on

one another for the interchange of intermediates required for this biosynthetic pathway (Lee and Heitman 2014).

Asexual spores are genetically identical to their parent cells and may be formed through the specific process of sporulation, or through the transformation of an existing cell. The asexual spores of basidiomycetes, ascomycetes, mucormycetes and chytridiomycetes are known as conidia, arthrospores or conidia, sporangiospores and zoospores (Table 1). Arthrospores are produced through the conversion of an existing cell, whilst conidia, zoospores and sporangiospores are formed through a specific process that produces new spores, known as sporulation or conidiation. Blastospores form in a budding manner, budding away from hyphae, swollen cells or vesicles. Specialized spore-producing cells known as phialides are also capable of producing blastospores.

The sexual spores of basidiomycetes, ascomycetes and mucormycetes are known as basidiospores, ascospores and zygospores, respectively. Sexual spores are usually formed via the fusion of hyphae, zoospores or gametangia of opposite mating types. This process may be initiated by the release of fungal hormones, as described for the mucormycetes (Austin, Bu'lock, and Gooday 1969). The genomic structure of the mating locus has been described for *Rhizopus* and Mucorales species and although sequence comparisons revealed locus conservation, the results enable increased phylogeny resolution (Gryganskyi et al. 2010).

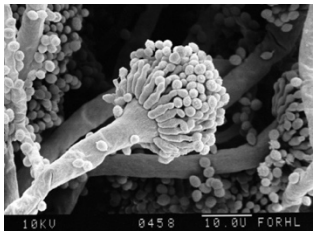
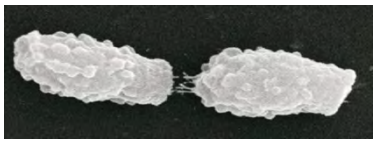
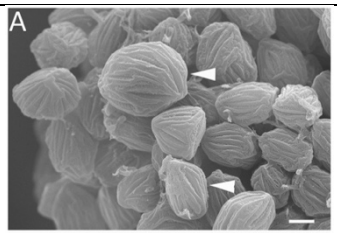
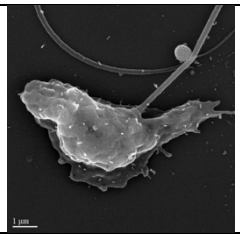
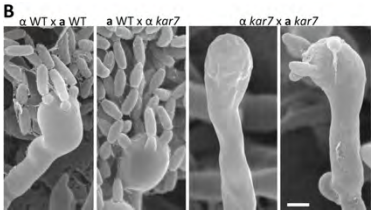
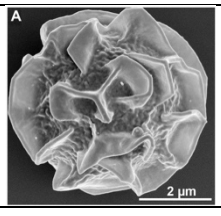
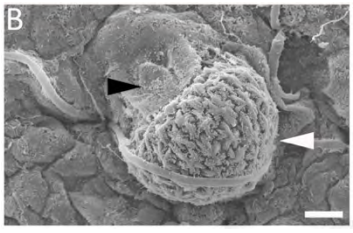
Class	Spore Type		Reproduction	Image Reference
Ascomycetes	Conidia on conidiophore: *Aspergillus fumigatus*		Asexual	*Aspergillus fumigatus*, conidia, close-up SEM. Credit: David Gregory & Debbie Marshall. CC BY.
Basidiomycetes	Conidia - *Cryptococcus neoformans*		Asexual	*Isolation and Characterization of Cryptococcus neoformans Spores Reveal a Critical Role for Capsule Biosynthesis Genes in Spore Biogenesis:* Botts et al. EukCell, 2009.
Mucormycetes	Sporangiospore – *Rhizopus delemar*		Asexual	*Structure, Function, and Phylogeny of the Mating Locus in the Rhizopus oryzae Complex:* Gryganskyi et al. PLOS ONE, 2010.
Chytridiomycetes	Zoospore - Bd strain JEL423		Asexual	*WASP and SCAR are evolutionarily conserved in actin-filled pseudopod-based motility:* Fritz-Laylin et al. The Journal of Cell Biology, 2017.
Basidiomycetes	Basidiospore - *Cryptococcus neoformans*		Sexual	*Function of Cryptococcus neoformans KAR7 (SEC66) in Karyogamy during Unisexual and Opposite-Sex Mating:* Soo Chan Lee, Joseph Heitman. EukCell, 2012
Ascomycetes	Ascospore - *Aspergillus fumigatus*		Sexual	Credit: Bryan Hansen. *Aspergillus fumigatus—What Makes the Species a Ubiquitous Human Fungal Pathogen?*: Kwon-Chung KJ, Sugui JA. PLoS Pathog, 2013.
Mucormycetes	Zygospore – *Rhizopus oryzae*		Sexual	*Structure, Function, and Phylogeny of the Mating Locus in the Rhizopus oryzae Complex:* Gryganskyi et al. PLOS ONE, 2010.

Table 1. Table describing fungal spore types.

Once spores are formed, they are usually kept suspended within sacs or fruiting bodies. These structures may contain thousands of spores and differentiate from fungal filaments, to which they remain associated. The number of spores contained in fruiting bodies can be described as a balancing act: maximizing the number increases spread of the species, increasing chances of survival, whilst too many may result in stalk collapse (Santorelli et al. 2008). Dispersion of spores from sacs is achieved through wind, water or animal disturbance. Release of some spores is also determined seasonally to coincide with the humidity and temperatures which provide an optimal climate for the germination of different species.

Germination is induced through favourable conditions and generally occurs in the following stages: isotropic growth or swelling, cell polarization, hyphal emergence and hyphal elongation (Figure 2) (Bonazzi et al. 2014). The process of germination culminates in the production of septate or aseptate hyphae depending on fungal species. Throughout isotropic growth, the spores can visibly be seen to swell. During this phase spores will take up water, whilst expanding and reorganizing their cell walls. Spores also increase their metabolic activity and may use external or internal energy sources in order to initiate transcription and translation (Griffin 1996; Novodvorska et al. 2016). Polarization of the cell wall following swelling determines where the hyphal tube will emerge and utilizes cell machinery such as the polarisome to support this process. This stage involves an extensive remodelling of the cell wall to enable hyphal extension. When hyphae emerge and extend, they grow to form what can be seen as a hyphal mat or matrix, in which the hyphae may overlap and even grow towards nutritional or light sources (Lucas, Kendrick, and Givan 1975; Dussutour et al. 2010).

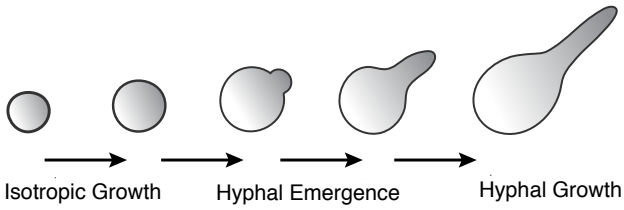


Figure 2. Schematic of germination. Adapted from Sephton-Clark & Voelz (2018).

Importance of spores and hyphae in pathogenicity and food spoilage

Although we are exposed to thousands of fungal spores every day (Moore-Landecker 2011), not every spore we encounter is pathogenic. Of those which are pathogenic, the developmental stage of the spore can affect the outcome of the encounter; whilst dormant spores may not be pathogenic, the germinated counter parts are often capable of causing disease in humans, animals or plants. To maintain dormancy, dispersed spores may employ nutrient and enzymatic compartmentalization, alongside the release of germination autoinhibitors, molecules which help regulate the maintenance of dormancy (Feofilova et al. 2012). Dormancy enables spores to avoid germination under conditions that may not be optimal, or could even be harmful to the germlings. Under suboptimal conditions the germling may perish due to environmental stress or lack of nutrients. Conversely, if spores stay dormant too long, they may miss a golden opportunity to exploit their environment for fungal growth and dissemination. As a result, the maintenance of dormancy is carefully regulated.

Cues which initiate germination may include the host environment, signalling spores to initiate pathogenic growth within or on a host. Once metabolically active, germinating spores

have the potential to express their repertoire of virulence factors. Damage to the host may be caused by the release of toxins, pathogenicity factors, or immunostimulatory components such as cell wall constituents. Plant pathogenic *Fusarium* spp. are known to produce and release phytotoxins which aid pathogenicity and invasion, through the induction of host cell death (Nishiuchi et al. 2006). The human pathogen *Aspergillus flavus*, is known to cause respiratory diseases, like many other *Aspergillus* sp. This may be through pulmonary infection in an immunocompromised individual, or simply through an allergic reaction induced by the inhalation of the conidia. *A. flavus* is also known to produce the mycotoxin, aflatoxin, which can lead to liver damage and even cancers, if the toxin is consumed (Hedayati et al. 2007). Similarly, *Candida albicans* produces candidalysin, a cytotoxic peptide which is toxic towards tissue, and causes damage during infection. Specifically, the hyphal form of *C. albicans* releases candidalysin, which aids dissemination and host invasion (Moyes et al. 2016).

In many cases, filamentous growth initiated during spore germination may be the underlying cause of disease. Filamentous growth can lead to tissue invasion and disruption which may prove fatal to the host. The lesser known but often more invasive *Aspergillus terreus* has been shown to produce accessory conidia, capable of increased germination rates when compared to the primary conidia produced by *Aspergillus* species, with accessory conidia forming hyphae within two hours of germination initiation. This increased germination rate may account directly for the aggressive pathogenicity of this species (Deak et al. 2009).

The emergence and extension of hyphae produced by plant pathogenic fungi, which is frequently accompanied by the release of extracellular degradative enzymes, is often key to infection. Infection caused by the wheat and barley pathogen *Fusarium graminearum* relies

on hyphal extension and the release of these enzymes in order to invade the host tissue (Zheng et al. 2012). Hyphal growth is also key to the infection caused by *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans.* Once the motile chytrid zoospores of the amphibian fungal pathogens *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* have made contact with the mucus membranes of their hosts, they are also known to utilise swelling and hyphal growth as a mechanism to invade host tissue (van Rooij et al. 2012).

Spore Composition

The spore cell wall

The cell wall of spores offers increased protection against environmental factors allowing survival of resting spores. The general structure and components within a spore cell wall are relatively conserved between fungal species. While fungal cell walls mainly consist of polysaccharides, with lipids and proteins usually accounting for a smaller percentage, the ratio of these components largely depends on species.

The composition of the *Mucor rouxii* spore wall has been well documented and is known to contain around 42.6% glucose, 16.1% protein, 10.3% melanin, 9.8% lipid, 9.5% chitosan, 2.1% chitin, 2.6% phosphate, 4.8% mannose and 1.9% glucuronic acid (Reyes and Bartnicki-garcia 1964). Similarly, the *S. cerevisiae* strain AM3 ascospore wall consists of large quantities of glucose (55%), mannan (17%), chitin/chitosan (9%), and protein (11%), along with smaller quantities of phosphates and organic acids (Briza et al. 1988). The cell walls of *Aspergillus oryzae* conidia show a much lower lipid content, at around 2%. However the carbohydrate content is similar at about 30% in *Aspergillus niger* (Sumi 1928; Feofilova et al. 1988), demonstrating the variability in ratio between species. The spore cell wall

of Aspergillus fumigatus has even been recorded to contain several lectins, capable of binding sialic acid and fucose, along with other sugars (Houser et al. 2013). Interestingly, the presence of some lectins has been shown to inhibit spore germination of *penicillia* (Barkai-Golan, Mirelman, and Sharon 1978). The conidial cell wall of *Aspergillus fumigatus* has also been shown to contain hydrophobic rodlets, known as RodA&B, thought to give structural support to the spore wall, as well as enhancing the adhesion of conidia to surfaces, and masking conidia from host immune recognition (Paris et al. 2003; Aimanianda et al. 2009).

The cell walls of many spore forming pathogenic fungi are also known to contain melanin. This pigment colours the spores brown, with an increased melanin content causing the spores to appear darker and most melanin mutants displaying an 'albino' phenotype. Fungal melanin consists of a highly complex structure, which exists in a stacked planar sheet structure and is likely formed from DOPA oligomers or polymers (Nosanchuck et al. 2015). The inclusion of melanin into fungal cells varies markedly between species. Melanin accompanies the hydrophobic rodlets in the outer layer of *Aspergillus fumigatus,* where it is thought to confer pathogenicity (Akoumianaki et al. 2016), and provide structural support (Pihet et al. 2009). The cell wall of an *Aspergillus fumigatus* mutant, which does not possess melanin, showed decreased electronegativity, hydrophobicity and a significant change in the structure of the conidial wall itself. This includes a loss of the outer hydrophobic rodlet layer which likely accounts for the loss of hydrophobicity (Bayry et al. 2014; Pihet et al. 2009). The accessory conidia of the highly pathogenic *Aspergillus terreus* have a wall which is compositionally very different to those of other *Aspergillus* species. The wall of *A. terreus* lacks the rodlet coating on the outermost layer, as well as the melanised underlayer, and also contains less ergosterol, demonstrating that variability even within a genus can be vast (Deak et al. 2009).

In addition, the cell wall of spores from the dimorphic human pathogen *Blastomyces dermatitidis* is known to contain melanin, which has been shown to offer protection against UV (Ultra Violet) (Nosanchuk et al. 2004), whilst antioxidant carotenes may also be present to provide protection against reactive oxygen species (ROS). In the opportunistic human pathogen *Cryptococcus neoformans*, melanin-containing vesicles known as fungal melanosomes are thought to deliver melanin to the cell wall, and once trapped form the layers seen in *C. neoformans* (Eisenman et al. 2005, 2009). The presence of melanin in *C. neoformans* is associated with an increase in virulence. It is thought that it provides protection against reactive oxygen species (ROS) which phagocytic cells use against pathogens (Moore-Landecker 2011; Schnitzler et al. 1999).

Spore compartmentalization and dormancy factors

The structure of spores is, in part, responsible for their ability to survive in extreme environments. It should be noted that in general spore walls, structurally, are very sturdy, although prone to morphing when under severely dehydrating conditions (Sarmiento et al. 2006). By storing compounds which will be used in the initial stages of germination separately from the enzymes which will catabolize them to release energy for the initiation of germination, spores maintain dormancy and energy stores (Dijksterhuis et al. 2007). For example, the storage molecule trehalose is highly abundant in the cytoplasm of dormant *Aspergillus nidulans* conidia. However, it is not metabolized in dormant conidia, indicating a lack of free trehalase enzymes. Trehalose is rapidly mobilized and used as an energy source to fuel germination (Elbein 1974; Svanström et al. 2014; Novodvorska et al. 2016).

In addition, dormancy factors can contribute to the maintenance of spores in a resting state. In *Rhizopus oligosporus* the autoinhibitor, nonanoic acid, maintains dormancy through a pH

regulated germination inhibition mechanism (Breeuwer et al. 1997). A transcriptomic approach which focused on dormant spores of *A. niger, A. fumigatus* and *A. oryzae* revealed that the autoinhibitor bZIP-type transcription factor AftA plays a significant role in maintaining dormancy. *aftA* mutants begin the process of germination much earlier than wild type conidia, and before the correct nutrients are available. Regulators like AftA are thought to be a common mechanism used by spores to maintain dormancy, with AftA involved in the regulation of dormancy and germination through interaction with conidia-associated genes such as the *calA-family* genes which encode a thaumatin-like protein (Hagiwara et al. 2016).

Water availability and metabolic activity

The maintenance of little or no metabolic activity allows spores to preserve dormancy. Metabolic activity of 'dormant' *Aspergillus niger* conidia measured in an aqueous environment was seen to be severely reduced when compared to germinated conidia. However, these readings may not present the true metabolic activity of dormant spores, as the presence of water alone has been recorded to trigger germination in *A. niger* (Teertstra et al. 2017; Novodvorska et al. 2016). Upon germination, spores rapidly take in water (Turgeman et al. 2016), and maintaining a low water content is thought to aid spore survival of desiccation. The water content of *Aspergillus oryzae* conidia has been recorded to be as low as 17.8%, a third of the reported percentage for *S. cerevisiae* yeast cells (Illmer, Erlebach, and Schinner 1999). It is thought that the lower the water content, the better equipped the spores are to survive extreme heat, although the thermostability of other spore components such as proteins and lipids will also play a role in this context (Sumi 1928; Nicholson et al. 2000).

The Spore Germination Program

Spore polarization

Prior to hyphal emergence, polarization of the spores occurs. In many fungi this process is reliant upon polarizomes, a protein complex which regulates polarity through its effects on actin, chitosomes and microvesicles which support the synthesis of chitin (Ruiz-Herrera and San-Blas 2003). This reliance appears conserved across fungi; work done by Kamada et al. (1991) in *Mucor rouxii* spores demonstrates that chitosomes act as a reservoir for chitin synthase throughout growth, as well as during dormancy (Kamada, Bracker, and Bartnicki-Garcia 1991). The recruitment of polarizome components and polarization-essential proteins has been well studied; during germination the actin cytoskeleton of *S. cerevisiae* spores is regulated by polarizome components Spa2p, Pea2p, Bud6p, Bni1p, Msb3 and Msb4. Over the course of germination, these components, along with Ras2p, regulate the formation and polarization of actin patches prior to polarized growth (Kono et al. 2005; Park and Bi 2007). The GTPase Cdc42p (cell division cycle 42) has been shown to be involved in regulation and recruitment of the polarizome, as well as the GTP binding proteins known as septins (Loeb et al. 1999; Bassilana, Blyth, and Arkowitz 2003; Momany and Talbot 2017). This conserved process of polarizome recruitment may also be accompanied by coiled coil cytoskeletal proteins locating to the cell wall. These proteins are thought to act as stress bearing structures to limit damage done by weakening the cell wall through polarized growth (Fuchino et al. 2013). In *P. discolor*, this process is followed by the formation of an ergosterol cap at the point of polarized growth (Van Leeuwen et al. 2008). Small GTPases, such as Rho1p, are also involved in the regulation of germination of *S. cerevisiae* (Kono et al. 2005). These hydrolytic enzymes have been shown to be involved in cytoskeletal arrangement, exocytosis and cell wall composition of the yeast. Cytoskeletal rearrangement also appears crucial to the

polarization of *Magnaporthe oryzae.* The polarization of the plant pathogen *Magnaporthe oryzae* has been shown to be regulated by the transcription factor Tpc1 (Transcription factor for Polarity Control 1) which interacts with the pathogenicity factor Mst12, that regulates infectious growth downstream of Pmk1 (mitogen-activated protein kinase 1) as well as NADPH oxidase (G. Park et al. 2002). Tpc1 is dependent upon MAPK signalling for normal regulation of cytoskeletal components, specifically F-actin components required for plant invasion (Galhano et al. 2017). Kinase signalling is also implicated in polarization; the pathogenic yeast *Penicillium marneffei* relies upon the p21-activated kinase pakA, which localizes to the site of germination and plays a major role in the cAMP-PKA pathway, to regulate polarization (Boyce and Andrianopoulos 2007). Kinases are also important for polarization in *S. cerevisiae*; Cdk1 (Cyclin dependent kinase 1) and the cyclins Clb1-4 promote isotropic growth of the cells. The accumulation of Clb2 specifically within the cytoplasm is thought to stabilize the switch between isotropic growth and polarization (Machu et al. 2014). RAS signalling also appears important for germination regulation; the G-protein alpha-subunits GasA and C are implicated in the regulation of *P. marneffei* germination, both dependently and independently of RasA (Zuber, Hynes, and Andrianopoulos 2003). GasA and C mutants also show defects in asexual development, as well as conidial yield (Boyce and Andrianopoulos 2007; Boyce, Hynes, and Andrianopoulos 2005; Pérez-Sánchez et al. 2010; Zuber, Hynes, and Andrianopoulos 2003). Many pathways involved in polarization regulation appear broadly conserved across filamentous fungal species, however it is likely there are also many species specific germination processes (Huang and Hull 2017).

The polarization process in *Mucor rouxii* is impaired by the presence of cAMP phosphodiesterase inhibitors, revealing that protein kinase A plays a role

in the differentiation of *M. rouxii* spores. This is further evidenced by germ tube emergence correlating directly with the amount of active PKA present, as threshold PKA levels result in germ tube emergence (Pereyra, Mizyrycki, and Moreno 2000). PKA is also important for the proper polarization of *Neurospora crassa,* and appears crucial to germination of *Aspergillus fumigatus* conidia (Zhao et al. 2006; Bruno et al. 1996).

The mechanism of polarization and initial germination appears to be broadly conserved across filamentous fungal pathogens (Boyce and Andrianopoulos 2015; D'Souza and Heitman 2001; Fortwendel 2016), however with this conservation there appears to be cross functionality: genes required for normal germination are frequently found to have various other functions, often in asexual development and the production of conidia. For example, the histidine kinase DRK1/drkA found in *Blastomyces dermatidis*, *Histoplasma capsulatum* and *P. marneffei* is known to be required for sporulation, virulence, cell wall integrity and production of infectious spores (Nemecek, Wuthrich, and Klein 2006; Boyce et al. 2011).

Hyphal outgrowth and extension

Germ tube emergence follows swelling and is a result of polarized growth, with the hyphal tip emerging from the site of polarization. Upon hyphal production, hyphae of most *Fusarium* species will immediately penetrate agar under laboratory conditions, although a few species produce hyphae that first grow along the top before penetration. As pathogenesis is often initiated by conidial germination and germ tube production, the more penetrating the species, the more pathogenic (Ruiz-Roldan et al. 2010; Petraitis et al. 2013b). For example, it is thought that the pathogenicity of *Fusarium verticillioides* depends on the *FPH1* (frustrated philade, conidiogenesis regulator) linked *SIG1* (surface vs. invasive germination), which regulates the invasive germination phenotype,

responsible for pathogenicity (Glenn, 2006).

Regulation of Germination

To exit dormancy and establish growth, appropriate internal or external signals are required. Germination can be initiated by the removal of dormancy factors, or by the introduction of environmental cues. Similarly to the process of seed germination, the removal of dormancy factors such as the dehydrin-like proteins DprA and DprB in *Aspergillusfumigatus* may induce germination (Hoi et al. 2011), whilst the removal of the autoinhibitor methyl 3,4-dimethoxycinnamate is essential for the germination of the plant pathogen *Uromyces phaseoli* (Macko et al. 1970; Hogan 2006). Although this induction method may trigger germination, it may not necessarily support full outgrowth of spores.

External cues capable of initiating germination and regulating growth include temperature, light, nutrient availability, water availability, pH, quorum sensing molecules and osmolarity (Alavi et al. 2013; Turgeman et al. 2016; Nguyen Van Long et al. 2017). Whilst species such as *Aspergillus niger* can start germination when in contact with water alone, the initiation of germination in *Rhizopus delemar* under starvation conditions induces atypical morphology, fragmented DNA and increased susceptibility to cell death (Turgeman et al. 2013; Novodvorska et al. 2016). Light responses have been well studied in plants, although they do not all possess germinating spores. Dependent on the wavelength, light is also capable of both inducing and inhibiting germination in fungi (Franklin and Quail 2010; Röhrig, Kastner, and Fischer 2013; Possart, Fleck, and Hiltbrunner 2014; Aron Maftei et al. 2014; Idnurm and Heitman 2005; Brunk et al. 2015).

The nutritional environment and germination

An appropriate nutritional environment to support fungal growth is essential for initiation of spore germination. The following sections will discuss the organic and inorganic nutrient signals that can induce spore germination and sustain growth.

Organic nutrients

Organic cues are one of the most commonly used germination initiation factors for fungi. Whilst carbon and nitrogen sources are generally indispensable for growth, macronutrients known to trigger germination in various fungi are manifold and species-specific. Essential nutrients for germination and activation of a higher metabolic rate in *Rhizopus oligosporus* include glucose, phosphates and a mixture of amino acids, with leucine capable of triggering germination (Thanh, Rombouts, and Nout 2005). Although *Aspergillus niger* may start to germinate in water alone, D-tagatose, D-lyxose, and 2-deoxy-D-glucose will all trigger germination, though they will not support outgrowth. For outgrowth D-glucose, D-mannose, or D-xylose is required (Hayer, Stratford, and Archer 2013). However, not all carbon sources are capable of triggering germination. For example D-galactose, L-glucose, and D-arabinose have all been shown to be incapable of triggering germination in *Aspergillus niger*, whilst they may support outgrowth if a complementary triggering sugar is present (Hayer, Stratford, and Archer 2013). This supports the notion that different stages of germination may be carried out regardless of whether conditions will support full germination (Hayer, Stratford, and Archer 2013). Similarly, spores of *Cryptococcus neoformans* are able to germinate on minimal water agarose medium, suggesting nutritional cues are not needed to trigger germination for this species (Velagapudi et al. 2009). *Blastomyces dermatitidis* spores will germinate on a low

glucose substrate, but can also utilize complex carbon sources from decomposing matter, a useful trait for exploiting carbon sources available in the environment. Additionally, *B. dermatitidis* requires a nitrogen source such as allantoin, creatinine, quanidoacetic acid, guanidine or cysteine. Interestingly, *B. dermatitidis* is also capable of growth at extremely high ammonia concentrations, of up to 42-62mmol/l. This is particularly significant as the growth of most soil inhabiting fungi will be inhibited at 2.1-4.2mmol/l. The ability to grow in such extreme conditions provides *B. dermatidis* with an environmental niche which it may exploit to increase its ubiquity (Baumgardner and Laundre 2001; Baumgardner 2009). The composition of the carbon source can even lead to opposing effects on germination, whilst L/D-leucine and/or unsaturated long chain fatty acids will induce germination in *Microsporum gypseum*, saturated short chain fatty acids are inhibitory towards germination (Barash, Conway, and Howard 1967).

Sulphur, a constituent key to life and a component of a few key amino acids, is required for the growth of many fungal species (Marzluf 1993). It enables strengthening and proper folding of protein structures through disulphide bridges, and is key to the functionality of many essential enzymes such as permeases and proteases. A source of sulphur such as biotin, thiamine or thioctic acid is required for the formation of *Histoplasma capsulatum* yeast cells. Whilst it is not required for the formation of hyphae, both mycelial and yeast forms utilise sulphur metabolism. It has been suggested that the sulphur requirement of the dimorphic fungus *Histoplasma capsulatum*, specifically cysteine, is temperature dependent, indicating the sulphur metabolism may be important for morphology and pathogenicity (Howard et al. 1980; Maresca and Kobayashi 1989).

Inorganic nutrients

Alongside macronutrients, micronutrients are also required by many fungi to initiate germination and sustain growth. For example, studies investigating calcium metabolism in *Sporothrix schenckii* showed that the presence of exogenous calcium stimulated mycelial production, with hyphal emergence corresponding to calcium uptake (Rivera-Rodriguez and Rodriguez-del Valle 1992).

Iron is essential for the proper growth of many fungi (Tamayo et al. 2014; Philpott 2006; C. Zhang 2014). Although too little iron may result in growth defects, too much can also be extremely toxic. Tight regulation of iron homeostasis is therefore essential. External siderophores are often used to scavenge iron from the surrounding environment, whilst internal siderophores store iron and limit toxicity. *Aspergillus* species employ the two transcription factors SreA and HapX to regulate iron homeostasis via siderophores, such as the intracellular siderophore SidC. *Aspergillus nidulans* conidia lacking SidC are known to be more susceptible to oxidative stress and exhibit delayed germination when grown in reduced iron environments (Eisendle et al. 2006). Interestingly, genes coding for the biosynthesis of triacetylfusarinine C, a common fungal siderophore, are absent in *Aspergillus niger*, indicating that there are many homeostatic mechanisms which are implicated in maintaining the correct metal homeostasis (Franken et al. 2014). The siderophore rhizoferrin is used by Mucorales species to scavenge iron from the surrounding environment. When Mucorales spores are germinated in iron limited conditions, they can be seen to produce atypical hyphae (Lewis et al. 2011; Kousser et al. 2019) and iron availability is known to regulate virulence in various Mucorales species (Ibrahim 2011). Notably, Mucorales species such as *Rhizopus delemar* may also bind iron provided by exogenous siderophores, such as the iron overload treatment deferoxamine, increasing spore germination.

In turn, this leads to higher susceptibility to mucormycosis in individuals who have elevated iron levels within their blood e.g. those with uncontrolled diabetes, patients receiving blood transfusions or those suffering from hemochromatosis. The combination of increased iron levels and treatment with the siderophore deferoxamine creates an optimal growth environment for the spores of Mucorales species (Boelaert et al. 1993; Gebremariam et al. 2016; Spellberg et al. 2016; Spellberg, Edwards, and Ibrahim 2016; Ibrahim, Spellberg, and Edwards 2016).

Micronutrients are often required by spores to overcome environmental and host stresses. Spores utilize enzymes such as catalase and superoxide dismutase (SOD) to avoid damage by reactive oxygen species (ROS). These enzymes are dependent on the presence of transition metals, namely Zn and Cu, to be able to perform their redox functions. Levels of SOD and catalase were shown to increase in *Aspergillus niger* grown at extreme temperatures, demonstrating their roles in survival in harsh conditions (Abrashev et al. 2005). SOD1, 2 and 3 are all required for the correct germination and outgrowth of *Aspergillus fumigatus*, whilst deletions of individual SOD proteins alone leads to growth perturbations and increased sensitivity to ROS (Lambou et al. 2010; Plante et al. 2017). Similar work done in *Neurospora crassa* showed that catalase is important for the survival and viability of conidia. Catalase-1 mutants showed an increased susceptibility to hydrogen peroxide and a decreased ability to germinate after exposure to light, when compared to the wild type control (Wang, Yoshida, and Hasunuma 2007).

The acquisition of micronutrients from low availability environments allows many fungal pathogens to survive and proliferate within hosts (Ballou and Wilson 2016). Nutritional

immunity is recognized as a way in which a host may limit the growth of a pathogen. However, pathogens have developed high specificity scavenging systems to combat this. Using high affinity chelators regulated by transcription factors such as ZafA (*Aspergillus fumigatus*), Csr1 (*Candida albicans*) and other Zap1 orthologues to regulate zinc homeostasis, pathogenic fungi may scavenge zinc from the host environment (Böttcher et al. 2015; Moreno et al. 2007). ZafA is required for the correct germination of *Aspergillus fumigatus* (Moreno et al. 2007), whilst Zap1 orthologues are thought to contribute towards virulence of fungi. Similarly, copper homeostasis has been shown to be important for the pathogenicity of fungi. Copper is also essential to the germination of *Schizosaccharomyces pombe*. If lacking copper transporters Ctr4 and 5, germination is halted at hyphal production, whilst the copper transporter Ctr6 has also been suggested to play a role in germination. Ctr6 is localised to the spore membrane at the end of the sporulation process, and spores lacking Ctr6 show decreased viability when grown in a low copper environments, than those with a functioning Ctr6 (Plante et al. 2014).

Micronutrients are not only required for the initial growth of pathogenic fungi, but often they are also implicated in ability of fungi to switch between dimorphic growth forms, as is the case for *Blastomyces dernmatidis*. *B. dermatidis* is known to produce a siderophore which aids in pathogenicity. Although it is capable of growth without supplementary ferric iron, *Blastomyces* requires exogenous magnesium and calcium for hyphal growth (Giles and Czuprynski 2004). The GATA transcription factor *SREB*, found frequently amongst fungal species, has been shown to regulate siderophore production in *Blastomyces dermatidis*, as well as lipid droplet formation, triacylglycerol and ergosterol synthesis. As lipid droplets are thought to be used as energy sources for *Blastomyces dermatidis* when transitioning from yeast to mycelial growth, *SREB* is also essential for hyphal growth (Gauthier et al.

2010; Marty et al. 2015). *Histoplasma capsulatum*, another dimorphic pathogen, utilizes a siderophore for pathogenicity and iron scavenging within host cells. *Histoplasma capsulatum* has been shown to scavenge iron from host ferritin/fe-transferrin with the *SID1* encoded ferric reductase. Without the iron scavenger, *Histoplasma capsulatum* is rendered incapable of replicating within host macrophages (Newman and Smulian 2013). *Histoplasma capsulatum* also utilises the transition metal requiring extracellular SOD and catalase enzymes to survive host ROS. As a result, decreasing the levels of iron and zinc available to *Histoplasma capsulatum* leads to higher susceptibility to macrophage killing (Garfoot and Rappleye 2016).

Germination Regulation via pH, Temperature, Light and Environmental Gases

Several exogenous factors, other than nutrient availability, are known to regulate germination of fungi. The abiotic factors discussed below may be important signals that aid pathogenic fungi to exploit their host environments at choice moments, or they may be signals that it is not yet a suitable environment in which to germinate.

pH

pH has been well established as a factor which influences germination in many fungal species and many pathogenic fungi grow best at the pH of their host environment. The host environment may also be liable to pH change. Once phagocytosed by host phagocytes, fungi are often subject to drastic pH changes within the phagosome, as the phagocytes attempt to disarm the phagocytosed fungi through fusion and fission events with both endosomes and lysosomes. Often, a matured phagosome will degrade the captured microbe, therefore maturation arrest (or phagosome escape) is a tactic often employed by fungi such as *C. neoformans*, to avoid degradation (Smith, Dixon, and May 2014; Yates, Hermetter, and Russell 2005; Gresnigt et al. 2018).

The germination of the Mucorales species *Rhizopus delemar* is induced at lower pHs; this is true in both artificial growth media, along with *in vivo* replicating conditions (Turgeman et al. 2016). The pH drop of the blood of diabetics undergoing a ketoacidic phase has even been linked to an increased susceptibility to mucormycosis, as the decreased pH in turn leads to an increased availability of iron and beta-hydroxybutyric acid for the fungus (Artis et al. 1982; Gebremariam et al. 2016). The pH dependent induction of germination in *Rhizopus delemar* is known to be regulated by two aquaporins (*RdAQP1* and *RdAQP2*). The optimal pH for germination initiation was found to be between pH 4-5 (Turgeman et al. 2016).

Other fungi are capable of growing at a vast range of pH's. The human fungal pathogen *Sporothrix schenckii* is found ubiquitously in soils and can grow between pH 3.5-9.4 (Tapia Noriega et al. 1993), whilst *Candida albicans* is capable of colonizing host environments of extreme pH's and growing in pH's from 2-10 (Odds 1988). Many fungi rely on the zinc finger transcription factor PacC, and the signalling pathway in which it is implicated, to sense the pH of their surroundings. The PacC signalling pathway has also been shown to effect germination in *Aspergillus nidulans*. Conidia lacking PacC components showed decreased growth, when compared to the wildtype, whilst the acquisition of iron is also thought to be dependent on PacC in *A. nidulans* (Bertuzzi, Schrettl, Alcazar-Fuoli, Cairns, Muñoz, et al. 2014). *A. nidulans* further relies on the signal transduction components encoded by the *PalA-I* genes to respond to environmental pH (Denison 2000). Given a suboptimal pH, fungi may modulate the pH of their surrounding environment through the release of pH altering molecules. Through the secretion of organic acids and ammonia, fungi are able to either increase or decrease the pH of their surroundings (Cornet and Gaillardin 2014; Vylkova 2017).

Temperature

In some fungal species, temperature has been shown to effect germination more than other factors. The germination of *Cryptococcus neoformans* was shown to be more reliant on temperature than it is on nutrient availability (Forsythe, Vogan, and Xu 2016). The small GTPase RAS1 has been shown to regulate the filamentous growth of *Cryptococcus neoformans* through the mitogen-activated protein kinase (MAPK) and RAS specific signalling pathways in response to shifting to higher temperatures (Alspaugh et al. 2000). *Neurospora crassa* mcb strains which lack the cAMP dependent protein kinase are incapable of polarized growth at higher temperatures, demonstrating that the complex triggers for germination are interlinked (Bruno et al. 1996). Interestingly, *Aspergillus nidulans* RasA, which is also involved in the polarization of conidia, regulates the germination of conidia in response to nutrients through possible interactions with heat sensitive factors encoded by spore germination-deficient (*sgd)* genes. These temperature dependent factors are also known to be important for germination and polarized growth (Osherov and May 2000). Studies investigating the impact of temperature on *A. fumigatus* germination revealed that over the course of germination higher temperatures (37°C) upregulated carbohydrate, lipid and secondary metabolism pathways, whereas lower temperatures (24°C) upregulated RNA metabolism and processing (Sueiro-Olivares et al. 2015).

Whilst spore production is often induced in suboptimal conditions as a survival mechanism, some fungi, such as the blight- causing fungus *Exserohilum monoceras*, require specific temperatures in order to produce spores. The optimum temperature for *Exserohilum monoceras* growth and spore production is between 27°C and 28°C (Zhang and Watson 1997).

Light

Environmental factors such as light play a role in the regulation of spore germination and growth in some species. Light induces germination and hyphae formation in arbuscular mycorrhizal species such as *Gigaspora gigantea*, but will inhibit germination and growth of many other fungal species, such as *Puccinia graminis* and *Aspergillus fumigatus,* dependent on the wavelength (Nagahashi, Douds, and Buee 2000). The crop pathogen *Puccinia graminis* showed inhibited germination when exposed to both white and far red light, whilst germination occurred normally in dark conditions (Lucas, Kendrick, and Givan 1975). *Aspergillus fumigatus* conidia showed decreased germination rates when exposed to blue light, red light and far red light (Fuller et al. 2013; Röhrig, Kastner, and Fischer 2013).

Light is sensed by fungi through photoreceptors which interact with and bind to fungal chromophores, ultimately transmitting and eliciting a cellular response to light. Light responses through photoreceptors have been studied extensively in the fungal plant pathogen *Neurospora crassa*. It has been shown that both conidia production and release can be regulated by the presence of blue light, through the *con-6* and *con-10* light responsive genes (Linden, Rodriguez-Franco, and Macino 1997). A whole transcriptome response to light exposure revealed that transcripts *con-6* and *con-10* were not only upregulated upon light exposure, but also play a role in the germination of *N. crassa* conidia (Wu et al. 2014). Similarly, *con-6* and *10* homologues *conF* and *J* were found to induce conidia formation in *Aspergillus nidulans*. A double mutant also harboured germination defects, demonstrating the role of blue and red light to be involved in both conidia formation and germination, which appears to be conserved across filamentous fungi (Suzuki et al. 2013). It has been

suggested that the light signalling pathways, such as the cellular response to blue light, in *Neurospora crassa* may be dependent on internal ROS, as SOD1 mutations showed increased light dependent accumulation of metabolites, suggesting ROS factors resulting from light exposure may act as alternative signals to transmit the signalling responses to light (Yoshida and Hasunuma 2004; Belozerskaya et al. 2012). In order to overcome the oxidative stress which light induces, *Neurospora crassa* upregulates the production of antioxidants and protective photopigments in response to light exposure (Wu et al. 2014). Light exposure also increased the resistance of *A. fumigatus* conidia to UV and ROS, demonstrating the ability to respond in a protective manner to potentially harmful stimuli (Fuller et al. 2013; Röhrig, Kastner, and Fischer 2013). Alternate mechanisms such as proton pumps are also activated by light; the light activated proton pump, CarO, is involved in the formation of *Fusarium fujikuroi* hyphae, with hyphal growth rates decreased under light conditions (Brunk et al. 2015; Garcia-Martinez et al. 2015).

The connection between light and germination has been exploited in both medicine and agriculture (Lucas, Kendrick, and Givan 1975; Schmidt-Heydt et al. 2011; Baltazar et al. 2015; Trzaska et al. 2017). Pulsed light containing UV spectra decreases the ability of spores to germinate on food sources such as wheat grain, providing a useful food spoilage preventative measure (Aron Maftei et al. 2014).

Gases

Atmospheric gases are most commonly used within food packaging to inhibit fungal germination, to prevent food spoilage. Through [O_2] reduction, or [CO_2] increases (15-20%), spoilage by species such as *Rhizopus stolonifer* is successfully controlled (Barkai-Golan 2001).

Although higher CO_2 concentrations often result in germination inhibition, germination is supported in *Fusarium roseum* at up to 32% CO_2 (Wells and Uota 1970), and *Rhizopus oligosporus* germination is supported by CO_2 when O_2 becomes limited (de Reu et al. 1995).

Signalling Molecules

The signalling molecules known as quorum sensing molecules are capable of regulating growth in both plant and human pathogenic fungi. One of the best-studied examples is the quorum sensing molecule farnesol, a secondary metabolite produced by *Candida* species when in stationary phase, capable of modulating the innate immune response (Leonhardt et al. 2015) and inhibiting *Candida albicans* hyphal growth at high concentrations (Piispanen et al. 2011). Farnesol is capable of regulating the growth phase of *Candida albicans* populations, as well as biofilm formation (Ramage et al. 2002). Even at low cell densities, a condition which usually induces hyphal growth in *Candida albicans*, it will not form hyphae if the quorum sensing molecule farnesol is present (Kruppa et al. 2004). Furthermore, farnesol, produced in higher quantities by *Candida albicans* grown between 37 and 40 °C, is known to inhibit the production of hyphae by disrupting the degradation of the hyphal growth repressor Nrg1. Nrg1 must be both down regulated via the cAMP-PKA pathway, as well as degraded for *Candida albicans* to produce hyphae, a complex regulation which controls the morphogenesis of *Candida albicans* and the resulting pathogenicity (Lu et al. 2014).

Farnesol has also been shown to inhibit the germination of the common plant pathogen *Fusarium graminearum*, with higher concentrations of purified farnesol inducing apoptosis (Semighini, Murray, and Harris 2008). Similarly, the presence of farnesol was

shown to impair growth of *Aspergillus nidulans* and induce apoptosis like characteristics in the hyphal form of *Aspergillus niger* (Semighini et al. 2006).

Whilst bacteria have been shown to modulate germination through nutrient competition (Kousser et al. 2019), the presence of the bacterial and fungal cell wall component GlcNac is also capable of inducing the switch to filamentous growth in *Blastomyces dermatidis, Histoplasma capsulatum* and *Candida albicans*. This response *in Histoplasma capsulatum* is thought to be mediated by the GlcNac transporters NGT1/2, required for the filamentous response (Gilmore et al. 2013), whilst the DNA-binding containing *RON1* is involved in regulating this switch in *C. albicans* (Naseem et al. 2017).

Hormones produced by both plants and fungi are also capable of regulating the germination of select fungal species. Gibberellic acids have been shown to stimulate the germination of the plant pathogen *N. crassa*, whilst auxins show a dose dependent modulation of germination. At low concentrations, auxins promote germination of the plant pathogen *Fusarium delphinoides*, whilst at high concentrations it is inhibitory towards germination (Kulkarni et al. 2013; Tomita, Murayama, and Nakamura 1984). The antagonist to gibberellic acids, abscisic acid, has been shown to promote germination of the rice blast fungus *M. oryzae* (Spence et al. 2015). Jasmonic acid was shown to decrease germination of *Fusarium oxysporum* spores, whilst the germination of corn pathogen *Harpophora maydis* can be subdued by the presence of sialic acid (Krol et al. 2015; Degani, Drori, and Goldblat 2015). The gaseous hydrocarbon and plant hormone, ethylene, is released by plants and known to initiate the ripening of fruits. Ethylene is also capable of triggering germination of the plant pathogen *Colletotrichum musae*, so that germination occurs simultaneously with fruit ripening. Upon germination, the fungus releases extracellular cutinase; this enzyme hydrolyses the waxy cutin layer coating the

plant, enabling pathogen access to the cuticle of the plant. Other plant pathogens, such as *Fusarium solani pisi*, even store the cutinase enzyme in the spores, enhancing access to the plant upon spore contact and germination (Kolattukudy et al. 1995).

Plant hormones have also been shown to modulate the growth of some human fungal pathogens. For example, sialic acid is capable of decreasing hyphal growth of *Aspergillus flavus*. However, it is striking that plant hormones have mainly been seen to regulate the germination of classical plant pathogens which rarely cause human infections. This suggests fungal plant pathogens may have evolved or diverged to produce hormones which are almost identical to those mentioned above, allowing fungi to regulate the growth of the plants which they rely on for nutrients and leading to a dynamic co-regulation of growth between fungi and their hosts.

Materials and Methods

This work has been adapted from the following articles: "Pathways of Pathogenicity: Transcriptional Stages of Germination in the Fatal Fungal Pathogen Rhizopus delemar"(Sephton-Clark et al. 2018) and "Host-pathogen transcriptomics of macrophages, Mucorales and their endosymbionts: a polymicrobial pas de trois" (Sephton-clark et al. 2019). For both I conceived, designed and performed the experiments, collected the data, performed the analysis and interpretation, wrote the manuscript, completed revisions, and prepared the figures.

Microbial Culture

Fungal Culture

Spore Isolation

Spores were harvested from 10 day old cultures maintained on sabouraud dextrose agar plates by washing with 10ml of phosphate buffered saline (PBS) (Sigma-Aldrich). Spores in PBS solution were centrifuged for 3 minutes at 3000 rpm and washed with 5ml of PBS. The pellet was resuspended in 3-5ml of PBS and the concentration of spores in the suspension calculated by counting with a haemocytometer.

Spore Growth

Sabouraud Dextrose Broth

Spores harvested as above were grown in Sabouraud (SAB) broth (10 g/liter mycological peptone, 20 g/liter dextrose), sourced from Sigma-Aldrich, at a range of temperatures (RT to 37°C, protocol dependent), at 100-250 rpm.

Sabouraud Dextrose Agar Plate

100-200 spores, harvested as above, were spread evenly onto SAB agar plates. Plates were incubated lid side up away from light at RT for 2-3 days, before being turned (lid side down) and incubated in the same conditions for 7-8 days.

Endosymbiont Curing

To cure spores of their respective bacterial endosymbionts, spores were cultured with ciprofloxacin, as described in Itabangi et al. 2019 (Itabangi et al. 2019). Briefly, spores were passaged for a month in 60 µg/mL ciprofloxacin and cultured on ciprofloxacin plates prior to use. Once cured, spores were sub-cultured at least twice in ciprofloxacin-free media before use.

Bacterial Culture

Bacterial endosymbiont species, isolated by Herbert Itabangi, were grown in lysogeny broth (also referred to as Luria-Bertani media) (LB), sourced from Sigma-Aldrich, at 37°C, for 12 hours, with 250rpm shaking prior to processing for genomic DNA extraction.

Germination Phenotyping

Spore Germination Assay

Live cell Imaging

Spores were harvested as above and used at $1x10^5$ Spores/ml in 200µl of Sabouraud dextrose Broth. Images of the spores were taken every 10 minutes over the course of 24 hours with brightfield microscopy. Images were taken at 20X objective on a Zeiss Axio Observer. Images were analysed with ImageJ V1.

Flow Cytometry

Spores were harvested as above and used at a concentration of $2x10^7$ Spores/ml in 200µl of SAB. Spores were incubated for between 0-6 hours, then fixed in 4% paraformaldehyde (PFA), sourced from Sigma-Aldrich and prepared according to manufacturer's instructions, before staining with either calcofluor white (CFW) or Fluorescein isothiocyanate isomer I (FITC). Spores stained with 250-1000 µg/ml of CFW were kept at RT, for 20 minutes, prior to washing with PBS. Spores stained with 100 µg/ml FITC were kept at RT, for 30 minutes, prior to washing with PBS.

XTT Assay

The XTT assay was used to measure metabolic activity of spores and germlings (Antachopoulos et al. 2006; Moss et al. 2008). To germinating spores, XTT sodium salt and menadione were added as described by Antachopoulos et al. After 80 minutes, optical density readings at 450nm were taken, these reading correspond to metabolic activity. Readings were

also taken at 600nm to determine fungal biomass. Readings were corrected by the appropriate controls during analysis.

Exosome Release

Spores were harvested as above and grown in SAB broth for 0-6 hours at 25°C. The extracellular vesicles were isolated according to manufacturer's instructions Exo-spin™. Extracellular vesicle (EV) size was determined with dynamic light scattering (dls), a method which determines the size of particles dispersed in a liquid, based on the detection of laser beam scattering.

Genomic DNA Extraction, Sequencing and Analysis

Genomic DNA Extraction

Fungal DNA Extraction

Spores germinated in SAB broth at 37°C, 100-200 rpm shaking, for 12 hours were pelleted at 3000rpm, and washed in PBS. To washed spores 1ml of Qiagen DNEasy PowerLyzer Microbial Kit lysis buffer (Solution SL) was added. The mixture was bead beaten at 6500 rpm for 2 rounds (30 seconds per round). Samples were immediately placed on ice post beating and DNA was extracted from the resulting solution as per Qiagen DNEasy PowerLyzer Microbial Kit instructions (column based extraction). Nanodrop measurements were taken to determine DNA quality, and agarose gel electrophoresis (1%) indicated DNA integrity.

Bacterial DNA Extraction

Bacterial cells grown in LB broth at 37°C, 100-200 rpm shaking, for 12 hours were processed according to the of Qiagen DNEasy PowerLyzer Microbial Kit (column based extraction). Nanodrop measurements were taken to determine DNA quality, and agarose gel electrophoresis indicated DNA integrity.

Genomic DNA Sequencing

Fungal Sequencing

Library preparation and sequencing of genomic DNA was performed by MicrobesNG. Briefly, Nextera XT Library Prep Kit was used for library preparation and pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina. Libraries were

sequenced on the Illumina HiSeq using a 250bp paired end protocol. 30X coverage was obtained for all fungal species sequenced.

Bacterial Sequencing

Library preparation and sequencing of genomic DNA was performed by MicrobesNG. Briefly, Nextera XT Library Prep Kit was used for library preparation and pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina. Libraries were sequenced on the Illumina HiSeq using a 250bp paired end protocol. 5X coverage was obtained for all bacterial species sequenced. Species identification was carried out with Kraken(Wood and Salzberg 2014).

Genome Sequence Analysis

Rhizopus microsporus Genome Assembly

Raw reads obtained from the MicrobesNG protocol above were quality checked with FastQC (Andrews). Reads were assembled with SPAdes (Bankevich et al. 2012), using the *R. microsporus* assembly (Mondo et al. 2017) as a reference (-untrusted contigs).

Rhizopus microsporus Variant Identification

Raw reads obtained from the MicrobesNG protocol above were aligned to the *R. microsporus* assembly (Mondo et al. 2017) with bwa (Li and Durbin 2010). Samtools (Li et al. 2009) and Picard (Broadinstitute) were used to extract alignment metrics. Haplotypecaller (McKenna et al. 2010) was used to identify single nucleotide polymorphisms (SNPs) in the aligned reads. SNPs identified in the aligned reads were used to replace their alternatives in

the reference genome with GATK (FastaAlternateReferenceMaker) (McKenna et al. 2010). Subsequent reads generated via RNA-Seq were aligned to this hybrid.

Comparative Genomics

Fisher's exact test was used to detect enrichment of Pfam terms between *Rhizopus delemar* and *Rhizopus microsporus* (*R. delemar* & *R. microsporus*), terms with a corrected (FDR, false discovery rate) P value of < 0.01 were considered significant. Orthologue genes of *R. delemar* and *R. microsporus* were identified using blast+. R (version 3.3.3) was used to carry out hypergeometric testing of KEGG and GO terms to determine enrichment. Orthologue genes of *R. delemar* and *Aspergillus niger* were identified using blast+.

Rhizopus delemar Genome Annotation Update

The genome of *R. delemar* was reannotated by incorporating the RNA-Seq data via BRAKER (version 2.1.0) (Hoff et al. 2016), this was fed into the Broad Institute annotation pipeline, which removed sequences that overlapped with repetitive elements, numbered, and named genes as previously described (Haas et al. 2011). Completeness of annotation was analyzed with BUSCO (version 3) (Simão et al. 2015). Duplicated gene pairs were identified based on parameters outlined by Ma et al (Ma et al. 2009) with Python (version 3.5.5). GO term enrichment of duplicated gene pairs was determined in R (version 3.3.3). Pathway Tools (version 21.0) was used to obtain information on specific pathways (Karp et al. 2016).

Transcription and Inhibition of *Rhizopus delemar* Germination

Germination RNA-Seq

RNA Isolation

Total RNA was extracted from *R. delemar* spores (harvested as above) which germinated in SAB broth for 0,1,2,3,4,5,6,12,16 and 24 hours. To extract Total RNA, the washed samples were immediately immersed in 1ml of Trizol and lysed via bead beating at 6500 rpm for 2 rounds, 30 seconds each. Samples were then either immediately frozen at -20°C and stored for RNA extraction, or immediately placed on ice for RNA extraction.

All further work was done on ice to inhibit RNases. After lysis via bead beating, samples were moved to clean Eppendorf tubes and 0.2ml of chloroform was added for every 1ml of Trizol used in the sample preparation. Samples were incubated for 3 minutes, then spun at 12,000 G at 4°C for 15 minutes to separate out the aqueous and organic phases.

The aqueous phase, containing RNA, was placed into a fresh tube and the interphase and organic phase containing proteins and DNA were stored at -20°C for any further work. To the aqueous phase, an equal volume of 100% EtOH was added, before the samples were loaded onto RNeasy RNA extraction columns. The columns were spun at 8000g for 30 seconds at 4°C and the flow through discarded as RNA should now be bound to the silica column. 700µl of buffer RW1 was added to remove and carbohydrates, lipids or proteins bound to the column. It should be noted that at this point, only RNA longer than 200bp will remain bound to the membrane, therefore if regulatory, small non-coding RNA etc... is also being isolated, this method will not be suitable. The column was spun at 8000g for 30 seconds at 4°C and the

flow through discarded. The washing buffer RPE was then added to the column to remove any contaminating salts which may affect results seen with the NanoDrop, or any other equipment which relies on measuring absorbance to determine quantity and quality of RNA. 500µl of the buffer was added to the column, followed by a spin at 8000g for 30 seconds at 4°C, the flow through discarded. Another 500µl of the buffer was added to the column, followed by a spin at 8000g for 2 minutes at 4°C, the flow through discarded. The RNA was then eluted in two rounds, each using 30µl of RNase free dH_2O to elute RNA by spinning the columns at 8000g for 1 minute at 4°C. 3µl aliquots of each sample was then taken for testing with the Nano drop, whilst the remainder of the sample was immediately frozen and stored at -20°C.

Three biological repeats were performed and the quality of the resulting RNA was checked by Agilent to ensure all RIN scores were above 8, indicating that there was minimal RNA degradation within the sample and a good ribosomal RNA ratio (Schroeder et al. 2006). 1ug of Total RNA was used for library preparation.

The following Library preparation was performed in accordance with the NEBNext pipeline. mRNA was isolated with NEB's Poly(A) magnetic isolation module. The isolated mRNA was then fragmented to around 200bp with Mg^{2+} ions. From this, ds cDNA was produced and underwent PCR amplification and indexing. The quality of the library was checked by Agilent BioAnalyzer and the concentration was determined by qPCR prior to sequencing.

Samples were sequenced using the Illumina HiSeq platform. 15 samples were loaded per lane, with 140 million reads expected per lane. 100bp paired end sequencing was employed (2X100bp).

Data Analysis

FastQC (version 0.11.5)(Andrews) was employed to ensure the quality of all samples, a Phred value of over 36 was found for every sample. Hisat2 (version 2.0.5) (Kim, Langmead, and Salzberg 2015) was used to align reads to the indexed genome of *R. delemar* (JGI: PRJNA13066) (Ma et al. 2009). HTSeq (version 0.8.0) was used to quantify the output (Anders, Pyl, and Huber 2015). Trinity and edgeR (version 3.16.5) were then used to analyze differential expression (Grabherr et al. 2009). Pathway Tools (version 21.0) was used to obtain information on specific pathways (Karp et al. 2016). Cytoscape (version 3.5.1) was used to analyse co-transcriptional networks.

Germination Inhibition

Inhibition Assessment of Targets Determined via RNA-Seq

Images of $1X10^5$ spores/ml in SAB were taken every 10 minutes to determine germination characteristics. Images were taken at 20X objective on a Zeiss Axio Observer. Calcofluor white (CFW), fluorescein isothiocyanate (FITC) (Sigma-Aldrich), and the ROS stain carboxy-H2DCFDA (6-carboxy-2=,7=-dichlorodihydrofluorescein diacetate [C400]; Invitrogen) were incubated with live spores, according to the manufacturer's instructions, prior to imaging. To assess inhibition, spores were incubated with either: 1 to 5 mM hydrogen peroxide, 1.5 to 10 nM antimycin A (Sigma-Aldrich) or Nikkomycin Z 120ug/ml prior to imaging. Bright-field and fluorescent images were then analyzed using ImageJ V1.

Strathclyde Natural Compound Library

Spores were harvested as above and used at a concentration of $1x10^5$ Spores/ml in 200ul of SAB. To measure metabolic activity the XTT assay was used (Antachopoulos et al. 2006; Moss et al. 2008). The compound A10 was added to germinating spores at concentrations of 1000, 100, 75, 50, 25, 10, 1, and 0 ug/ml. To control for effects introduced by the extract solvent (DMSO), DMSO was added to samples at an equal volume to the highest volume of A10 extract used. 3% Acetic Acid was used as a positive control. To measure metabolic activity and inhibition the XTT assay was used (Antachopoulos et al. 2006; Moss et al. 2008). Optical density readings at 450nm were taken at 80 minutes after the addition of the XTT mix. Readings were also taken at 600nm to determine biomass.

Rhizopus-macrophage interactions

Macrophage

Macrophage Culture

Macrophages from the J774.A1 cell line were cultured in Dulbecco's Modified Eagle Medium (DMEM), (complemented with 10% foetal bovine serum, 1% penicillin, 1% streptomycin and 1% L-glutamine), sourced from Sigma-Aldrich. Macrophages were grown at 37°C, in 5% CO2.

In vitro Phagocytosis assay

Macrophages were incubated for one hour in serum-free DMEM prior to infection. Spores were pre-swollen in SAB (2 hr for *R. delemar*, 4 hr for *R. microsporus*). Washed spores were incubated at 5:1 MOI with 1 x 10^5 macrophages as described in Itabangi et al (Itabangi et al. 2019), to ensure that >95% of macrophages contained one spore or more. After a 1 hour of incubation, excess spores were washed off the surface and the macrophages were incubated for a further 2 hours, before processing for RNA-Seq experiments. For live cell imaging experiments, images were taken starting immediately after the excess spores were removed.

Phagocytosis Live Cell Imaging

Time course images were taken to determine how LPS pre-treatment (100ng/ml) (Myers, Tsang, and Swanson 2010) of macrophages, and Nikkomycin Z pre-treatment (120ug/ml over the course of swelling in SAB) of spores effected phagocytic outcome. Images were taken at 20x on a Zeiss Axio Observer, with images taken every 5 minutes. Bright-field and fluorescent images were then analysed using ImageJ V1.

RNA-Seq

Bulk *Rhizopus* RNA-Seq

RNA was extracted from spores which had either been incubated with the macrophages, or incubated in DMEM for the equivalent time period. To remove the macrophages, triton at 1% was used to lyse the macrophages, the resulting solution was then centrifuged for 3 min at 3,000 rpm, and washed, leaving only spores. The DMEM control also received the same treatment. To extract total RNA, the washed samples were immediately immersed in TRIzol and lysed via bead beating at 6,500rpm for 60s. Samples were then either immediately frozen at −20°C and stored for RNA extraction or placed on ice for RNA extraction. After lysis, 0.2 ml of chloroform was added for every 1 ml of TRIzol used in the sample preparation. Samples were incubated for 3 min and then spun at 12,000 g at 4°C for 15 min. To the aqueous phase, an equal volume of 100% ethanol (EtOH) was added, before the samples were loaded onto RNeasy RNA extraction columns (Qiagen). The manufacturer's instructions were followed from this point onwards. RNA quality was checked by Agilent, with all RNA integrity number (RIN) scores above 7 (Schroeder et al. 2006). One microgram of total RNA was used for cDNA library preparation. Library preparation was done in accordance with the NEBNext pipeline, with library quality checked by Agilent. Samples were sequenced using the IIlumina HiSeq platform; 100-bp paired-end sequencing was employed (2 × 100 bp) (>10 million reads per sample).

Single Cell Macrophage RNA-Seq

For single cell sequencing experiments, macrophages were infected with fungal spores, as outlined above. Uninfected macrophages, used as a negative control, were treated in the

same manner and underwent mock washes and media changes at identical time points to infected macrophages. Macrophages were isolated and released from the bottom of their wells with accutase, as per manufacturer's instructions (Technologies). Once in solution, the macrophages were loaded onto the 10X genomics single cell RNA sequencing pipeline for single cell isolation and library preparation. In total, 1082 single cells were sequenced. The libraries were sequenced on the IIlumina Sequencing Platform.

RNA-Seq Data Analysis

For the bulk RNA-Seq data, FastQC (version 0.11.5)(Andrews) was employed to ensure the quality of all samples. Hisat2 (version 2.0.5)(Kim, Langmead, and Salzberg 2015) was used to align reads to the indexed genome of *R. delemar RA 88-880* (PRJNA13066)(Ma et al. 2009) and the indexed genome of *R. microsporus* (Mondo et al. 2017). HTSeq (version 0.8.0)(Anders, Pyl, and Huber 2015) was used to quantify the output. Trinity and edgeR (version 3.16.5) were then used to analyse differential expression (Grabherr et al. 2009). For the single cell RNA-Seq data, the 10X genomics analysis pipeline (Loupe Cell Browser V 2.0.0, Cell Ranger Version V 2.0.0) was used to align reads to the *mus musculus* genome (version MM10), and quantify the output. For single cell analysis, the samples were then aggregated using this pipeline, to allow comparisons between samples.

Other

Rhizopus delemar Protoplast Formation

R. delemar spores were harvested as above and incubated in 10ml of SAB broth at 37°C until germ tubes roughly equal in length to the diameter of the spores had formed. The germlings were then spun at 3000 RPM for 3 minutes and the supernatant removed. The germlings were washed in PBS and resuspended on 10ml of 0.6M KCl, with the addition of 50mg of glucanex. The germlings were incubated at 30°C, 100RPM and checked periodically for protoplast formation using brightfield microscopy. The bodies that formed were imaged with brightfield microscopy. To check for protoplast formation, the resulting bodies were immersed in deionised water to check for lysis. Protoplasts formed were incapable of germinating.

Zebrafish

Macrophage isolation and RNA Extraction

Zebrafish husbandry and infections were performed by Aleksandra Jasiulewicz. Zebrafish larvae expressing either mCherry macrophages (mCHerry+), GFP neutrophils (GFP+) or wildtype (AB) were used in the following work. The Zebrafish Larvae, infected and uninfected, were suspended in fixation solution (10ml 4% PFA solution + 5ml 3% Sucrose), on ice for 10 minutes. 1ml of the fixation solution was used for every 10 larvae. The fixation solution was then removed and the larvae were dissociated in 1ml of 0.25% Trypsin (preheated to 37°C) per 10 larvae, the mixture was incubated at 37°C for 10 minutes, mixing via pipetting periodically. To terminate dissociation, 10% FBS and 1ul of 1M $CaCl_2$ were added per 1ml of trypsin to the mixture. The solution was then spun at 800G for 3 minutes to pellet the dissociated cells and remove the dissociation media. The cells were resuspended in an

appropriate amount of PBS for sorting (1ml per 10 larvae) and kept on ice until sorting. Sorting was carried out using a BD FACSaria sorter, cells were sorted based on fluorescent signal when compared to the signal obtained from wild-type (AB) zebrafish cells. The sorted cells were then kept on ice (for no longer than 2 hours) until RNA extraction. Total RNA was extracted following the Recoverall guidelines, modifying the protease digestion step to 3 hours at 50° (Hrvatin et al. 2014).

Chapter 2: Mucorales Spore Germination Characterisation

This work has been adapted from the book chapter "Spore Germination of Pathogenic Filamentous Fungi" (Sephton-Clark and Voelz 2017), for which I performed the literature search, wrote the manuscript, completed revisions, and prepared the figures. Selected results have been previously published in and "Pathways of Pathogenicity: Transcriptional Stages of Germination in the Fatal Fungal Pathogen Rhizopus delemar" (Sephton-Clark et al. 2018) for which I conceived, designed and performed the experiments, collected the data, performed the analysis and interpretation, wrote the manuscript, completed revisions, and prepared the figures.

This chapter will introduce the varying germination phenotypes observed across Mucorales species. Results obtained through live cell imaging, FACS and extracellular vesicle isolation demonstrate that species within the Mucorales order exhibit a range of germination rates and physiologies. Phenotypic heterogeneity to this extent has implications for diagnostic testing, pathogenicity and phylogenetic assignment.

Germination phenotype diversity

Large variation is seen in spore physiology between fungal species; ungerminated spores may range in diameter from 2.8 to 8.85um (Reponen et al. 2001; Li et al. 2011), and present in a variety of shapes from spherical to ellipsoidal (Reponen et al. 2001). Some spores require an initial swelling/isotropic growth phase prior to hyphal germ tube emergence, the timing of which also varies between fungal species.

Filamentous fungi rely upon germination to transform from spore form to filamentous form. Germination may occur at a variety of rates, and optimal germination conditions vary between species. *Aspergillus* species will germinate fully using a sugar cane bagasse substrate, by 13 hours. In comparison*, Rhizopus microsporus* will germinate under the same conditions in 11 hours, whilst *Myceliophthora thermophila* fails to germinate completely (Hassouni et al. 2007). For a single species such as *Aspergillus niger*, environmental requirements will also govern germination rate. Within an optimal temperature range (19-45°C) *A. niger* will germinate in 12 hours, however at a suboptimal temperature range (50-60°C), *A niger* fails to germinate (Hassouni et al. 2007). It is unclear how Mucorales spores germinate in relation to one another, and whether this impacts pathogenicity.

Over the course of germination, the fungal spore wall changes in a dynamic nature. Spore cell wall components are produced and organized by several cell wall associated enzymes including chitin synthase and deacetylase, β(1,3)-glucan synthase, α(1,3)-glucan synthase and Mannosyltransferase for polysaccharide production, with the corresponding transferase enzymes responsible for remodelling polysaccharide portions of the cell wall (Karkowska-

kuleta and Kozik 2015). These enzymes enable constant reorganisation over the course of germination. The production of melanin in *Aspergillus fumigatus* is known to require the ALB1, ARP1/2, ABR1/2, and AYG1 enzymes which make up the dihydroxynaphthalene melanin pathway (Tsai et al. 1999; Bernard and Latge 2001; Pihet et al. 2009). These enzymes are also important for restructuring and reorganizing the cell wall throughout germination. They also play large roles in the production of filaments, as well as the production of new spores themselves. The *Aspergillus fumigatus* genes chsC,G&E, all encoding for chitin synthases, are required for proper conidiation, as well as hyphae formation, whilst the functionality of chitin synthases also appears to be required for the acquisition of melanin to the cell wall of *C. neoformans* (Mellado et al. 1995, 1996; Aufauvre-Brown et al. 1997; Tsai et al. 1999; Beauvais et al. 2001; Anand, Yadav, and Yadav 2016; Tsirilakis et al. 2012). Although the genomes of Mucorales species show the presence of predicted cell wall remodelling enzymes, the roles which they play throughout germination is unknown.

The structural changes of germinating *Aspergillus fumigatus* conidia have been tracked to show both compositional and spatial changes over this time, a characteristic which is also observed in Mucorales spores (Figure 2,3,4) (Dague et al. 2008). Over the course of germination, a loss of hydrophobicity can be seen in *Aspergillus*, which is due to a rearrangement of the hydrophobic rodlet outer layer as the *Aspergillus* conidia swell. After 2 hours of germination, the hydrophobic rods form clusters, surrounded by hydrophilic polysaccharides, resulting in a heterogeneous *Aspergillus* coat (Dague et al. 2008). Work done in the plant pathogen *Phanerochaete chrysosporium* shows a similar trend, as the outer rodlet coat is disbanded over the course of germination (Dufrêne et al. 1999). The conidial cell wall of the pathogenic mold *Scedosporium boydii* also shows a decrease in hydrophobicity over the course of

germination, along with a decreasing electrostatic charge, an increased accessibility of polysaccharides and lectins within the cell wall and a remodelling of cell wall proteins. Conidial GPI anchored proteins are swapped for hyphae specific GPI anchored proteins over germination (Ghamrawi et al. 2015). Taken together, the highly dynamic restructuring of the cell wall of spores as they germinate is dependent on many enzymes and components, which appear to act cooperatively in order to provide structure and environmental protection. How these enzymes behave in Mucorales species throughout germination is unknown.

This cell wall remodelling may aid the release of extracellular vesicles (EV), which fungal species such as *Magnaporthe oryzae* release over the course of germination (Rybak and Robatzek 2019). Extracellular vesicles have been implicated in fungal pathogenesis (Panepinto et al. 2009; Yoneda and Doering 2006)(Alspaugh 2015), and have been reported to carry RNA, lipids, fungal cell wall components, complex sugars and enzymes (Albuquerque et al. 2008; Vallejo et al. 2011; Da Silva et al. 2015; Eisenman et al. 2009; Muxing Liu et al. 2018b). Recently Liu et al detected the presence of small RNAs in EVs isolated from *R. delemar* (Liu et al. 2018a). sRNAs are thought to play roles in modulating plant-pathogen interactions (Zhao, Liang, and Zhou 2018) and have the potential to modulate host immunity in response to infection, proving a potent potential virulence factor. Whilst fungal EVs often appear to carry cargo implicated in pathogenesis, EVs from *Candida albicans* have been shown to offer 'protection' in a *Galleria mellonella* infection model (Vargas et al. 2015). Despite their role, it remains unclear where EVs originate, and exactly how they traverse the fungal cell wall. Whilst Rodrigues et al propose the cytosol as a potential EV reservoir, Wolf et al. provide evidence for EV formation via membrane budding (Rodrigues et al. 2013; Wolf et al. 2014). Recent work has shown that the cell wall of *C. albicans,* and *C. neoformans,* is permeable to vesicles 60-80nm in size. The viscoelastic properties of the cell wall may therefore allow for EV traversal (Walker et al. 2018).

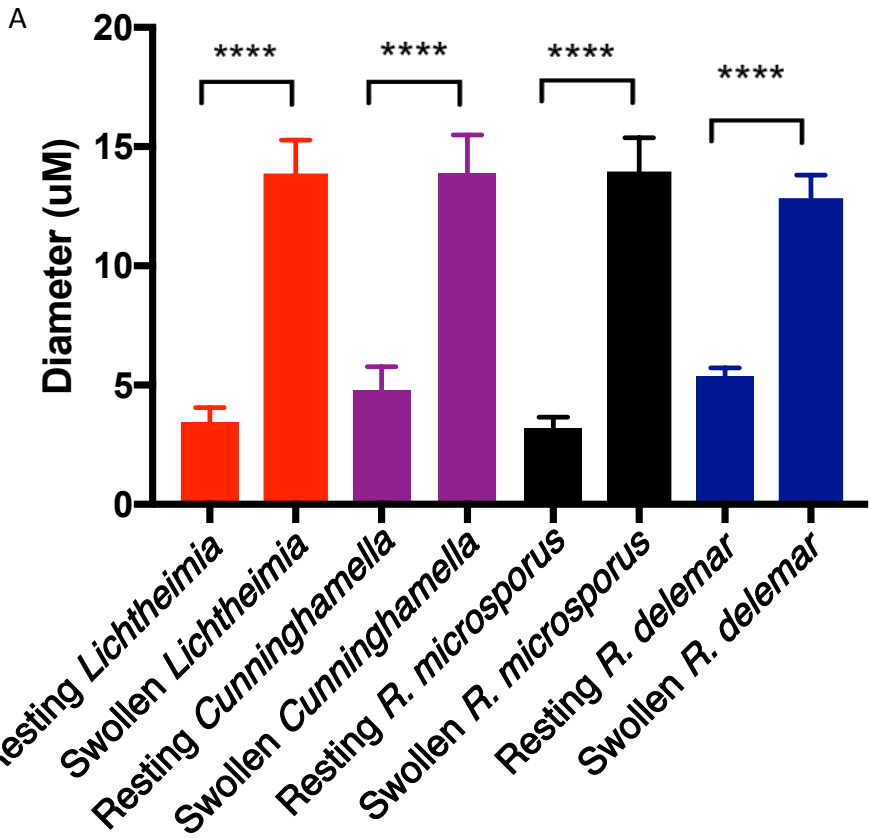
The mechanism by which EVs traverse the cell wall remains unclear, however several studies have revealed the presence of cell wall degrading enzymes within fungal EVs, offering a potential mechanism for this understudied process. The release of extracellular vesicles over the course of germination has been poorly studied to date, and remains unknown in *Rhizopus sp*.

The definition of germination varies widely between studies and fungal species, an approach which standardises the definition and measurement of germination would benefit the field. For the work presented here, germination is said to have been reached upon the formation of a hypha, the length of which is greater than or equal to the diameter of the spore body from which it emerges. The following results address gaps in the literature regarding comparative germination phenotypes, germination dynamics of the Mucorales cell wall and extracellular vesicle release.

Results

Cell Size and Germination Rate

The following results focus on germination phenotypes observed in *Rhizopus delemar, Rhizopus microsporus*, *Cunninghamella* and *Lichtheimia* (see Chapter1 Figure1, for phylogenetic reference). These species were chosen due to the high frequency at which they are isolated from mucormycosis cases, and access to clinical isolates of each species. Germination rates and cell size measurements were determined with live cell imaging. In all species, there is a significant difference between resting and swollen spore diameters (Figure 1a, $p<0.05$), whilst hyphal width and length remains similar between species (Figure 1b). *R. delemar* germinates at an increased rate compared to all other species (Figure 1c), consistent with reports that indicate *R. delemar* to be a rapidly germinating species, which might account for the frequent isolation of *R. delemar* from mucormycosis cases (Mingfu Liu et al. 2015).



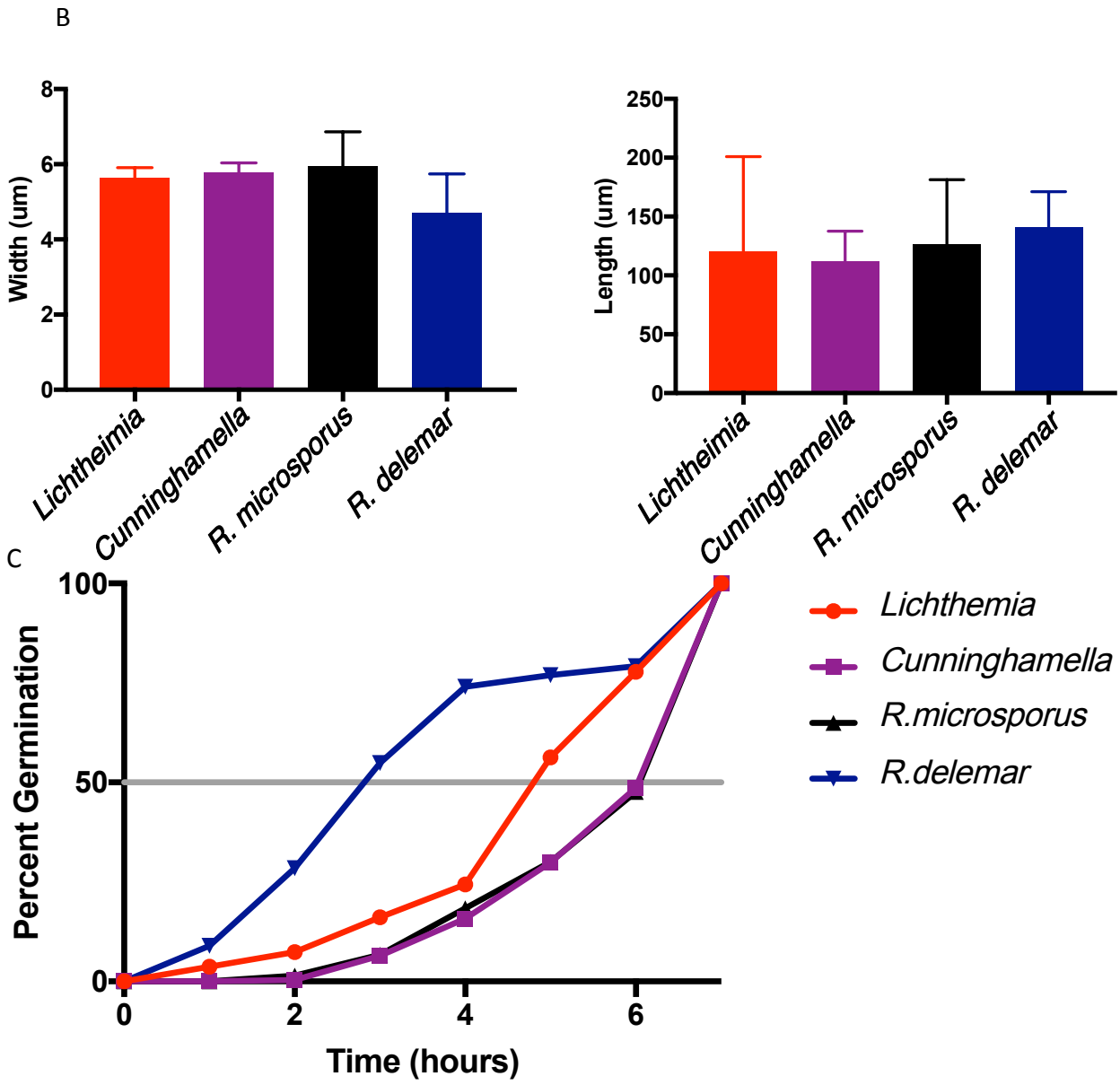


Figure 1. Germination phenotypes vary between Mucorales species. a) Diameter of resting and swollen spores of: *Lichthemia, Cunninghamella, R. microsporus* and *R. delemar*. N=3 biological repeats per species. 200 spores measured per repeat. Error bars show standard deviation. All resting vs. swollen pairings showed a significant difference between spore diameter when a Student's t- test was applied, significance represented by asterisk (****=p<0.05). b) Width and Length of hyphae produced by *Lichtheimia, Cunninghamella, R. microsporus* and *R. delemar* spores. c) Percent germination of *Lichtheimia, Cunninghamella, R. microsporus* and *R. delemar* spores over time (n=1)

Morphology and Cell Wall Dynamics

The cell wall alters over the course of germination in multiple fungal species (Dague et al. 2008; Dufrene et al. 1999). Multiple studies have focused on rodlet dynamics in *Aspergillus species* as a measure for cell wall restructuring, however the genome of *R. delemar* lacks genes with predicted functions in rodlet formation, and SEM images of *Rhizopus arrhizus* confirm a lack of rodlets (Wurster et al. 2017). Like other Mucorales species, the genome of *R. delemar* appears enriched for chitin synthases (Ma et al. 2009) and deacetylases (Chibucos et al. 2016), twice as many seen for many dikaryotic fungi (Ma et al. 2009). For this reason, cell wall chitin/chitinosan content was chosen as an initial measure of cell wall plasticity over germination. Flow cytometry revealed dynamic processes which take place within the cell wall over the course of swelling (Figure 2). Over the 6-hour course of isotropic swelling and germ tube emergence, CFW staining becomes more intense, indicating that more chitin/chitinosan is available to the stain. By 6 hours all species show an intense stain for CFW, and both *Mucor cbs* and *Mucor nrrl* (Figure 2. C,D) show slightly increased staining at 0 hours, indicating that *Mucor* species display more chitin/chitinosan on the resting spore surface.

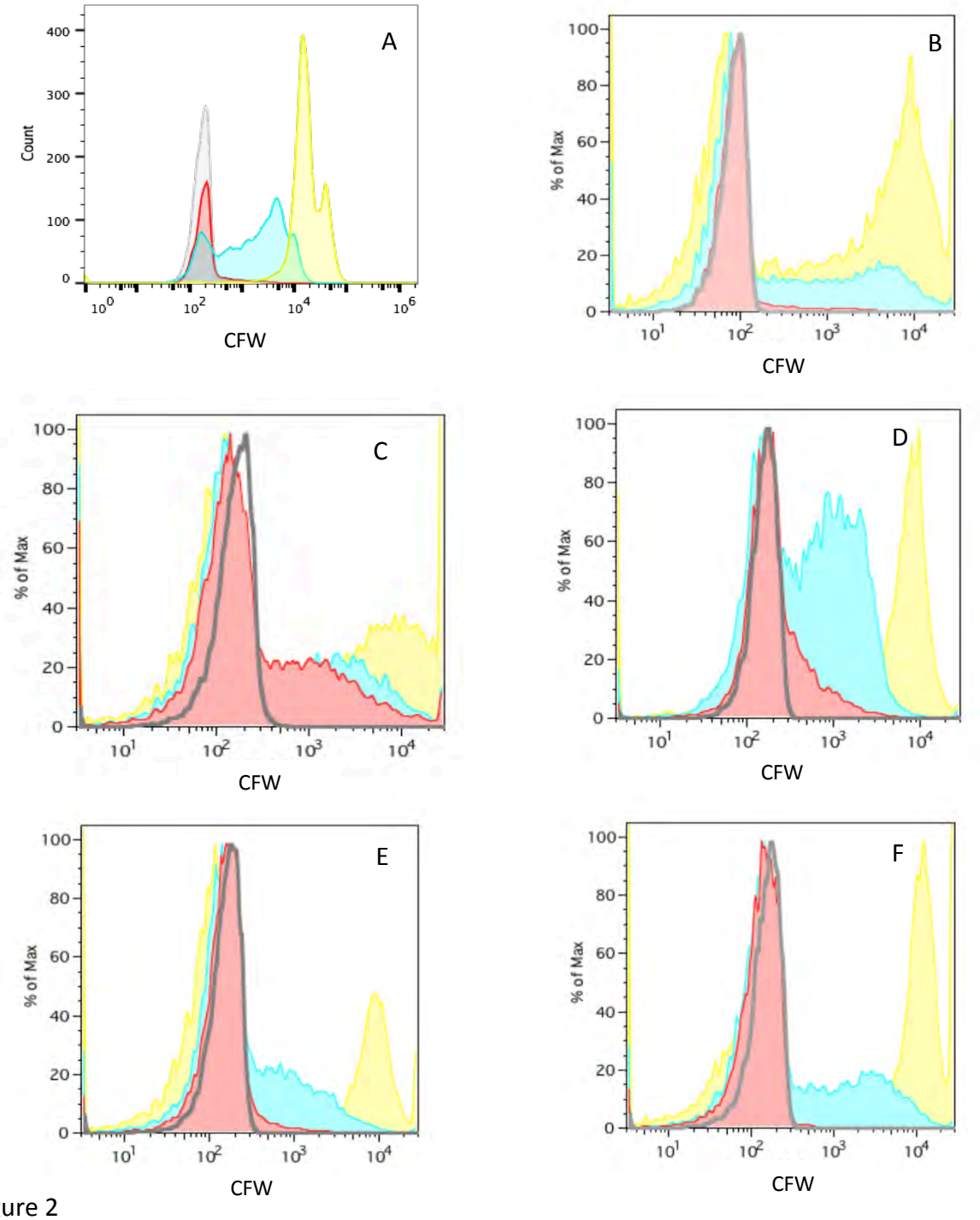


Figure 2

Flow cytometry data displayed as histograms with % of events (Y axis) vs. fluorescence intensity (X axis). Spores swollen for: 0 hours (red), 3 hours (blue) and 6 hours (yellow) in SAB broth. Unstained control is represented in grey. a) *R. microsporus* stained with CFW b) *R. delemar* stained with CFW c) *Mucor cbs* stained with CFW d) *Mucor nrrl* stained with CFW e) *Cunninghamella* stained with CFW f) *Lichthemia* stained with CFW

To better characterise the distinct phases of germination, *R. delemar* and *R. microsporus* were chosen for higher resolution phenotyping. Germination is characterized by three distinct transitions: dormancy to swelling, swelling to germ tube emergence, and the switch to sustained filamentous growth. For both species the switch from dormancy to swelling was triggered by exposure to rich media. Swelling, characterized by an isotropic increase in size, continued for 4 to 6 h (Figure 3). Once fully swollen, germ tubes emerged from the spore bodies. Most *R.delemar* spore bodies (75.5%, Fig. 1C) produced hyphae that exceeded the diameter of the spore body in length by 4-5 hours, whilst *R. microsporus* requires 6-7 hours to reach this point. At this time point, the spores were considered fully germinated. Hyphal growth was monitored up to the 24 hours post germination initiation for *R. delemar* to inform subsequent RNA-Seq studies.

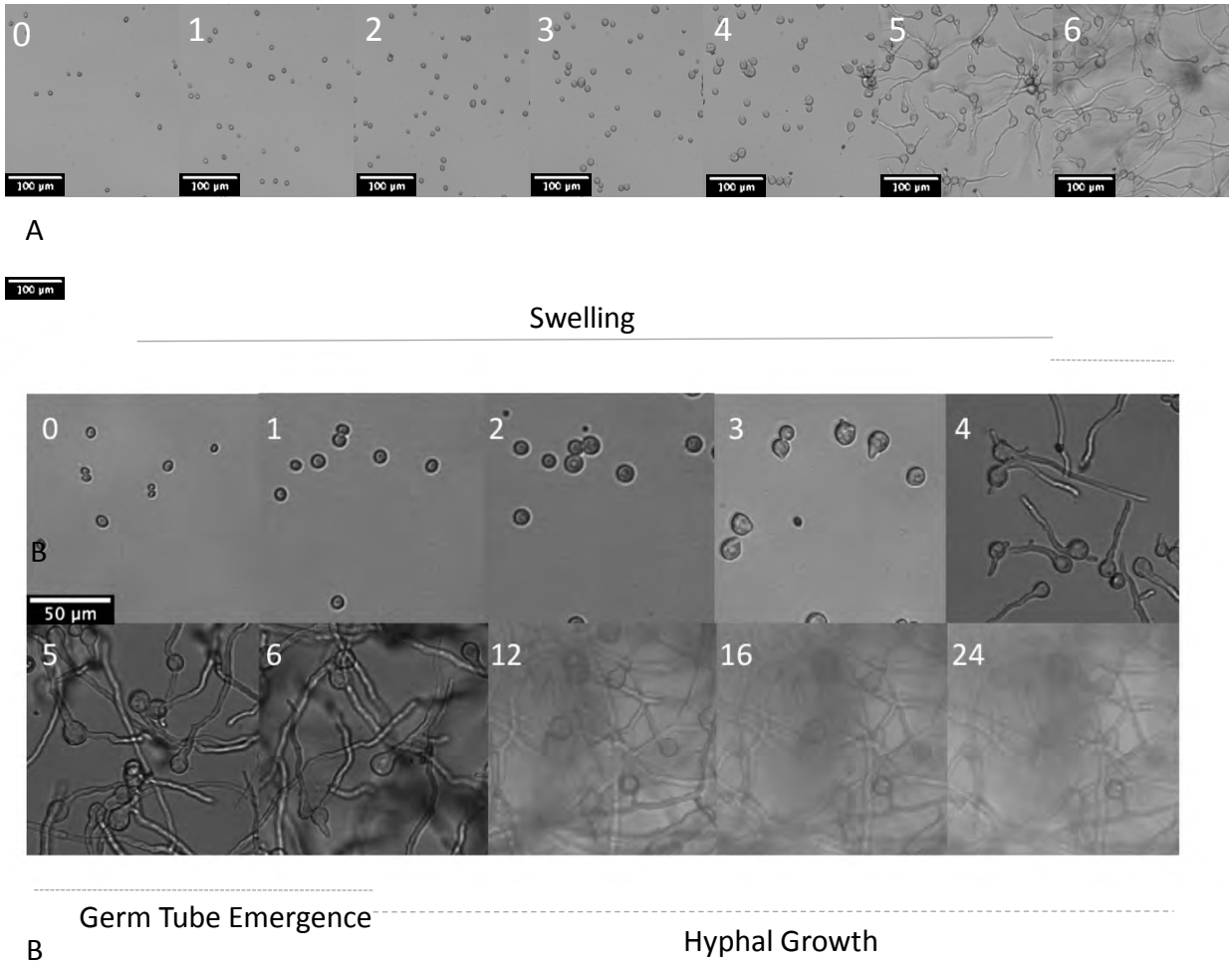


Figure 3. Germinating spores exhibit three distinct phenotypes a) Images taken from time lapse live cell imaging of *R. microsporus* spores that have germinated for 0-6 hours (left to right) b) Images taken from time lapse live cell imaging of *R. delemar* spores that have germinated for 24 hours.

To determine whether the increase in cell wall staining observed via flow cytometry was due to the emergence of hyphal buds/tips with high chitin/chitinosan exposure, *R. delemar* and *R. microsporus* spores were imaged after staining with CFW and FITC, to determine the location on cell wall restructuring. CFW stains specifically for chitin and chitinosan whilst FITC

specifically binds protein. Both species exhibit an increase in chitin/chitinosan and protein content uniformly across the spore body throughout germination (Figure 4. A,B). *R. delemar* appears to display a reduced quantity of chitin/chitinosan and protein in its hyphal protrusions (Figure 4a), indicating the process of cell wall restructuring may be especially important during the initial stages of germination.

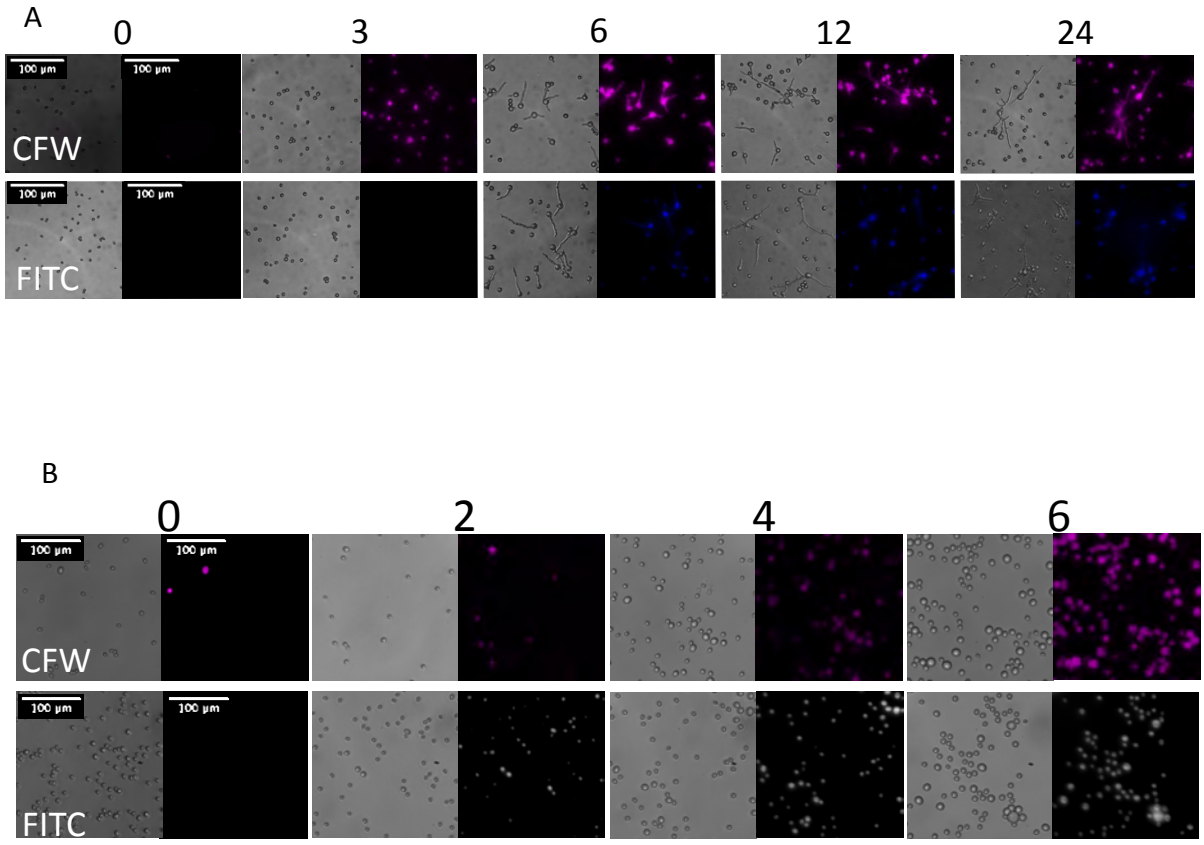


Figure 4. Cell wall dynamics. A) *R. delemar* spores germinated for 0, 3, 6, 12, and 24 h, stained with calcofluor white (CFW) and fluorescein (FITC). B) *R. microsporus* spores germinated for 0, 2, 4 and 6 h, stained with calcofluor white (CFW) and fluorescein (FITC).

Extracellular Vesicle Release

To determine whether Mucorales species also release extracellular vesicles (EVs) over germination, as has been reported in other species (Rodrigues et al. 2008), an EV isolation protocol was applied to the supernatant of germinating *R. delemar* spores. EVs were successfully isolated and the size of these particles determined. The concentration of EVs isolated increases post germination initiation (Table 1) and after 4 hours, the size of the particles released increases significantly, with the average size for 0-2 hours being 2.74nm and the average size for 4-6 hours being 269nm (Figure 5), however the charge remains neutral (Table 2). pH has been shown to influence charges across EV membranes, therefore influencing EV ability to fuse with external cells via micropinocytosis (Yáñez-Mó et al. 2015). As germinating *R. delemar* spores are able to germinate in a range of pHs, and may influence the pH of their microenvironment as they do so, effective EV cargo delivery may be altered depending on germination stage or environment.

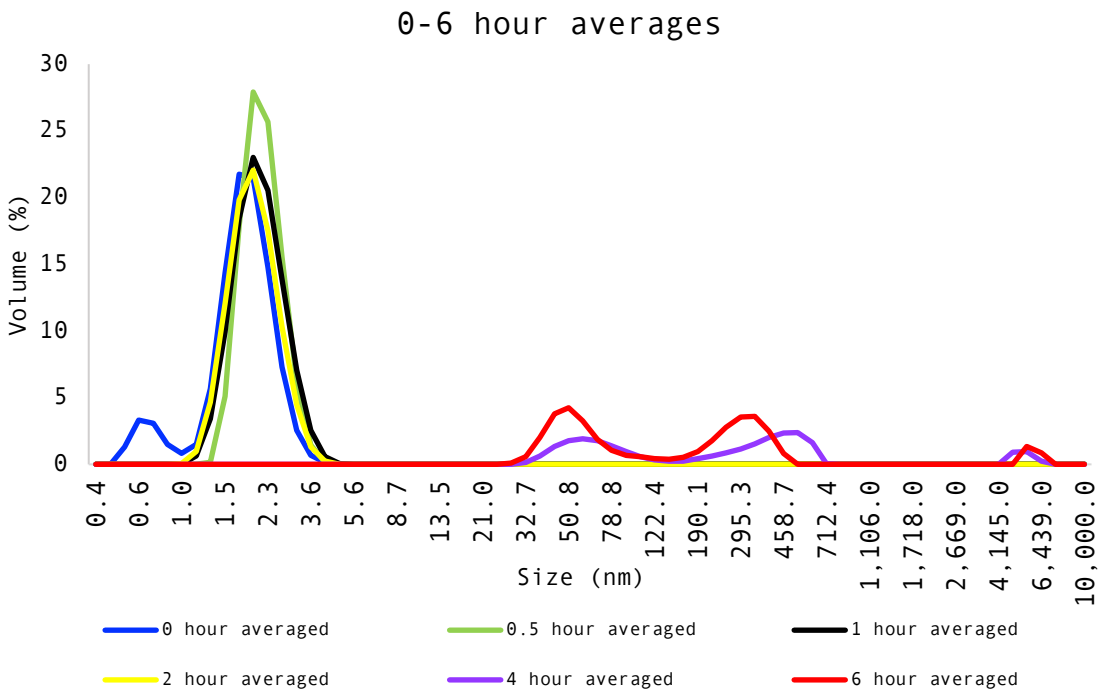


Figure 5. Extracellular vesicles are detected from spores which have germinated for 4-6 hours. Graph showing extracellular vesicle (EV) size profiles of EVs isolated from *Rhizopus delemar* spores germinated for 0-6 hours, data derived via dynamic light scattering (dls) analysis. Dls allows the size of particles dispersed in a liquid to be derived from the scattering of a laser beam.

Condition	Average Concentration (particles/spore)
0 hour	3.78
1 hour	10.40
2 hour	7.87
4 hour	8.96
6 hour	16.99

Table 1: showing the concentrations of EVs from spores germinated in SAB, recorded by nano sight.

Condition	Average charge (mV)
0 hour	-19.75
0.5 hour	-12.83
1 hour	-11.43
2 hour	-11.48
4 hour	-11.49
6 hour	-12.06
SAB control (no spores present)	-14.82

Table 2: showing the charge of the isolated EVs from spores germinated in SAB, recorded by zetasizer. By applying an electric field to particles analyzed by dynamic light scattering, the zeta potential (potential difference across phase boundaries, measured in mV) was determined.

Conclusions

The species studied here display similar spore and hyphae sizes throughout germination. Conversely, germination timing is varied between Mucorales species (Figure 1c). Despite timing differences, the germination phenotype of swelling, germ tube emergence and finally hyphal growth appears conserved across the species studied. Trends in cell wall component (chitin/chitinosan and protein) exposure varies between species. Closely-related species exhibit similar exposure patterns to one another over germination (Figure 2: *Mucor cbs* and *Mucor nrrl*), however these patterns are distinct from other species. Finally, germinating *Rhizopus delemar* spores were found to release extracellular vesicles after 4-6 hours (Figure 5). Small particle peaks (1-4nm) were also detected from spores that germinated for 0.5-2 hours, however given the size, these peaks likely represent artifacts or cell wall components. These results and the potential for further work will be discussed here.

Discussion

The phenotypic variation over germination highlights the broad phylogeny of the Mucorales order, which has been better described taxonomically through DNA based reclassification (Hoffmann et al. 2013; Gryganskyi et al. 2010; Abe et al. 2007). The species studied here display similar resting spore, swollen spore and hyphal sizes (Figure 1a,b), thus making diagnosis difficult through single time point imaging alone (Yang et al. 2016). However the differential germination dynamics of these species (Figure 1c, 2) might provide a cost effective alternative for diagnosis through multiple time point imaging and cell staining. All species appear to undergo cell wall remodelling during germination (Figure 2, 4), however the extent to which each species remodels is variable. Cell wall remodelling and unmasking in response to pH has been linked to pathogenicity in *Candida albicans* (Sherrington et al. 2017), and it may be possible that the variation in remodelling of these species is linked to variation in pathogenicity. The known Mucorales pathogenicity factor CotH resides within the cell wall of pathogenic Mucorales species, and unmasking of this factor over germination may result in increased pathogenicity. This would support the observation that swollen Mucorales spores are more pathogenic than ungerminated ones (Chibucos et al. 2016). Mucorales cell wall remodelling may occur in response to pH changes, as we see for the pH dependent recruitment of aquaporins over germination (Turgeman et al. 2016). The EV data shown here adds to the sparse literature on fungal EV release over germination. It is likely that these EVs contain sRNAs (Liu et al. 2018b), however they may include other components such as enzymes or pathogenicity factors which might aid environment dependent colonisation. Both seedlings and neutrophils have been shown to release EVs capable of interacting with their respective fungal pathogens (*Sclerotinia sclerotiorum* and *Aspergillus fumigatus*) to inhibit growth (Regente et al. 2017; Shopova et al. 2019).

It is possible that *Rhizopus delemar* might release EVs in a similar manner to ensure spore survival and propagation, despite environmental responses to the spores.

Further Work

This field would benefit from further work focusing on the impact of environment on germination. Most fungal germination studies use rich media, however this does not reflect the 'real world' environments which many fungi have evolved to inhabit. Studies which address germination phenotypes in a range of 'realistic' media (soil, blood, guano etc.) would greatly increase our understanding of how these pathogens interact with their surroundings, and advance our understanding of their pathogenesis. This work would also benefit from an in-depth analysis of the precise alterations which occur in the Mucorales cell wall over germination, and any molecules they may secrete (eg EVs). High resolution mass spectrometry and scanning electron microscopy would allow these areas to be explored further.

Chapter 3: Hallmarks of the Mucorales genome

Results have been previously published or adapted from the following articles: "Pathways of Pathogenicity: Transcriptional Stages of Germination in the Fatal Fungal Pathogen Rhizopus delemar"(Sephton-Clark et al. 2018) and "Host-pathogen transcriptomics of macrophages, Mucorales and their endosymbionts: a polymicrobial pas de trois" (Sephton-Clark et al. 2019). For both I conceived, designed and performed the experiments, collected the data, performed the analysis and interpretation, wrote the manuscript, completed revisions, and prepared the figures.

This chapter will introduce characteristics of the Mucoralean genome. Results describing genome assembly, annotation and comparisons of two Mucorales species, *Rhizopus delemar* and *Rhizopus microsporus*, show the conservation of a core set of fungal features, but divergence between the two genomes in enrichment of substrate binding, transporter activity, endosome activity and DNA repair.

Mucoralean Genomics

Previously *Rhizopus oryzae* was reclassified into two species based on the ability to produce either fumaric-malic acid or lactic acid. Strains capable of producing fumaric-malic acid were reclassified as the species *Rhizopus delemar.* This reclassification led to two distinct clusters, when ribosomal DNA from both *Rhizopus oryzae* and the newly proposed *Rhizopus delemar* was used to determine a phylogenetic relationship between the two (Abe *et al.* 2007). This work is supported by findings from Chibucos et al (Chibucos *et al.* 2016), who identify two distinct clusters (Figure 1) within *Rhizopus* species, based on whole genome sequences.

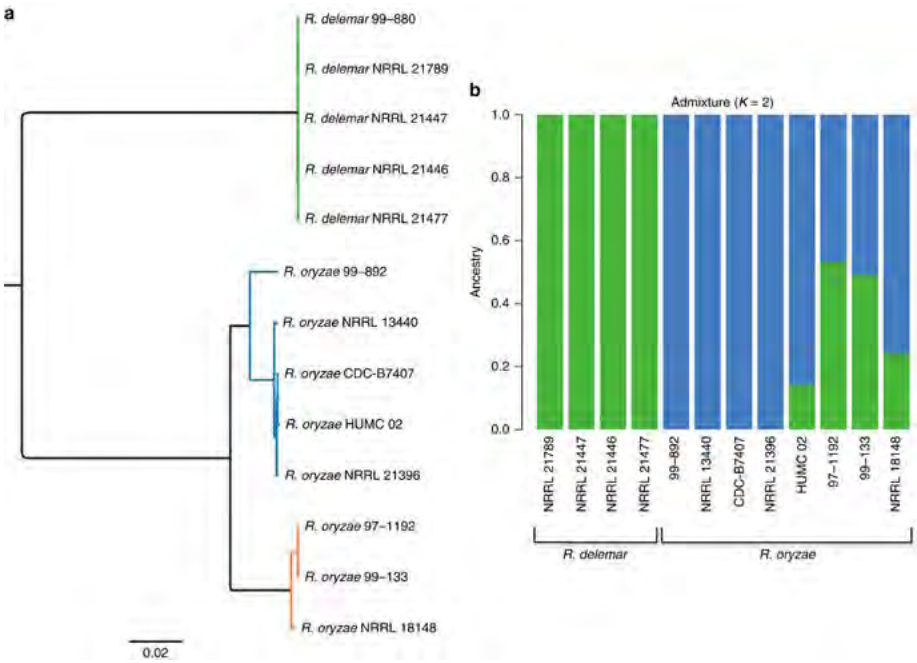


Figure 1. Figure and legend from "*An integrated genomic and transcriptomic survey of mucormycosis-causing fungi*", Chibucos et al. 2016 (Chibucos *et al.* 2016). a) SNP-based whole-genome maximum-likelihood phylogeny of all 13 *R. delemar* and *R. oryzae* strains. All nodes have a bootstrap value of ≥97 out of 100. b) Population structure inferred using the program Admixture. Values represent fraction of population ancestry denoted by colours: green (*R. delemar*) and blue (*R. oryzae*).

Prior to reclassification, the genome of *Rhizopus oryzae 99-880* (now *Rhizopus delemar 99-880*) was sequenced and assembled from whole genome shotgun library sequencing data by Ma et al. (Ma *et al.* 2009). Although transcriptomic data was not available for assembly and annotation, the resulting assembly identified the 45.26Mb genome to be repetitive (20% transposable elements), and consisting of 13,895 protein coding genes. A whole genome duplication event, followed by subsequent gene duplication events, indicated by the segments of duplicate gene pairs scattered throughout the genome, resulted in multiple gene family expansions. These gene families have roles in ergosterol biosynthesis, cell growth, signal transduction, protease secretion and subtilase secretion (Ma *et al.* 2009). Notably, analysis revealed a lack of the common siderophore producing non-ribosomal peptide synthases, highlighting *R. delemar's* reliance on Rhizoferrin for iron scavenging. This is especially relevant in the context of this species' requirement for iron in order to germinate, and its implications in pathogenesis (Ibrahim *et al.* 2007; Andrianaki *et al.* 2018; Kousser *et al.* 2019).

The chitin deactelyase family is also expanded in *R. delemar,* compared to non-early divergent fungi, as it is for other mucoralean species. There appear to be 2,658 core clusters present in the 19 Mucorales species included in the analysis carried out by Chibucos et al. We see that there is a range of genome sizes in this study, with the smallest genome consisting of 22.3Mb, whilst the largest is 96.7Mb. Highly pathogenic Mucorales species from this study were found to contain a unique set of 174 genes, conserved in *Rhizopus* species but lacking in non-*Rhizopus* species and thus labelled as putative virulence genes. Amongst the virulence genes conserved across the Mucoralean family, CotH, which codes for a surface protein uniquely expressed by Mucoralean species, is present in varying copy numbers between Mucorales

species. The genomes of *Rhizopus delemar, Rhizopus oryzae* and *Rhizopus microsporus* and *Lichtheimia corymbifera* all contain over 6 CotH genes, whilst other Mucorales species contain fewer than 6. The number of CotH genes present has been linked directly to pathogenicity, however species containing fewer than 6 copies, such as *Mucor circinelloides,* may still cause mucormycosis (Chibucos *et al.* 2016). It is clear that CotH plays an important role in virulence, however it does not account for all interactions between Mucorales species and host.

Aside from CotH, few key pathogenicity genes/characteristics have been identified within all Mucorales species. Around 54% of clinical Mucorales isolates are were found to harbour bacterial endosymbionts from the Burkholderia family in a study carried out by Ibrahim et al. (Ibrahim *et al.* 2008), and though previous work demonstrated that the presence of these endosymbionts did not impact infection of endothelial cells (Ibrahim *et al.* 2008), recent work by Itabangi et al (Itabangi *et al.* 2019) has shown that the bacterial endosymbiont within *Rhizopus microsporus* impacts the fungus' interaction and regulation of the host immune system. A bacterial endosymbiont also impacts sexual reproduction in *R. microsporus* (Mondo *et al.* 2017), demonstrating the unique relationship crucial to development and pathogenicity between *Rhizopus microsporus* and its bacterial endosymbiont.

Although several genome sequences exist for *Rhizopus microsporus* (Horn *et al.* 2015; Mondo *et al.* 2017) a comprehensive analysis of the genome is not available. It is unknown whether *Rhizopus microsporus* underwent a whole genome duplication, similarly to *Rhizopus delemar*. The following results will provide a thorough analysis of both the *Rhizopus microsporus* and *Rhizopus delemar* genome, and include results based on comparative genomics analysis carried out to compare the two species.

Genome Statistics

	Size (Mb)	GC%	Protein	rRNA	tRNA	Gene
Rhizopus delemar	45.32	35.6	17,459	5	239	17,703
Rhizopus microsporus	25.53	37.7	10,891	Not predicted	70	10,959

Results

Resulting Characteristics of the Reannotated *R. delemar* Genome

We utilized our RNA-Seq data to revise the current annotation of the available *R. delemar* genome (revised GFF file available at: https://github.com/psephtonclark/RhizDeleAnno), using BRAKER 2.1.0 (Hoff *et al.* 2016) to improve gene structures and incorporate these into an updated annotation. Compared to the previous annotation (Ma *et al.* 2009), this updated set included 475 new predicted genes, 370 new protein family domains (Pfam terms), 103 new pathway predictions (KEGG-EC), and 96 new transmembrane domains (TMHMM terms) (Figure 2). The updated annotation was assessed for completeness with BUSCO v3 (Simão *et al.* 2015) and was shown to include a good representation of expected core eukaryotic genes, with minimal missing BUSCOs (benchmarked universal single-copy orthologues) (2%)

Re-Annotation of *R.delemar* Genome

Annotation	Vesper Annotation	Vesper Annotation with RNA Seq data incorporated
No. Genes	17,038	17,513
No. Pfam	11,742	12,112
No. EC	2,094	2,197
No. tmhmm	2,238	2,334

R.delemar Genome Annotation Including RNA Seq Data:
Summarized benchmarks in BUSCO notation:
C:88%[D:36%],F:9.8%,M:2.0%,n:1438
1266 Complete BUSCOs
744 Complete and single-copy BUSCOs
522 Complete and duplicated BUSCOs
142 Fragmented BUSCOs
30 Missing BUSCOs
1438 Total BUSCO groups searched

Figure 2. Table displaying genome annotation statistics, comparing the original *R. delemar* annotation (column 1, 'Vesper Annotation') and the annotation updated with our RNA-Seq data (column 2, ''Vesper Annotation with RNA Seq data incorporated'').

Biochemical Pathways Present in *R. delemar*

A custom genome database for *R. delemar* was created with PathoLogic to allow for PathwayTools analysis of the reannotated *R. delemar* genome (Database available at https://github.com/psephtonclark/rhior3cyc). From this, metabolic, signalling and transporter pathways can be visualised with the PathwayTools GUI (Figure 3). This annotation classified genes with predicted or known functions into the following categories: Enzymatic reactions (2061), Transport reactions (13), Activation/inactivation pathways (4), Biosynthesis pathways (166), Degradation pathway (93), Detoxification pathway (8), Precursor Metabolism (20), Macromolecule Modification pathway (4), Metabolic cluster (6), Superpathways (31). This annotation also identified 4377 enzymes, 93 transporters and 1517 compounds. These

pathways and molecules have been visualized in Figure 3. All pathway predictions are based on genomic information. Annotations of model fungal species inform these predictions, therefore the predictions will be more accurate, the more closely related the species are. Key metabolic pathways identified include:

- Carbohydrate Biosynthesis and degradation(sugar, polysaccharide, oligosaccharide)
- Amino Acid Biosynthesis and degradation (polyamine, proteinogenic, modification, β alanine biosynthesis, L-citrulline biosynthesis, L-Ornithine)
- Fermentation and alcohol degradation
- Respiration
- Glycolysis
- TCA Cycle
- Lipoate biosynthesis
- NAD/NADP metabolism
- Polyprenol biosynthesis
- Porphyrin compound biosynthesis
- Tetrahydrobiopterin biosynthesis
- Tetrapyrrole biosynthesis
- Vitamin biosynthesis
- Secondary Metabolite biosynthesis and degradation (Phenylpropanoid, Sugar derivative, Terpenoid)
- Lipid biosynthesis and degradation (choline, FA, phospholipid, sphingolipid, sterol)
- Aminoacyl-tRNA charging
- Nucleoside/tide biosynthesis and degradation
- Aldehyde degradation

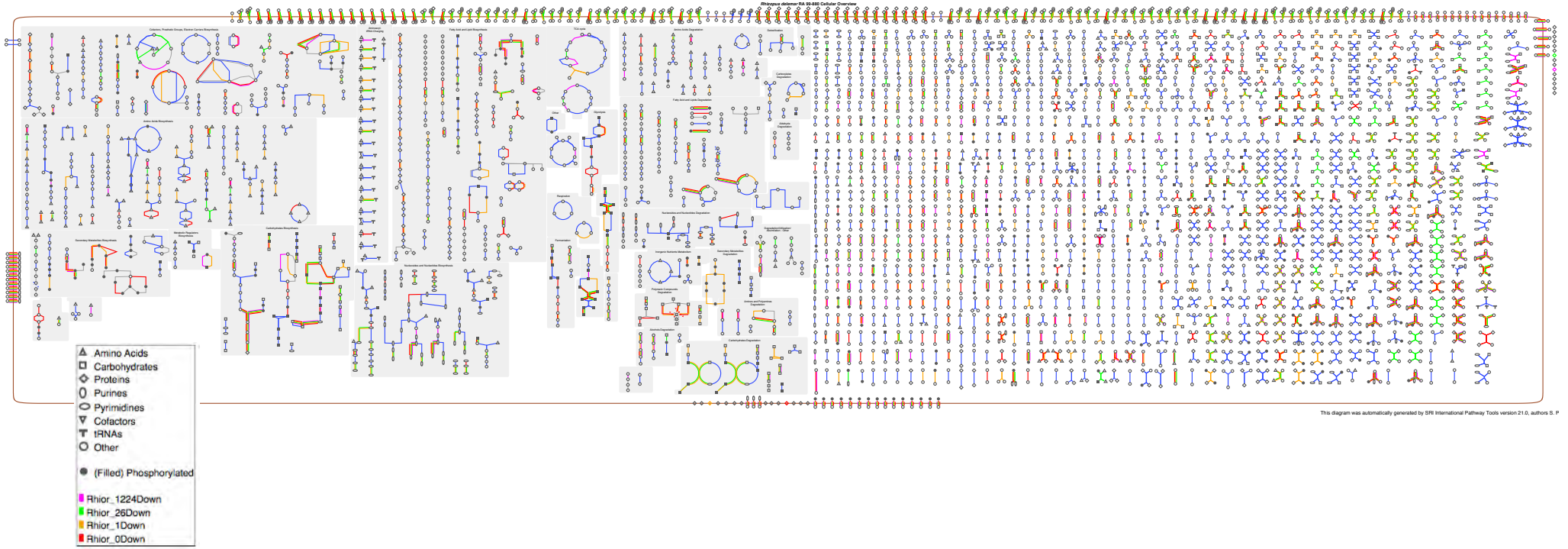


Figure 3. Pathway visualization based on the *R. delemar* genome. The PathoLogic database required to generate this can be found in the online appendix (https://github.com/psephtonclark/rhior3cyc). This annotation was developed via the following method: predicted gene functions were incorporated into the PathoLogic annotation. Pathways were predicted as described by Karp et al (Karp *et al.* 2002), and can be visualized with the PathwayTools GUI. For each prediction there is information available detailing compound structure, enzymatic reactions and other metabolic functions. Metabolomic and transcriptomic data can be overlaid onto the annotation, thus pathways up/down regulated (condition dependent) will be highlighted in various colors (this example shows pink, green, orange and red representing pathways down regulated in hyphal, swelling, germination initiation - 1hr, and resting spores - 0hr, respectively). △= amino acids, □ = carbohydrates, ◇ = proteins, 0 = purines, ⬭ = pyrimidines, ▽ = cofactors, T = tRNA's, ◯ = other

R. delemar WGD Enrichment

Previous work determined *R. delemar* is likely to have undergone a whole genome duplication, followed by multiple individual gene duplication events (Ma *et al.* 2009). The preservation of duplicated genes suggests a specific function for each gene in the pair may have developed. Enriched functions of duplicated gene pairs which occur throughout the genome were determined by testing for significant enrichment of pair GO terms. The genome appears enriched for gene pairs with predicted functions in succinate metabolism, pigment metabolism, cell cycle, amine metabolism, glycosylation, carbohydrate metabolism, cofactor metabolism, sulphur metabolism, coenzyme metabolism, RNA polyadenylation, asparagine metabolism, mitochondrial transport and cellular component biogenesis (Figure 4).

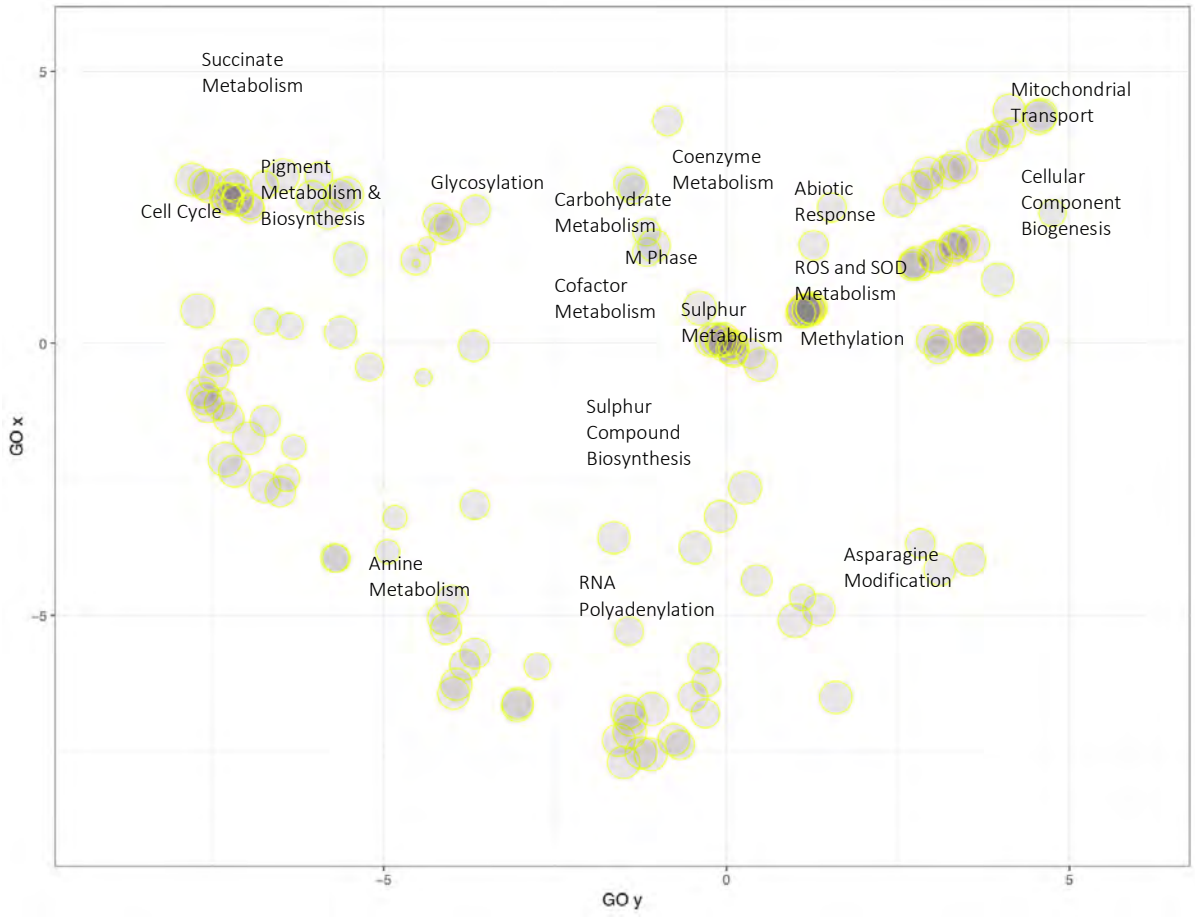


Figure 4. Plot representation of GO terms of duplicated gene pairs enriched in the *R. delemar* genome. The X and Y axis represent semantic space based on ReviGO. The size of the dots correspond the enrichment significance (P Value, Hypergeometric Enrichment).

R. microsporus Genome Assembly and Statistics

A genome assembly of *R. microsporus* was produced with SPAdes, using short read data as input. For this, Mondo et al's assembly was taken as a reference (Mondo *et al.*, 2017), producing an assembly with 4778 contigs. With a GC content of 37.7% (Figure 5), our assembly appeared identical to that produced by Mondo et al. The low resolution of the assembly produced (4778 contigs) is likely due to the lack of long read information and likelihood of a highly repetitive genome, which increases the difficulty of contig joining when relying on short read data alone. As Mondo et al's assembly achieved a higher resolution (131 scaffolds) by making use of long read data, I used this assembly, but replaced nucleotides identified as SNPs (Figure 6) in our *R. microsporus* strain, to produce a reference genome specific to the *R. microsporus* strain used throughout the project (University of Birmingham *R. microsporus* strain). Briefly, I did this by aligning the raw reads obtained through short read sequencing to the assembly produced by Mondo et al. I then called variants present when comparing the sequence of the assembly to the aligned reads. I then inserted the variant nucleotides from our strain into the assembly, in place of the previous nucleotides. In total, 6132 SNPs were identified and replaced (Figure 6).

	Scaffolds
# contigs	4778
# contigs (>= 1000bp)	1096
# contigs (>= 5000bp)	577
# contigs (>= 10000bp)	491
# contigs (>= 25000bp)	313
# contigs (>= 50000bp)	172
# Total length (>= 0bp)	30030542
# Total length (>= 1000bp)	25509162
# Total length (>= 5000bp)	24582835
# Total length (>= 10000bp)	23970496
# Total length (>= 25000bp)	20979206
# Total length (>= 50000bp)	15886454
Largest contig	235405
Total length	27890444
Reference Length	25972395
GC%	37.77
N50	61180
NG50	66773
N75	25555
NG75	31831
L50	138
LG50	123
L75	311
LG75	261
# N's per 100 kbp	1.33

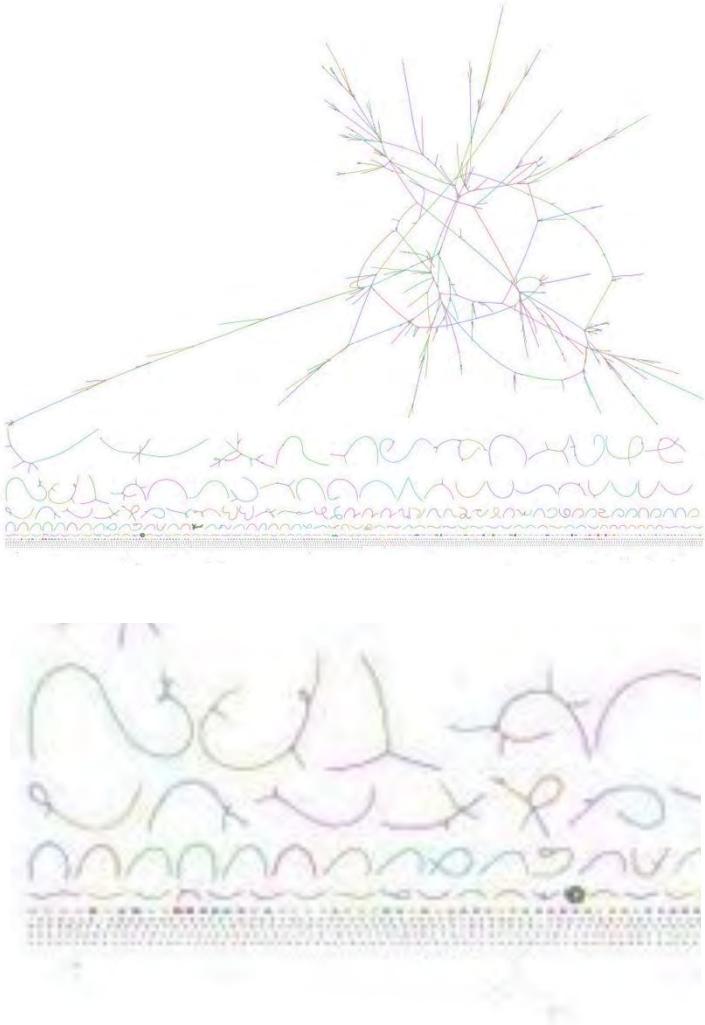
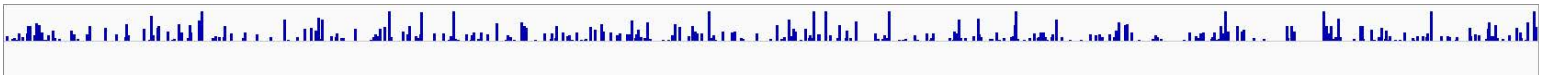


Figure 5. Genome statistics and visual representation for the genome assembly of *R. microsporus*, produced with SPAdes, based on short reads only. All contigs are displayed and demonstrate the poor quality of the assembly. A small section of the assembly has been magnified to display the large number of short contigs incorporated into this fractured genome assembly.



Scaffold 1

Scaffold 131

number of SNPs:	6132
number of MNPs:	0
number of indels:	486

Figure 6. Single nucleotide polymorphisms and indels identified when comparing Mondo et al's assembly to aligned reads representing the UoBirmingham *R. microsporus* strain. The IGV graph (top) demonstrates that Variants (blue columns corresponding to genome location of variants) were identified across the entire genome. To generate this, the UoB *R. microsporus* VCF file (variant call data) was aligned against the Mondo et al *R. microsporus* genome. Variants highlighted in blue.

Comparative Genomics

R. delemar genes with homology to genes in *R. microsporus* were identified via blast+. A comprehensive list of these genes can be found in the online appendix (https://github.com/psephtonclark/RhizoOrthologues). 8,356 genes were identified with a bit score > 90 (E Value < 1 x 10^{-20}) and a percentage identity match of over 60% (Pearson, 2013). 2,121 highly conserved genes were found (identity % > 80%), and those with an identity percentage of over 90% (234 genes) had roles in ribosome structure, translation, protein metabolism, phosphorus metabolism, organelle structure, transcription, respiration, ATP metabolism, sugar metabolism, catalytic activity, ion transport and binding.

Fisher's exact test identified enriched Pfam terms when the genome contents of *R. delemar* and *R. microsporus* are compared. Compared to *R. microsporus,* the genome of *R. delemar* is

enriched for genes with protein domains (PFAM) associated with ion binding, carbohydrate derivative binding, nucleic acid binding, cytoskeletal protein binding, poly(A) binding, NAD+ ADP-ribosyltransferase activity, protein kinase C activity, translation initiation factor binding and inorganic phosphate transmembrane transporter activity (Figure 7). *R. microsporus* is enriched for genes with protein domains corresponding to nucleoside phosphate binding, early endosome activity and DNA repair complex activity (Figure 7).

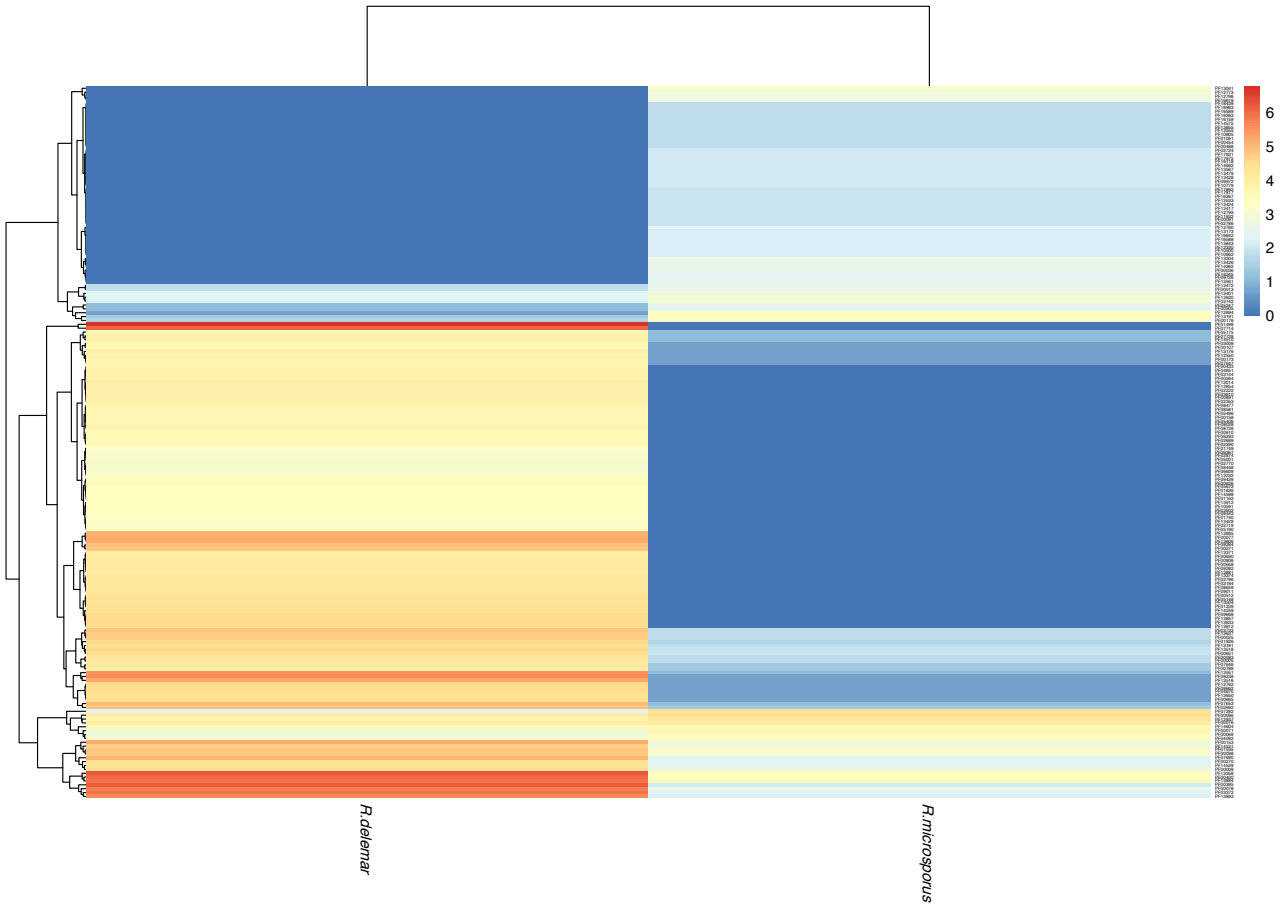


Figure 7. Pfam terms enriched (FDR corrected P Value < 0.01) in *R. delemar* vs *R. microsporus* genomes, with colour representing Log10 Count. A list of the top 50 most significant Pfam terms can be found in the appendix (ComPfam.pdf).

Comparison with *Aspergillus* genomes

R. delemar genes with homology to genes in *Aspergillus niger* were identified via blast+. A comprehensive list of these genes can be found in the online appendix (https://github.com/psephtonclark/Orthologues). 13,613 genes were identified with a bit score > 80 (E Value < 1 x 10^{-20}), of these 2,494 genes had a percentage identity match of over 50% (Pearson, 2013). Highly conserved genes (155 with identity % > 80%) had roles in ribosomal processes, translation, transcription, protein metabolism, enzyme activity (kinase, transferase, hydrolase), ion transport and amine processing.

Conclusion and Discussion

Through incorporation of RNA-Seq data and short read data, an improved *R. delemar* genome and updated *R. microsporus* genome (specific to the UoB *R. microsporus* strain) were produced. The extra *R. delemar* annotation and duplicated genome information will inform further RNA-Seq analysis, whilst RNA-Seq analysis of the UoB specific *R. microsporus* is now possible. Pathway analysis of the *R. delemar* genome revealed predicted pathways, which show minimal missing eukaryotic components (Figure 2). As expected, there appears to be greater conservation between the *R. delemar* and *R. microsporus* genomes, than between the *R. delemar* and *A. niger* genomes. *R. delemar* does however appear enriched for many more functions when compared to *R. microsporus*, a trait which may be explained by the larger genome size and likely whole genome duplication event. Unsurprisingly, the conserved fungal gene core shared by *R. microsporus, R. delemar* and *A. niger* is highly populated with genes predicted (GO) to play roles in protein synthesis, a general process highly conserved across filamentous fungi (amongst other kingdoms). Species-specific genes likely offer an advantage

to that species, which may be environment dependent. For example, the enrichment of endosome activity in the rice seedling blight fungus *R. microsporus* (Lackner and Hertweck, 2011) may aid plant pathogenesis, as it does in *U. maydis* (Bielska et al. 2014). The *R. delemar* genome appears enriched for phosphate transportation, which may support growth in low micronutrient conditions. This is consistent with *C. albicans* reliance upon phosphate transport in stress and starvation conditions (Ikeh et al. 2017).

Further work

To better our understanding of the Mucoralean genome, DNA sequencing of 'ultra-long' reads, via Oxford Nanopore or PacBio technologies, would allow resolution of highly repetitive regions, resulting in chromosomal level genome detail. This would inform better gene predictions and, combined with a whole genome CRISPR-Cas9 knockout library, allow for high quality functional gene annotations. Knockout library analysis may also reveal novel drug targets, potentially increasing our ability to treat mucormycosis.

Chapter 4: Transcriptional States of Germination

This work has been adapted from the book chapter "Spore Germination of Pathogenic Filamentous Fungi" (Sephton-Clark and Voelz 2017), for which I performed the literature search, wrote the manuscript, completed revisions, and prepared the figures. Results have been previously published in "Pathways of Pathogenicity: Transcriptional Stages of Germination in the Fatal Fungal Pathogen Rhizopus delemar" (Sephton-Clark et al. 2018) for which I conceived, designed and performed the experiments, collected the data, performed the analysis and interpretation, wrote the manuscript, completed revisions, and prepared the figures.

This chapter will introduce transcriptional regulation of germination in a range of fungal species. Results will describe the transcriptional landscape of *Rhizopus delemar* over germination. This fine scale RNA-Seq study sheds light on pathways highly regulated over the course of germination, identifying pathways which could be targeted to inhibit germination, providing new treatment options for mucormycosis. Dormant spores are shown to be transcriptionally unique, containing a subset of transcripts absent in later developmental stages. A large shift in the expression profile is prompted by the initiation of germination, with genes involved in respiration, chitin, cytoskeleton, and actin regulation appearing to be important for this transition. A period of transcriptional consistency can be seen throughout isotropic swelling, before the transcriptional landscape shifts again at the onset of hyphal growth. In addition, I compare germinating *R. delemar* spores (transcriptionally) to germinating *Aspergillus niger* spores. The results identify a core set of orthologous genes which show similar expression patterns over germination; however germinating *R. delemar*

also exhibits a uniquely expressed set of genes. These results provide us with a greater understanding of the regulation of germination and highlight processes involved in transforming *R. delemar* from a resting spore state to a hyphal mass.

Germination Regulation

During spore germination, previously dormant spores adapt a metabolically active state that is characterized by rapid changes in the transcriptional landscape, metabolism, cell wall composition and cell physiology. Together these changes lead to isotropic growth which is defined by swelling of the spore and a marked increase in spore size, eventually leading to hyphal growth. The transcriptional, biochemical and physiological changes that take place over this time course will be addressed species by species in the following section.

Aspergillus species

Transcriptional profiling of germinating *Aspergillus niger* shows the largest differences in gene expression between resting conidia and germlings (Novodvorska et al. 2013; van Leeuwen et al. 2013). Conidia having germinated for 2 hours (T2), though barely reaching isotropic growth, show the greatest change in abundances of transcripts of all major classes, when compared to resting conidia. Dormant conidia were enriched in transcripts involved in protein synthesis, whilst T2 conidia contained transcripts involved in protein synthesis, energy production, cell cycle, transcription and translation. Most strikingly, many transcripts present in resting conidia were completely depleted in all other stages studied, demonstrating that the largest transcriptional shift appears at the beginning of germination. T4 conidia showed little alteration in the transcriptional landscape, compared to T2, however metabolic transcripts, such as the NADPH-dependent carbonyl reductase homologue, appear to be the most upregulated when compared to T2 (van Leeuwen et al. 2013).

Conidial Transcripts

It has been suggested that resting *Aspergillus spp.* conidia contain vast quantities of pre-packaged messenger RNA molecules, ready to be translated into proteins and cell components essential for growth and metabolism, once dormancy is broken (Lamarre et al. 2008; Novodvorska et al. 2016). This could explain the vast decrease in transcripts seen between resting and germinated conidia, accounting for the rapid conidial germination response. Others have argued that the large quantity of transcripts present in resting conidia are instead an artifact of conidiation and are degraded upon germination (van Leeuwen et al. 2013).

Transcripts present in large amounts in dormant conidia, which were swiftly depleted, play roles in the degradation of internal trehalose stores, as well as mannitol metabolism, signalling and hydrophobin production. This combination of stored transcripts are likely useful for both the initiation of germination, as well as conidiation, which would support a combination of the models proposed above (van Leeuwen et al. 2013; Novodvorska et al. 2013, 2016). Transcripts for heat shock proteins and ROS protection were also found to be high in dormant conidia and depleted upon germination. Eight proteins involved in ROS protection were found to be specifically enriched. These include catalase A, thioredoxin reductase, and mitochondrial peroxiredoxin (Oh et al. 2010). A triple superoxide dismutase (SOD1,2&3) deletion in *Aspergillus fumigatus* resulted in delayed germination, whilst individual deletions resulted growth inhibition at high temperatures and sensitivity to ROS (Morales Hernandez et al. 2010; Lambou et al. 2010). This indicates stress response and ROS signalling is likely crucial to germination initiation.

A similar study analysing the transcriptional landscape of germinating *Aspergillus niger* elucidated further information on the metabolic changes during the early stages of dormancy disruption (Novodvorska et al. 2013). Dormant conidia were shown to possess many transcripts involved in carbon starvation, trehalose and mannitol biosynthesis. Trehalose mobilisation and utilisation occurs rapidly at the onset of germination in *A. nidulans*, as does mannitol utilisation (Thammahong et al. 2017; Novodvorska et al. 2013), making it likely that these transcripts were pre-packaged to aid germination initiation. Transcripts involved in glycerol synthesis and the metabolism of conidial sugar solutes were also abundant in the dormant conidia and are thought to be important in the immediate onset of germination to provide energy, as their presence may allow for the immediate access to sugars required for growth.

The presence of abundant metabolic transcripts and ROS response transcripts within dormant conidia supports the argument that conidia come with pre-packaged mRNA ready to mobilise carbohydrates and protect from ROS damage at germination onset. However, these transcripts may also be required for maintaining dormancy. Internal ROS has been shown to act as a germination signalling molecule in *Colletotrichum gloeosporioides* and therefor ROS responders (SOD, catalase etc.) might aid in dormancy maintenance (Li et al. 2018; Nesher et al. 2011)(van Leeuwen et al. 2013). Similarly, low concentrations of ATP have been shown to inhibit trehalase, thus inhibiting trehalose mobilisation and germination initiation (Thevelein, den Hollander, and Shulman 1982), therefore low levels of metabolic activity may be required for ATP production to maintain dormancy. For the above transcripts to play a role in maintaining *Aspergillus sp.* dormancy, translation would be

required; spores of *Saccharomyces cerevisiae* have been found to contain transcripts which are constantly translated throughout dormancy, and it has been suggested that this ongoing expression plays a role in long term spore survival (Brengues, Pintard, and Lapeyre 2002). If the purpose of conidial transcripts are to aid germination initiation, the mRNA may be capped and packaged to increase stability. Highly stable mRNA has been detected in dormant *S. cerevisiae* spores, these mRNAs were stable for several months, but rapidly degraded upon germination onset (Brengues, Pintard, and Lapeyre 2002). If transcripts within the conidia are remnants of conidiation, to reduce degradation there may be either a lack of degradative enzymes such as ribonucleases existing freely within the conidia, or these enzymes are kept compartmentalized, as many fungal species compartmentalise enzymes necessary for secondary metabolism (Kistler and Broz 2015).

I suspect that there is more than one explanation for the vast transcriptional shift upon germination initiation. Given that 'resting' *A. niger* conidia show metabolic activity (Novodvorska et al. 2016), it is likely that selected transcripts are translated during dormancy (Brengues, Pintard, and Lapeyre 2002). Selected transcripts might play a role in dormancy maintenance as autoinhibitors do, or by controlling ROS and ATP as previously discussed. As transcripts are rapidly degraded upon germination and play roles in trehalose mobilisation, it is likely that some transcripts are also pre-packaged (polyadenylated and capped) ready for germination initiation, as is seen for *S. cerevisiae* and *M. tuberculosis* (Brengues, Pintard, and Lapeyre 2002; Ignatov et al. 2015). There is less support for that argument that the transcripts found in dormant spores are artifacts of conidiation, as the longest lived mRNA in transcriptionally inactive *S. cerevisiae* has a half-life of under 100 minutes (Wang et al. 2002).

Germination Metabolism

Follow up work on the metabolic changes in germinating *Aspergillus niger* showed that dormant conidia suspended in water alone exhibited low levels of respiration (Novodvorska et al. 2016). This either suggests that a nutrient source is less essential for initiating the process of germination, as can be seen in *C. neoformans*, or that dormant conidia in fact maintain a low level of metabolism at all times, as is supported by Brengues et al (Brengues, Pintard, and Lapeyre 2002). Transcripts involved in oxidative phosphorylation and therefore respiration, were also shown to be present in dormant *A. niger* conidia (Novodvorska et al. 2016). Proteomics showed that at early time points, proteins containing the Has domain with potential aldehyde-lyase activity, proteins with potential enoyl-[acyl-carrier-protein] reductase activity and proteins with translation elongation factor activity were abundant. These proteins are suspected to play roles in carbohydrate metabolism, oxidative phosphorylation and translation, respectively (Novodvorska et al. 2016).

Initial germination relies on fermentation to derive energy, probably from internal carbon sources such as trehalose. Fermentation rapidly switches to respiration after 25 minutes of germination. After 60 minutes of germination, glucose uptake can be detected, suggesting conidia may use their internal carbon sources to support the initial stages of germination, accounting for the metabolic activity measured in water alone (Novodvorska et al. 2016). An array of glucose and hexose transporters (An02g03540, An15g03940, An05g01290, An16g08940, An02g01270) were upregulated during the early stages of germination, with the low affinity and high affinity transporters An02g03540 (an *A. nidulans* homologue) and a mstA homologue, respectively, appearing most upregulated at the early and later time points (Novodvorska et al. 2016). Several G protein coupled receptors were also highly transcribed

at the early stages of germination (Novodvorska et al. 2016). As G protein coupled receptors are known to play roles in signalling and nutrient sensing (Choi et al. 2015), it is unsurprising that several serine and threonine phosphatases, linked with glucose uptake, were also found to be upregulated at these time points.

The presence of sorbic acid prolonged the fermentation phase of germination in *A. niger*, altering the metabolism of several substrates including trehalose and ergosterol (Novodvorska et al. 2016). The germination of *Aspergillus* species is also reliant upon RAS/MAPKinase signalling along with cAMP/PKA signalling in response to nutrients. An upregulation of amino acid metabolism also appears necessary for germination, as proteins within germinating *Aspergillus fumigatus* show a specific requirement for lysine (Osherov et al. 2002; Lamarre et al. 2008).

Neurospora crassa

A transcriptional study of germination in the model organism and plant pathogen *Neurospora crassa* showed similar changes during isotropic growth and hyphal emergence to those described in *Aspergillus* spp. (Kasuga et al. 2005). *Neurospora crassa* will begin to germinate upon immersion in water with the correct salts and carbon sources. Vogel's medium is commonly used to initiate germination in laboratory conditions. This is a medium that contains: sucrose, water, Na_3 citrate, KH_2PO_4, NH_4NO_3, $MgSO_4$, $CaCl_2$, trace elements and biotin. Germination results in swelling of the irregular spores, followed by changes in the cell wall composition and hyphal formation, with most conidia possessing hyphae at around 4 hours (Kasuga et al. 2005). Biochemical analysis has shown that in the time immediately after germination induction, internal stores of carbon such as trehalose and glutamic acid are

released and degraded (Kasuga et al. 2005). Transcriptionally, major differences were seen between resting and germinated spores (Kasuga et al. 2005). Conidia-specific genes such as the light responsive *con-6* and *10*, as well as *8*, are enriched in resting conidia, but swiftly depleted after germination (Kasuga et al. 2005), whilst internal amino acid stores are also rapidly degraded following the initiation of germination. After 30 minutes of germination, processes such as glucan and chitin synthesis, DNA processing and heat shock protein production peaked, whilst after 1-4 hours of germination, germlings were enriched in transcripts involved in RNA synthesis, DNA processing, ribosome biogenesis, protein biosynthesis, amino acid biosynthesis and enzymes & proteins involved in cellular transportation (Kasuga et al. 2005). Interestingly, the peak in amino acid production correlates with the restoration of amino acid stores at around 3 hours post germination induction. Respiratory transcripts peaked after 8-16 hours of germination (Kasuga et al. 2005), correlating with the observation that oxygen consumption commences after 2-4 hours of germination (Kasuga et al. 2005; Schmit and Brody 1976).

Fusarium species

The conservation of cellular reprogramming during germination is further evidenced by the protein content present in germinating conidia of *Fusarium oxysporum*. At early time points (3 hours), germlings were enriched for proteins involved in metabolic processes, redox processes, RNA processing, transcription and translation. Later time points (7-11 hours) showed an increase in proteins associated with ergosterol biosynthesis (Deng et al. 2015). Several conidiation and germination essential proteins and factors are known to exist for the plant pathogen *Fusarium graminearum*. GEA1 (Germinated Ascospores 1) is essential for normal conidia production, as well as development of the ascus wall, in which the conidia

are contained (Son, Lee, et al. 2013). The gene *FgATG15* also appears to be essential for normal conidia formation, as mutants produce abnormally shaped conidia. In addition, *FgATG15* is required for the turnover of stored lipids, a process often essential for germination (Nguyen et al. 2011). Similarly to other filamentous fungi, the production of conidia in *Fusarium graminearum* relies upon the presence of an AbaA (*Aspergillus*) orthologue, known to be involved in the regulation of cell cycle pathways, regulating these processes through the conserved AbaA-Weta signalling pathway in *Aspergillus* spp. and *Fusarium* sp. (Son, Kim, et al. 2013).

In summary, the timings of general mechanisms such as translation, metabolism and DNA synthesis correspond well between *N. crassa* and *Aspergillus* spp. Interestingly, the timing of more specific processes such as heat shock protein production also appears conserved between species. It has been suggested that the presence of heat shock proteins at these early stages in *Neurospora crassa* aids the correct folding of newly synthesized proteins (Osherov et al. 2002; Kasuga et al. 2005). Similarities between transcriptional changes over germination have also been found when comparing data from this study to changes seen in germinating *D. discoideum* and *U. maydis* (Zahiri, Babu, and Saville 2005). Together with the similarities seen in *Aspergillus* spp., this suggests the broad transcriptional changes seen over the course of germination are highly conserved between fungi and other eukaryotes, indicating that a 'core germination program' which evolved prior to recent eukaryotic species divergence, may exist (Kasuga et al. 2005).

Mucorales

It has been shown that the fatty acid composition of *Mucor rouxii* is altered over the course of germination, with quantities of gamma linoleic acid increasing over

germination, whilst other fatty acids fluctuate over germination (Khunyoshyeng et al. 2002). The content of chitin synthase also increases over the course, predominantly under aerobic conditions, thought to provide chitin to the cell wall as the spores swell and the cell wall expands (Kamada, Bracker, and Bartnicki-Garcia 1991). It is known that *Mucor circinelloides* relies in part in the *Atf1/2* (putative transcription factors) pathway to germinate within phagocytes (Pérez-Arques et al. 2019), and aquaporin recruitment is required for germination of *Rhizopus delemar* (Turgeman et al. 2016). Aside from the transcriptional profiles described above, the transcriptional landscape of germinating fungi has been largely unexplored, and remains understudied for Mucorales species.

Results

In order to address this gap, I performed a transcriptional analysis of *Rhizopus delemar* spore germination. My phenotypic analysis of spore germination (Chapter 2, Figure 3b) established the temporal pattern for the development of spores from dormancy to filamentous growth. These dramatic morphological changes require vast cellular reprogramming. I performed transcriptional analysis of each stage outlined in this process (Chapter 2, Figure 3b). For high-resolution capture of the transcriptional regulation of spore germination, mRNA was isolated and sequenced from resting spores (0 h), swelling spores (1, 2, 3, 4, and 5 h) and during filamentous growth (6, 12, 16, and 24 h). Three biological replicates were produced for each time point, and mRNA from each sample was sequenced with Illumina HiSeq technology, with 100-bp paired end reads. Reads were aligned to the *R. delemar* genome (Ma et al. 2009), giving an average alignment rate of over 95% per sample, with an average of 68% (12,170 genes) of all genes expressed over all time points. Genes which were never expressed in this time course likely have roles in other processes, such as sexual reproduction, or may be expressed under specific conditions, such as nutrient starvation.

Transcriptional Trends over Germination

Principal-component analysis (PCA) of TMM (trimmed mean of M-values) normalized read counts per gene (Figure 1) showed that the biological replicates grouped closely together, with time points grouping into 3 clusters separated by time (principal component 1 [PC1]) and stage (PC2), as determined by k-means clustering).

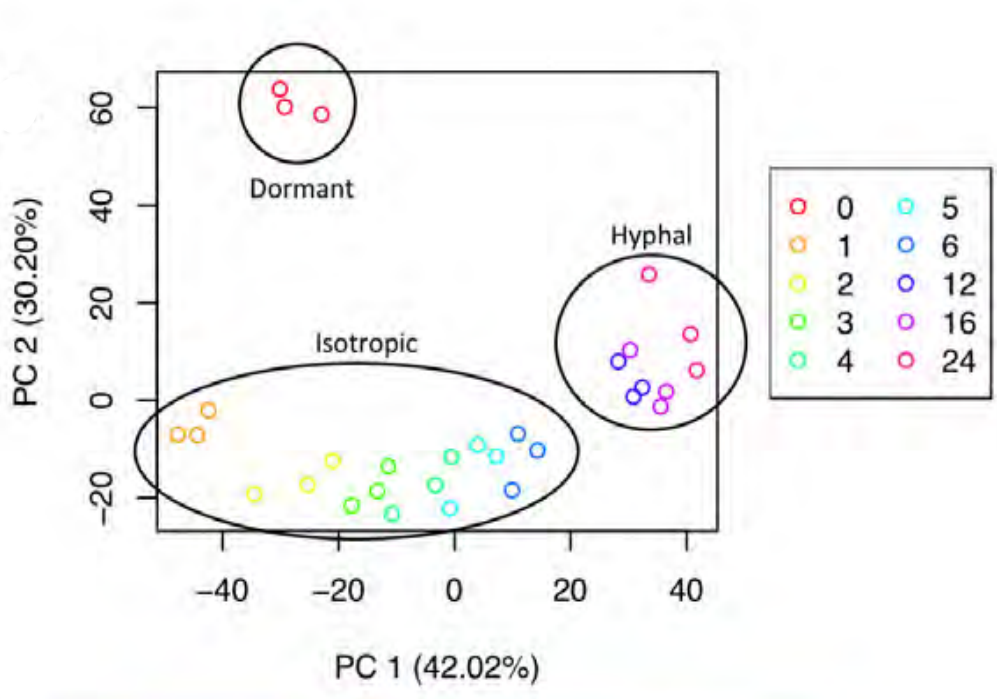


Figure 1. Principal-component analysis of 7,942 genes differentially expressed across all time points (n=3 for each time point; T = 0, 1, 2, 3, 4, 5, 6, 12, 16, or 24 h post-germination). Each time point is colour coded.

In examining the overall transcriptional profiles of our cells, we observed a set of 482 transcripts that were only expressed in ungerminated spores (Figure 2,3), representing 3.76% of total transcripts expressed in ungerminated spores (Figure 2). As a result, genes expressed in resting spores account for 71.5% of all genes in the genome, whereas the highest percentage of the genome covered by germinated spores is 68.8% (Figure 2). Resting-spore specific transcripts that were co-expressed (across all samples, n=3) have predicted roles in lipid storage and localization, as well as transferase activity on phosphorous-containing compounds (Figure 3). As these transcripts are absent in germinated spores, they may have roles in the maintenance of spore dormancy.

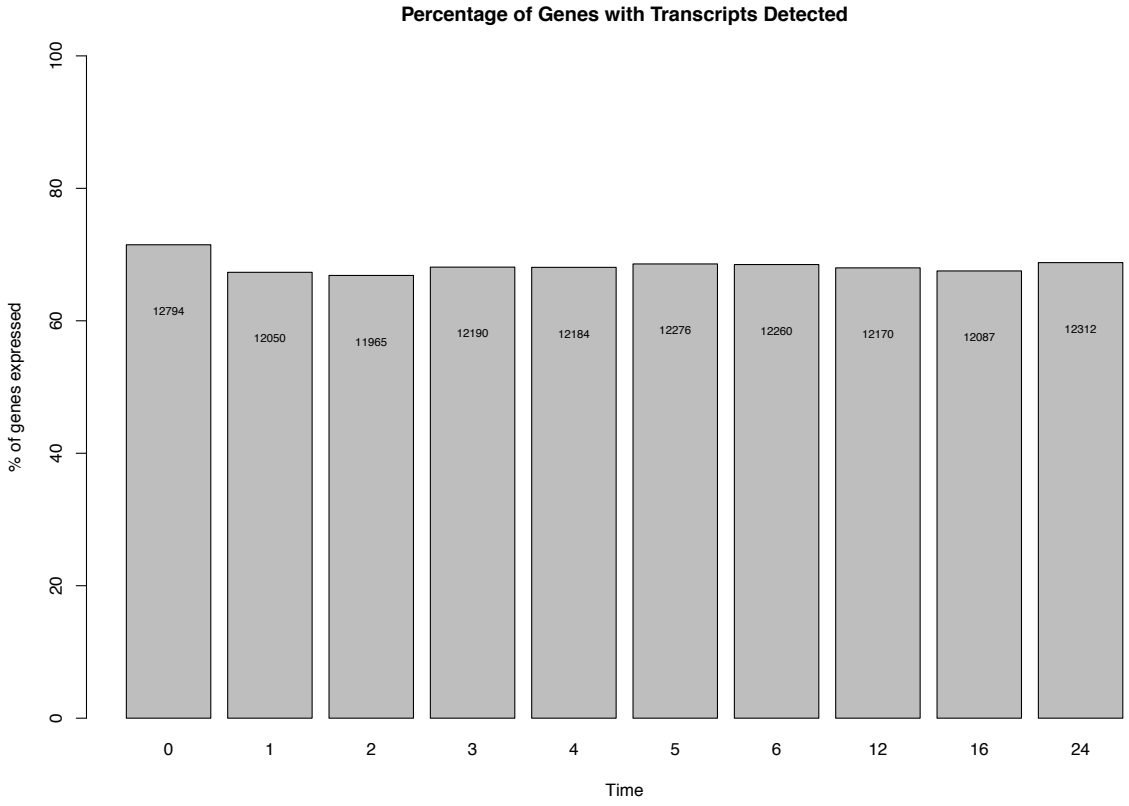


Figure 2. Number of genes expressed (10 or more transcripts present) at germination time points (t = 0,1,2,3,4,5,6,12,16 and 24 hours). Percentage was determined by dividing the number of genes expressed (10 or more transcripts) at each time point by the total number of genes within the genome, and multiplying by 100.

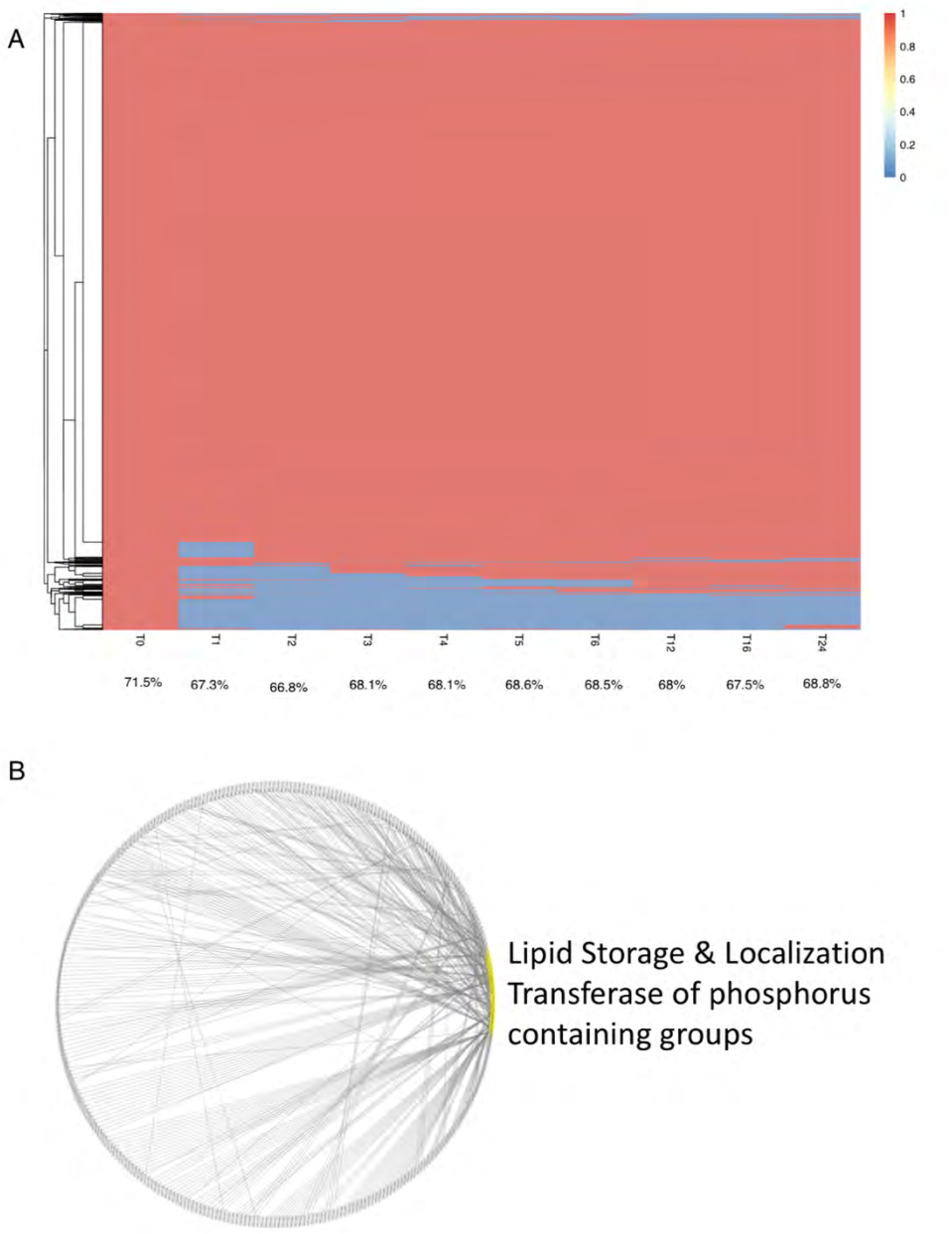


Figure 3. Resting-spore-specific expression. a) Heat map displaying the absence (blue) or presence (red) of 10 or more transcripts for a given gene over time. The average percentage of the transcriptome expressed at any given time point is given below. b) Co-expression between all T0 samples (n=3), where each node represents a gene only expressed in ungerminated spores. Nodes linked (by grey lines) to 10 or more others are highlighted in yellow, with their functions shown adjacent.

A series of analyses were performed to identify the transcriptional changes occurring during spore germination. Principle component analyses highlighted that the fungal transcriptome displayed a time-dependent shift across 3 major clusters corresponding to the phenotypic developmental stages swelling, germ tube emergence, and hyphal growth, indicating that spore germination is underpinned by progressive shifts in transcriptional regulation (Figure 4,5). The transcriptome of resting spores was distinct from that of all other developmental stages, changing dramatically between 0 and 1 h. Thereafter, the transcriptional profiles of swelling spores and of those developing germ tubes were distinct but clustered together (2 to 6 h). Furthermore, fully established filamentous growth was characterized by a specific transcriptional signature (12, 16, and 24 h) (Figure 4,5). Consistent with stage-specific transcriptional changes, progressive change in differential gene expression was observed during examination of the transcriptional profiles of each time point.

Differential Expression Throughout Germination

A total of 7,924 genes were differentially expressed across the entire time course (Figure 5a). Analysis of differentially expressed genes by k-means clustering identified seven major clusters of expression variation over time (Figure 5a,b). Genes in clusters 1 and 3 are expressed at low levels in resting spores, with abundance increasing upon germination (1 h) (Figure 4b). Both clusters are enriched (hypergeometric test, corrected P value of < 0.05) for transcripts with predicted roles in regulation of the cytoskeleton, protein metabolism, the electron transport chain, translation, and sugar metabolism (Figure 5c), suggesting these processes are important for germination initiation. Clusters 4 and 6 show gene expression levels moving from low to high over time, peaking during hyphal growth (Figure 5b). These clusters are enriched (hypergeometric test, corrected P value of < 0.05) for transcripts with

predicted functions related to kinase, transferase, transposase, and oxidoreductase activities, along with pyrimidine and phosphorous metabolism, stress response, transport, and signalling (Figure 5c). This is consistent with the established roles for these processes in starting and maintaining vegetative growth (van Leeuwen et al. 2013; Balmant, Sugai-guérios, and Coradin 2015; Yao et al. 2016). Cluster 5 contains genes that have high expression levels in both ungerminated spores and the hyphal form, but low levels during initial swelling (Figure 5b). Cluster 5 is enriched (hypergeometric test, corrected P value of < 0.05) for transcripts with predicted functions in regulation of the cytoskeleton, transferase and hydrolase activities, and phosphorous metabolism (Figure 5c). This suggests that these functions may be repressed during isotropic growth to maintain swelling. Clusters 7 and 2 contain genes with expression levels peaking in ungerminated spores (Figure 5b). These clusters are enriched (hypergeometric test, corrected P value of < 0.05) for transcripts with predicted functions relating to glycerone kinase, pyrophosphatase, transferase, hydrolase, and oxidoreductase activities, as well as cofactor and coenzyme metabolism, pyrimidine, sulfur, nitrogen, sugar, and aromatic compound metabolism. These clusters are also enriched for reduction-oxidation (redox) processes, respiration, and stress responses (Figure 5c). Notably, every cluster is enriched for transcripts involved in ion transport regulation, specifically potassium, sodium, and hydrogen ions. This suggests tight regulation of transmembrane transport of these particular ions is important for the survival of *R. delemar*.

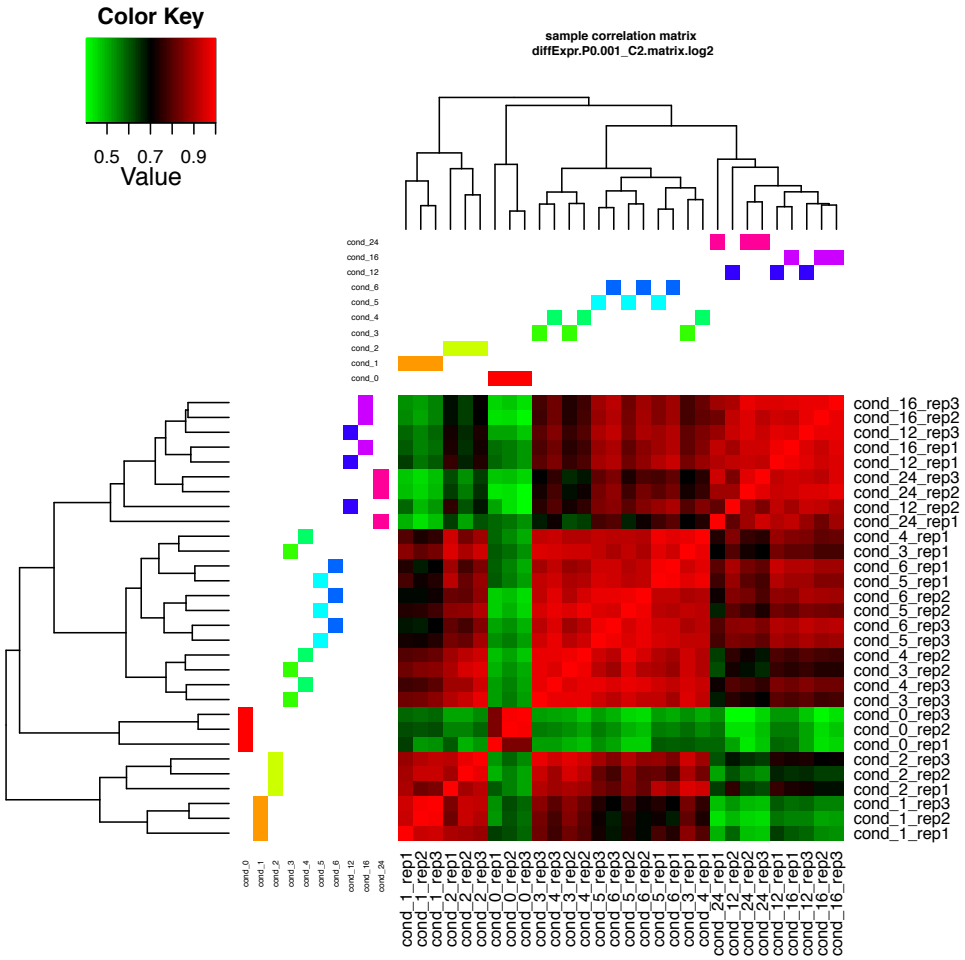


Figure 4. Heatmap displaying sample correlation. *R. delemar* samples (T0-T24, n=3) are hierarchically clustered based on gene expression patterns (Grabherr, Brian J. Haas, Moran Yassour Joshua Z. Levin, Dawn A. Thompson, Ido Amit, Xian Adiconis, Lin Fan, Raktima Raychowdhury, Qiandong Zeng, Zehua Chen, Evan Mauceli, Nir Hacohen, Andreas Gnirke, Nicholas Rhind, Federica di Palma, Bruce W., and Friedman 2013). Colours represent sample correlation, red = similar, green = dissimilar.

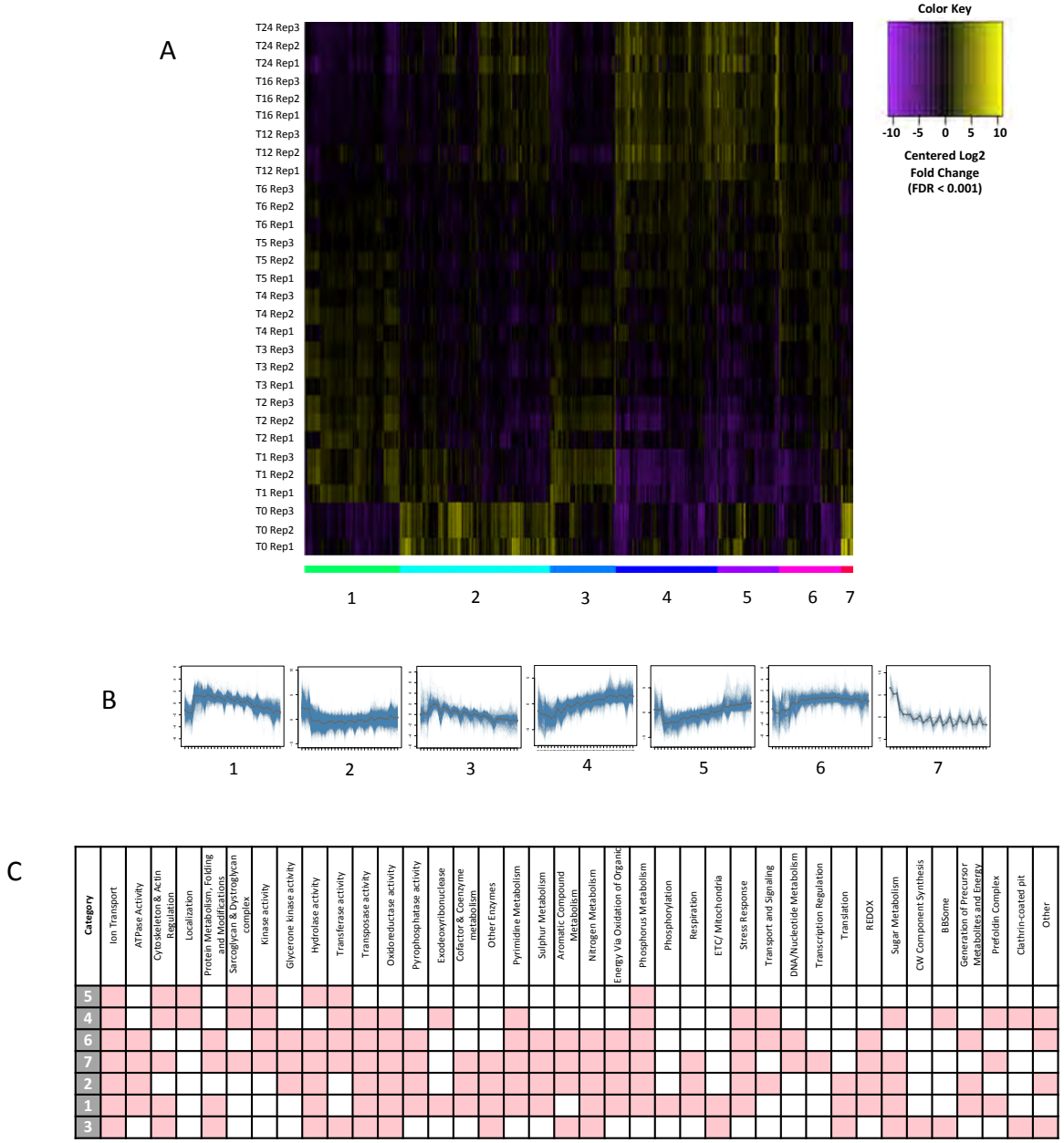


Figure 5 . Clustering of expression over time. a) Heat map displaying differentially expressed genes. Expression levels are plotted in log2, space and mean centred (FDR of < 0.001) across the entire time course. k-means clustering has partitioned genes into 7 clusters, as indicated by coloured bars and numbered graphs below the heat map. b) Graphs displaying cluster expression over time (0 to 24 h). c) Table displaying categories enriched (hypergeometric test, corrected P value of < 0.05), indicated in red, for clusters 1 to 7.

Ungerminated spores have a radically different expression profile from germinated spores (6,456 significantly differentially expressed genes; false-discovery rate [FDR] of < 0.001): this is reflected by the functions of transcripts enriched in ungerminated spores. By pairwise comparisons of differentially expressed genes between time points, the largest transcriptional changes were seen during the first hour of germination (3,476 genes upregulated and 2,573 genes downregulated [Figure 6a]). The large quantity of apparently down regulated transcripts may in fact represent transcripts which have been degraded, or immediately utilised upon germination initiation. Following this, there was a period of transcriptional consistency over the course of isotropic swelling, where few or no genes were found differentially expressed (Figure 6a). A noticeable shift in differential expression then bridges the beginning and later stages of hyphal growth (6 to 12 h [Figure 6a]). At the beginning of germination, an increase is observed in expression of transcripts with predicted roles in stress response, mitochondrial ribonucleases (MRP), the prefoldin complexes, organophosphate and sulfur metabolism, and transposase, ATPase, nucleoside triphosphatase, and glycerone kinase activities (Figure 6b). A decrease in expression of genes with predicted functions in the organization of the actin cytoskeleton, carbohydrate metabolism, translation initiation factors, hexon binding, and phosphodiesterase, arylformamidase, galactosylceramidase, and precorrin-2 dehydrogenase activities is also seen (Figure 6b). Notably, some categories are both positively and negatively regulated at the beginning of germination: transcripts predicted to have roles in ion channel activity and hydrolase and pyrophosphatase activities do not always trend together (Figure 6b). It is likely these processes may involve several regulatory mechanisms implicated in initializing germination.

After initiation (1 to 2 h), there is an overall trend of downregulation. The majority of transcripts that were upregulated at 1 h are downregulated at 2 h (Figure 6b), suggesting a

reorganization of the transcriptome upon germination initiation. Notably, metabolism of sulfur, organophosphate, and thiamine diphosphate remains downregulated at both 2 and 3 h. After the transcriptional stability during isotropic growth and hyphal emergence, transcripts with predicted roles in stress response, respiration, ATPase and nucleoside triphosphatase activities, and redox increase during early hyphal growth (Figure 6b). Between 6 and 12 h, the proportion of downregulated transcripts decreases, with hydrolase and pyrophosphatase activities appearing both up and downregulated.

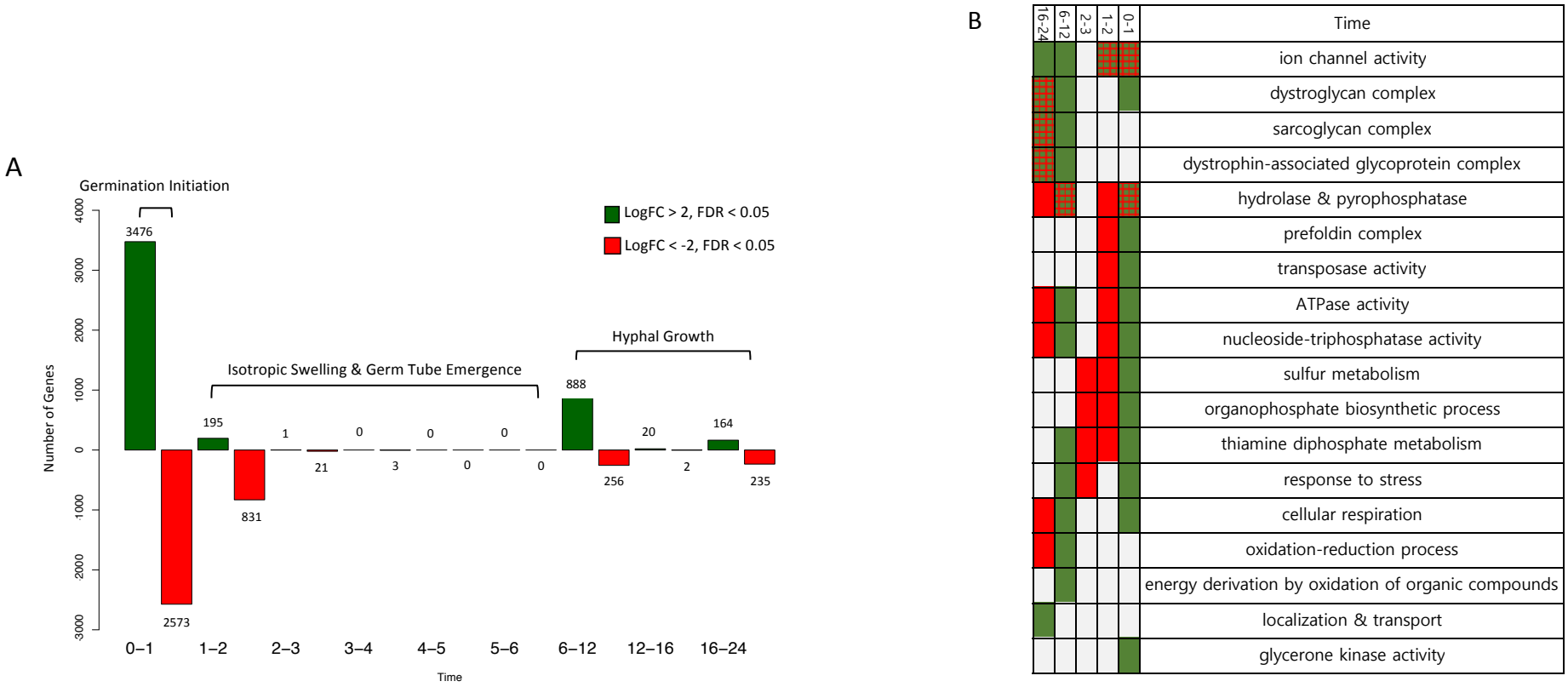
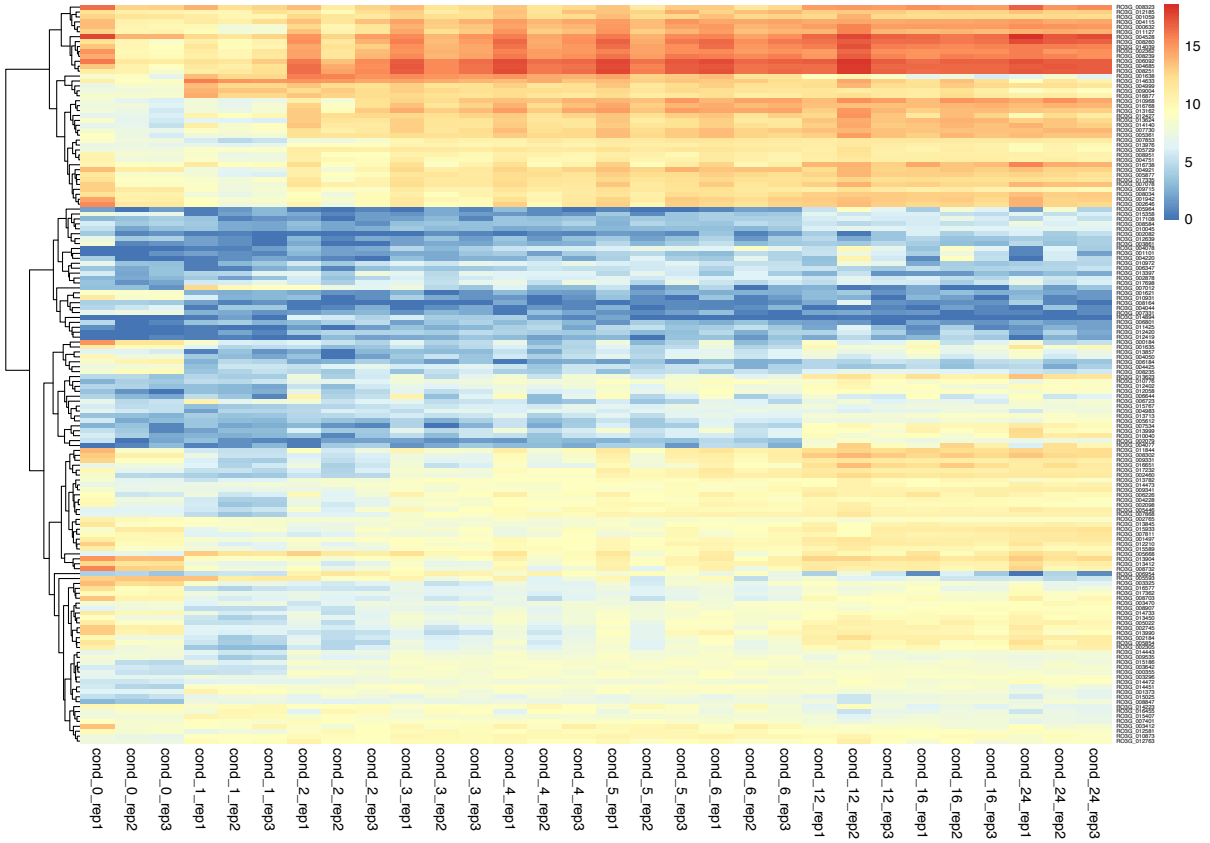


Figure 6. Differential gene expression over time. a) The number of genes significantly differentially expressed (multiple comparisons corrected P value of < 0.05, corrected via Benjamini-Hochberg method) between time points, shown over time. Green bars indicate genes with an increase in expression (log fold change [FC] of > 2), while red bars indicate genes with a decrease in expression (log FC of < -2). b) Enriched categories for the up or downregulated genes over time. Green boxes indicate an overall upregulation of this category, red indicates an overall downregulation and red-green hatching indicates mixed regulation of this category.

Metabolic Activity over Germination

Similar trends are observed when analysis of is carried out to determine the expression of genes with predicted functions (Pfam) in carbohydrate metabolism (Figure 7a), inorganic ion metabolism (Figure 7b), lipid metabolism (Figure 7c), amino acid metabolism (Figure 7d) and signalling (Figure 7e). Resting spore expression appears most similar to post-germination initiation expression. When carbohydrate metabolism is examined (Figure 7a) two subsets appear to gradually increase in expression over time, demonstrating the sustained requirement for respiration as biomass increases. Resting spores appear to express a subset of genes involved in inorganic ion metabolism (Figure 7b), which appear lowly expressed post germination-initiation, whilst other subsets are stably expressed throughout. Resting spores also express a subset of genes involved in lipid metabolism (Figure 7c), whilst other subsets appear highly expressed only after germination is initiated. This demonstrates the potential reliance of resting spore on stored lipids to initiate and sustain germination. Expression of genes with predicted roles in amino acid metabolism appears relatively stable over germination (Figure 7d), whilst other small subsets appear highly expressed within resting spores (0h), and again in filamentous form (12-24h). A slight increase in expression can also be noted at the onset of germination (1h), indicating spores rely on protein synthesis to kick-start germination and biomass production, as expected. Expression of genes with predicted functions in signalling show 3 distinct trends (Figure 7e): One subset appears highly expressed in resting spores (0h), a second subset is highly expressed at the onset of germination and throughout swelling(1-6h) and a third is highly expressed within filamentous forms (12-24h). These subsets correspond with the phenotypic stages outlined for germination of these spores, and demonstrate the reliance on unique signalling pathways to initiate and maintain these phenotypic stages.

A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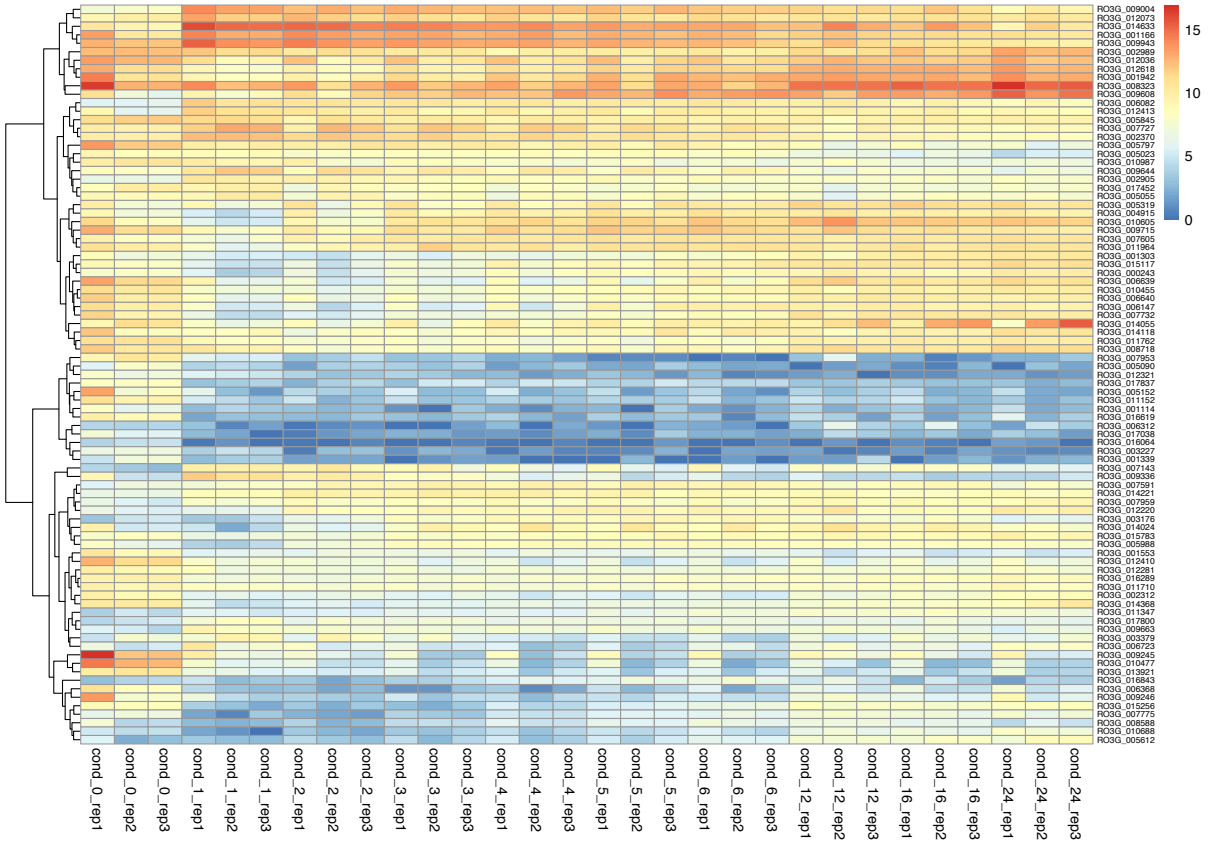
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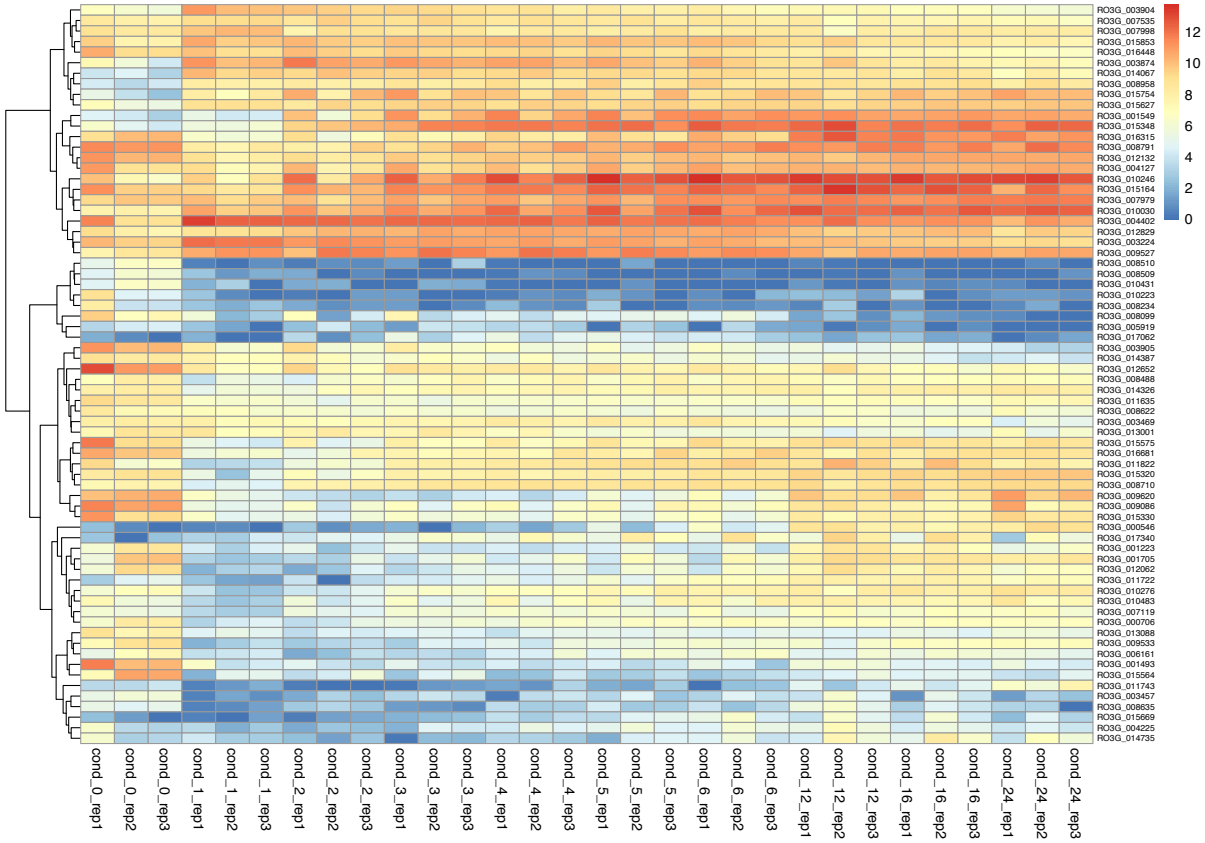
C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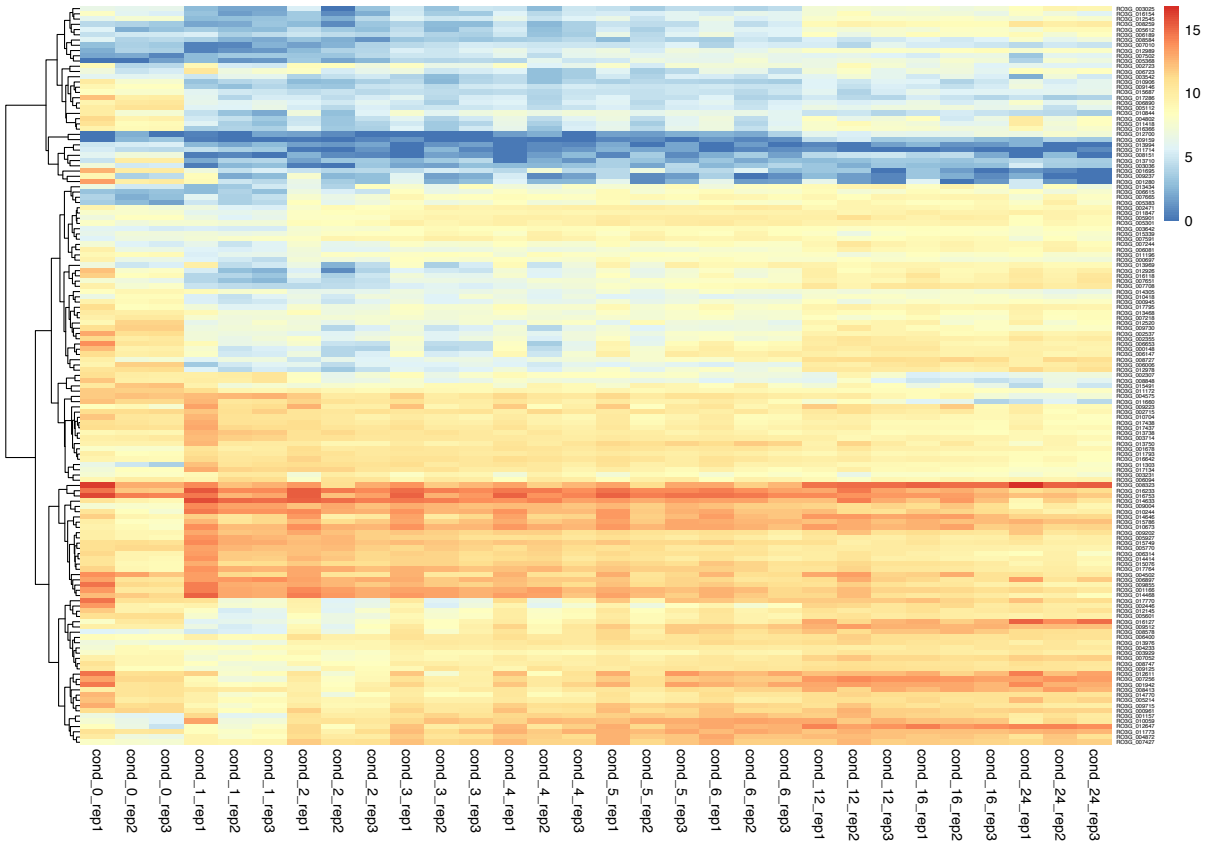
D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E

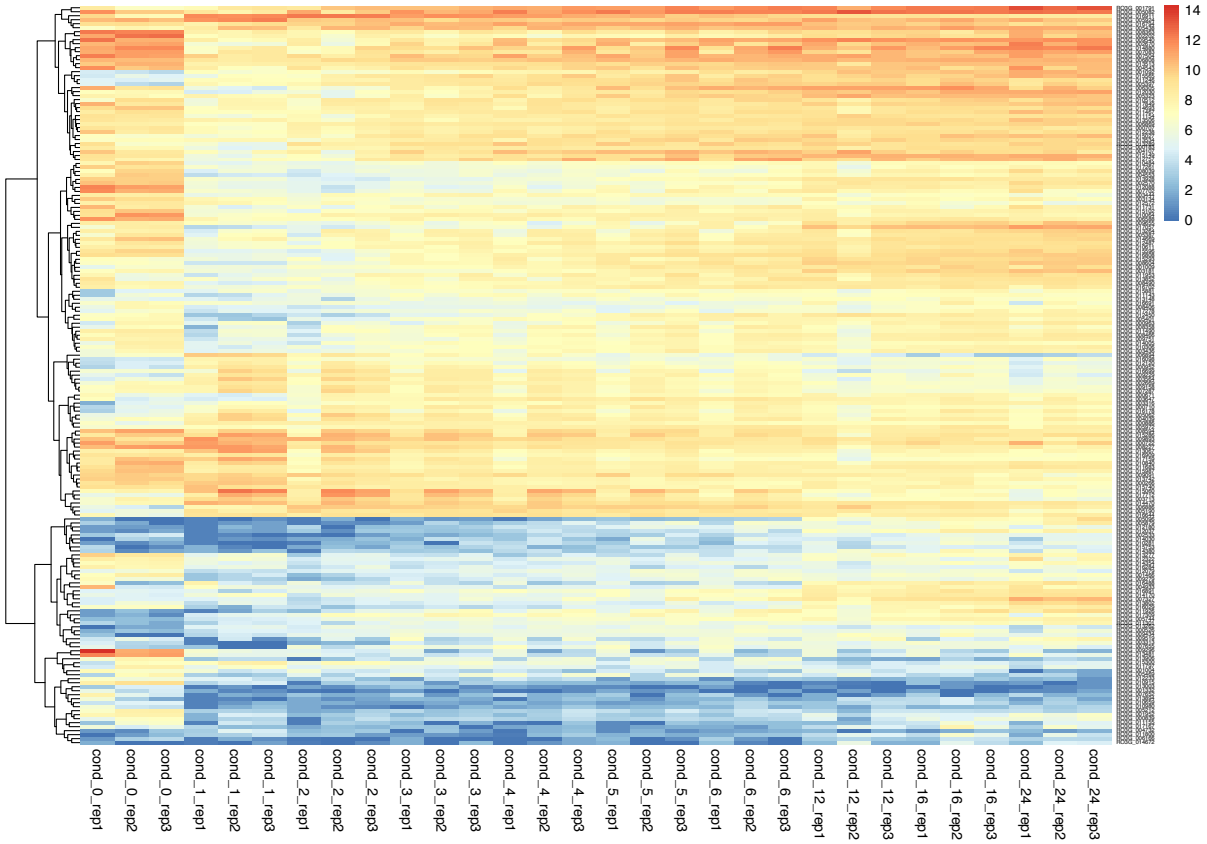


Figure 7. Heatmaps displaying expression of metabolic processes over germination (t = 0,1,2,3,4,5,6,12,16 and 24 h, n=3). a) Expression of genes with Pfam annotations involved in carbohydrate metabolism (expression = plotted in log2, FDR of < 0.001). b) Expression of genes with Pfam annotations involved in inorganic ion metabolism (expression = plotted in log2, FDR of < 0.001). c) Expression of genes with Pfam annotations involved in lipid metabolism (expression = plotted in log2, FDR of < 0.001). d) Expression of genes with Pfam annotations involved in amino acid metabolism (expression = plotted in log2, FDR of < 0.001). e) Expression of genes with Pfam annotations involved in signalling (expression = plotted in log2, FDR of < 0.001).

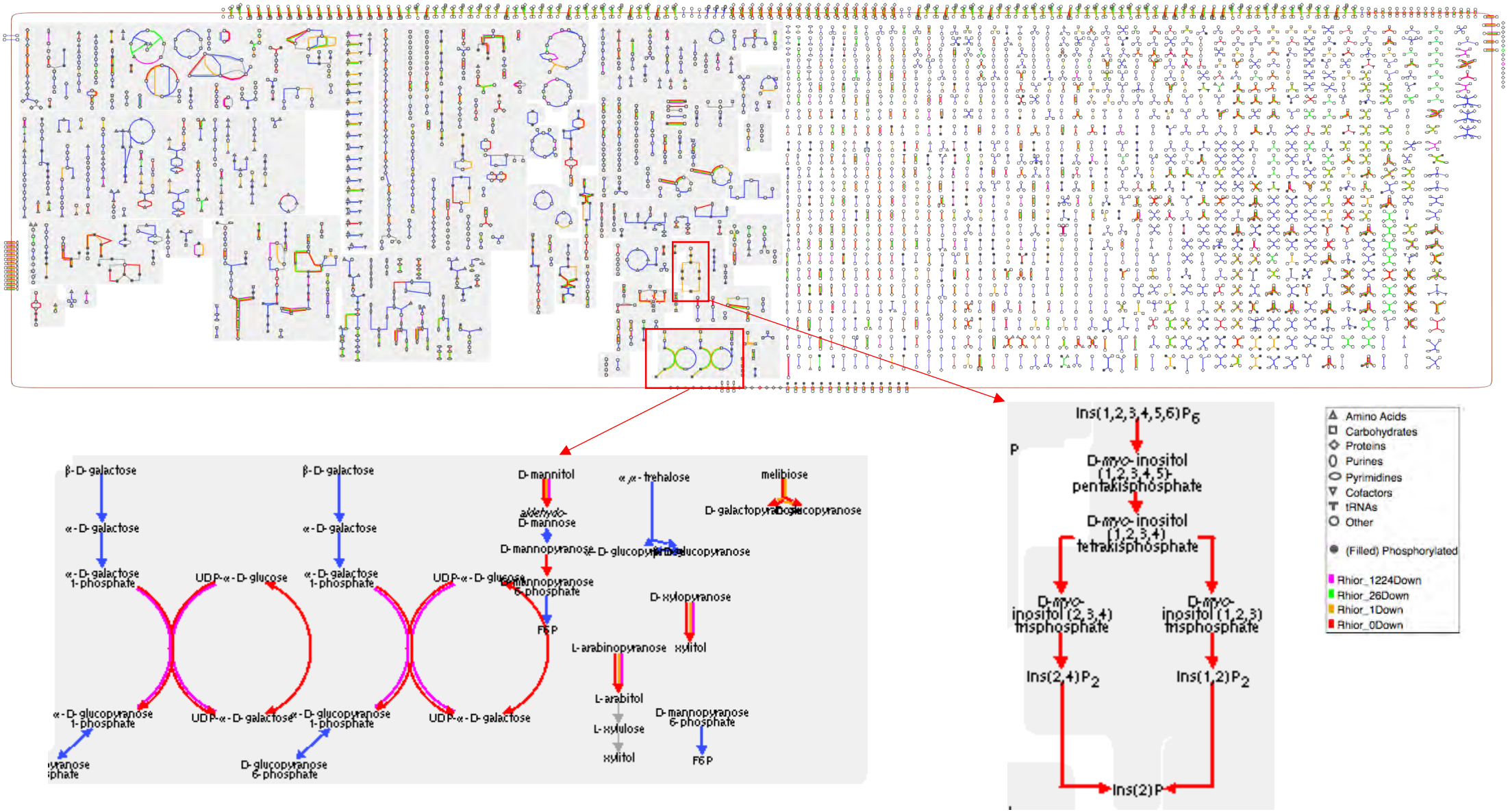
Pathways Upregulated at Alternate Germination Phases

PathwayTools annotation of the *R. delemar* genome allows analyses of individual pathway regulation over the course of germination. There are currently only 4 fungal genomes (*S. cerevisiae*, *C. albicans*, *E. dermatitidis* and *P. rubens*, available at: https://biocyc.org/organism-summary?object=CALBI) publicly available on biocyc, which have annotated PGDB's (PathoLogic Databases: format compatible with PathwayTools). This annotation is the first available (https://github.com/psephtonclark/rhior3cyc) for a Mucorales species.

Pathways which appear highly regulated during discrete germination phases (Figure 8) will be described here. The following pathways were found to be upregulated at resting (0h) and during hyphal growth (12-24h): protein glycosylation, chitin biosynthesis, trehalose degradation, ribonucleotide biosynthesis, glycolysis and galactose degradation. This is consistent with other findings which show chitin biosynthesis to be important for hyphal emergence and extension in *A. nidulans* (Yamazaki et al. 2008), protein turnover to be a hallmark of filamentous vegetative growth (Sun et al. 2017; Anderson et al. 2016) and respiration to be important for maintaining fungal biomass (Prasad, Kurup, and Maheshwari 1979). It should be noted that glycolysis also appears down regulated at 0-1 hours, indicating complex regulation of this process may be required to maintain dormancy and initiate germination. Galactose degradation appears downregulated throughout swelling (1-6h), highlighting its importance for initiation and hyphal growth.

Within resting spores (0h), hypusine and glyoxylate pathways are upregulated and NADP mitochondrial/cytosolic conversion appears to increase, whilst spermidine pathways also appear upregulated in both resting and initial spores (0-1h). Similarly, cysteine and glutamine

pathways are also upregulated at this time. Inositol degradation appears downregulated in resting and initial spores (0-1h), indicating inositol may be important at the onset of germination, whilst phyate degradation appears upregulated within resting spores (0h), indicating a role in dormancy maintenance. Xyoglucan and chitin degradation appear downregulated in resting spores (0h), along with peroxisome processes. This is consistent with conidial *A. niger*, which upregulates transcripts involved in amino acid metabolism and glyoxylate pathways, and downregulates peroxisome processes (Novodvorska et al. 2013). At germination initiation (1h), Fe-S cluster biosynthesis appears downregulated, whilst phyate degradation is upregulated. Within swelling spores (3-6h), cysteine and glutamine catabolic and metabolic pathways appear upregulated, whilst NADP conversion, CytochromeC, ATPase and NADPH oxidoreductase activities appear downregulated. This is consistent with *A. niger* germlings, which show upregulation of metabolic processes including amino acid processing (van Leeuwen et al. 2013). Within hyphal forms (12-24h), Fe-S cluster biosynthesis, tetrahydrofolate biosynthesis, oxoglutarate and isovalerate decarboxylation, Acetyl CoA metabolism, glycolysis, ATPase and peroxisome activity appear upregulated, indicating a reliance on aerobic respiration for hyphal growth. This is consistent with findings which show aerobic respiration to be important for the hyphal growth of many fungal species (Seto and Tazaki 1975; Lew and Levina 2004; Solaiman and Saito 1997; Watanabe et al. 2006).


β-D-galactose
α-D-galactose
α-D-galactose 1-phosphate
UDP-α-D-glucose
α-D-glucopyranose 1-phosphate
UDP-α-D-galactose
D-glucopyranose 6-phosphate
D-mannitol
aldehydo-D-mannose
D-mannopyranose
D-mannopyranose 6-phosphate
F6P
α,α-trehalose
D-glucopyranose
melibiose
D-galactopyranose
D-xylopyranose
L-arabinopyranose
xylitol
L-arabitol
L-xylulose
Ins(1,2,3,4,5,6)P6
D-myo-inositol (1,2,3,4,5) pentakisphosphate
D-myo-inositol (1,2,3,4) tetrakisphosphate
D-myo-inositol (2,3,4) trisphosphate
D-myo-inositol (1,2,3) trisphosphate
Ins(2,4)P2
Ins(1,2)P2
Ins(2)P
Amino Acids
Carbohydrates
Proteins
Purines
Pyrimidines
Cofactors
tRNAs
Other
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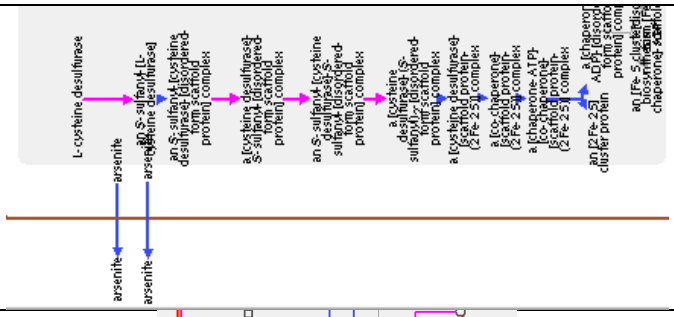
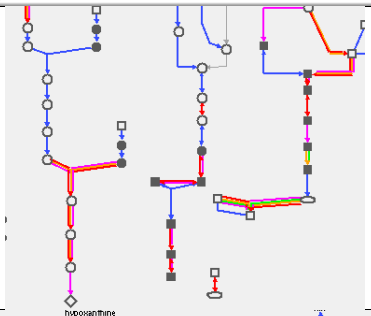
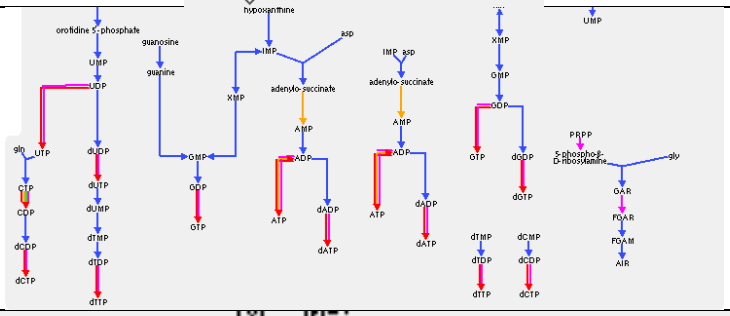
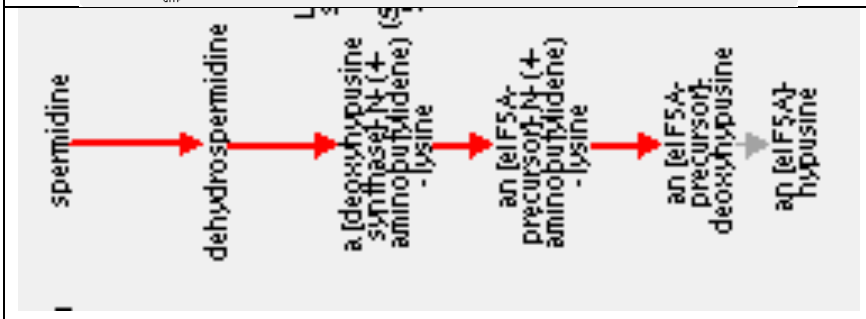
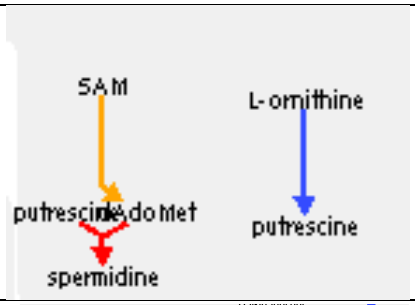
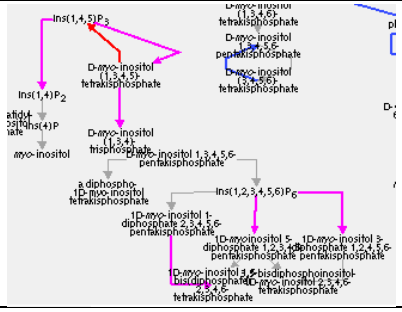
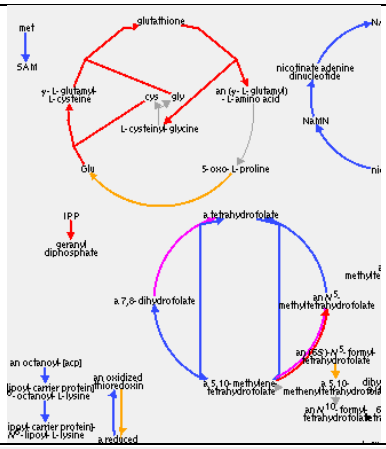
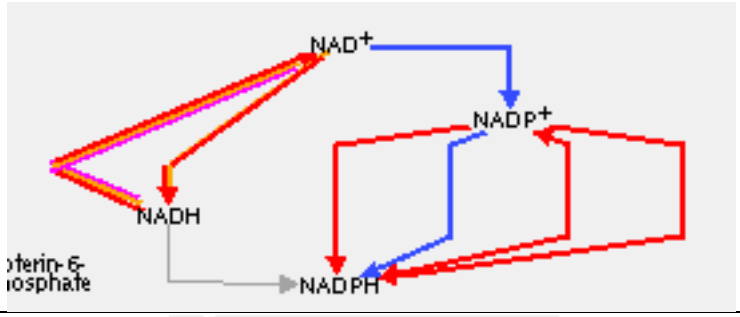
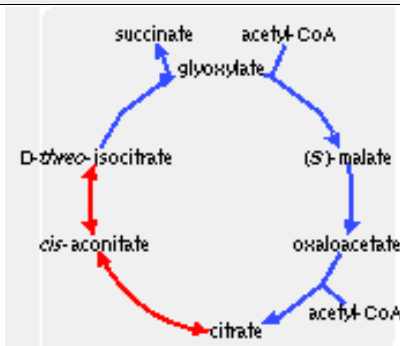
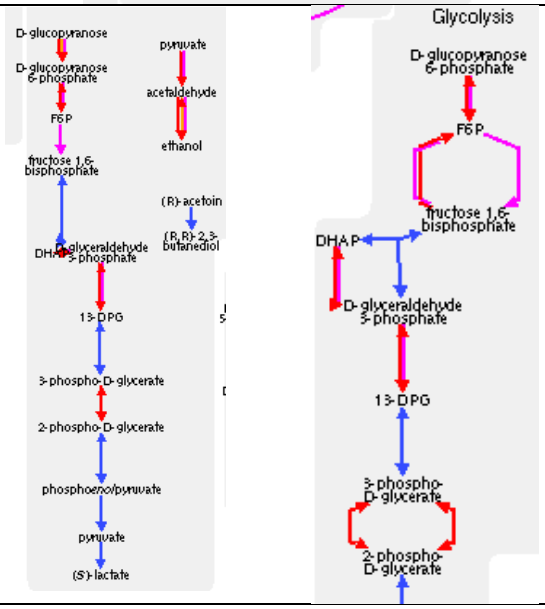
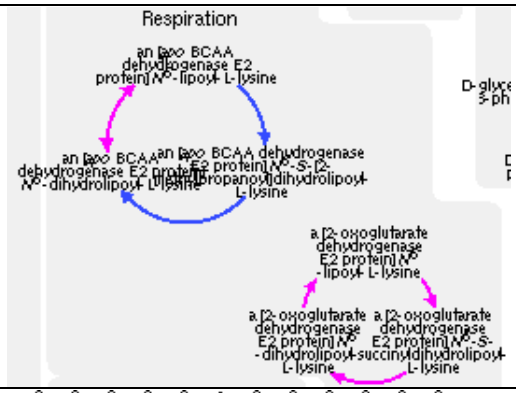
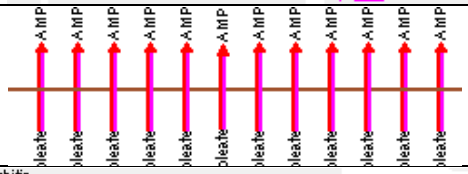
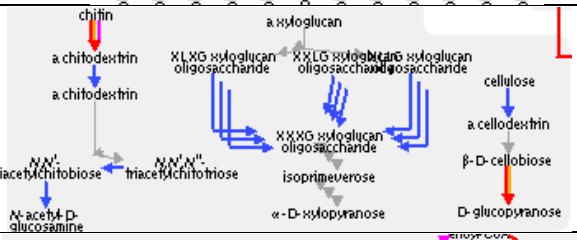
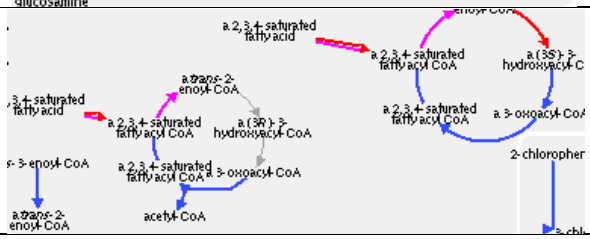
Rhior_26Down
Rhior_1Down
Rhior_0Down

	Up at Hyphal(12-24) Down at Resting and Initial (0-1):Fe-S Cluster Biosynthesis
	Up at Resting(0) and Hyphal (12-24): Protein Glycosylation, Chitin Biosynthesis and Trehalose Degradation
	Upat Resting and Hyphal (0, 12-24) Down at Initial and Mid(1-6): Ribonucleotide Biosynthesis
	Up at Resting(0): Hypusine Biosynthesis- AA only found in EIF5A, this interacts with Spermidine
	Up at Restingand Initial (0-1): Spermidine Biosynthesis- involvedin signal transduction and stress response
	Up at Hyphal (12-24) Down at Resting & Initial (0-1): Inisitol Pi Biosynthesis and Degradation

	Up at Resting and Initial (0-1), Down at Mid (3-6): Cysteine and Glutamine Pathways Up at Hyphal (12-24) and Down at Resting & Initial(0- 1): Tetrahydorfolate Biosynthesis
	Up at Resting (0)and Down at Mid (3-6): NADP Mitochondrial/Cytosolic Conversion
	Up at Resting (0): Glyoxylate Pathway
	Glycolysis 1 & 2: Upat Restingand Hyphal (0, 12-24), Down at Resting andInitial(0-1)

	Up at Hyphal (12-24): Oxoglutarate and Isovalerate decarboxylation (CoA Regeneration)
	Up at Hyphal (12-24): Acetyl CoA Ligase and Synthase
	Down at Resting(0): Xyoglucan and Chitin Degradation
	Up at Hyphal (12-24), Downat Resting and Mid (0, 3-6): Peroxisome

- Down at Mid (3-6): Cytochrome C, ATPase and NADPH Oxidoreductase
- Up at Hyphal (12-24): CoA Ligase
- Up at Hyphal (12-24): ATPase
- Up at Resting (0) and Down Mid (3-6): Myrosinase Production
- Down at Resting (0): Monoxygenase
- Down at Initial (1) & Mid (3-6): Glucoronosyl transferase : UDP generation
- Up at Hyphal (12-24), Down at Mid (3-6): Phosphatidylcholine transferase

Figure 8. Pathways differentially regulated over the course of germination. Differential pathway expression was determined by highlighting pathways which included genes significantly differentially expressed (multiple comparisons corrected P value of < 0.05) between time points. Genes with increased expression (LogFC > 2) and decreased expression (LogFC < -2) were selected for this analysis. Up and down regulated pathways were highlighted using the custom *R. delemar* pathologic annotation and PathwayTools.

Co-transcriptional Networks

To determine whether phenotypes expressed a core set of genes, co-transcriptional network analysis was performed. In total, resting spores and those germinated for up to two hours shared expression of 4,738 transcripts. These transcripts were enriched for processes which include cellular and primary metabolism, ribosome biogenesis, ncRNA processes, RNA metabolism and protein metabolism (Figure 9). This core set may be key to initiating cellular metabolism and protein processing which support the rapid morphological changes which occur at germination initiation. Swelling spores (3-6h) co-expressed only 109 transcripts (Figure 10), with roles in metabolism, RNA, tRNA and nucleotide processing and protein processing. Though much smaller, this core set appears similar in function to the one identified within 0-2h resting and swelling spores.

R. delemar in its hyphal form (12-24h) shares 1677 co-expressed transcripts. These had roles in metabolism, tRNA, RNA and nucleotide processing, protein processing and macromolecule processing (Figure 11). Again, the core set identified here appears highly similar in functionality to the core sets for resting and swelling spores. This analysis suggests there is a core set of transcripts expressed to maintain function of *R. delemar*. As all transcripts included in this analysis were identified to be significantly differentially expressed across the entire course of germination (and therefore not constituently expressed for the entirety of germination), it may be possible that alternate genes with similar functions are expressed throughout the germination of *R. delemar*. This mechanism would allow for regulation of these processes alternately at different morphological stages.

WGCNA & Cytoscape: 0-2 Hours, 4738 Nodes

P Value	GO Term
4.67E-05	gene expression
8.67E-05	primary metabolic process
8.67E-05	cellular macromolecule metabolic process
8.67E-05	ribosome biogenesis
8.67E-05	ncRNA metabolic process
9.31E-05	cellular metabolic process
2.51E-04	translation
2.51E-04	macromolecule metabolic process
3.90E-04	cellular process
3.90E-04	ribonucleoprotein complex biogenesis
3.90E-04	RNA modification
6.03E-04	cellular protein metabolic process
2.27E-03	rRNA processing
2.27E-03	rRNA metabolic process
2.74E-03	macromolecule biosynthetic process
3.50E-03	cellular macromolecule biosynthetic process
3.81E-03	protein metabolic process
6.07E-03	ncRNA processing

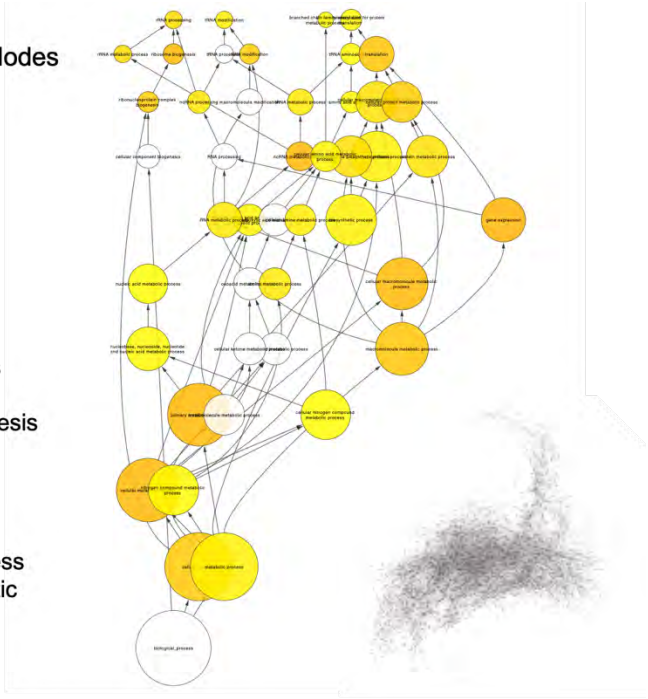


Figure 9. Co-transcriptional network of genes (grey) and network enrichment visualisation (yellow) expressed between 0 and 2 hours post germination. Yellow nodes represent enriched predicted functions (GO term, P < 0.05), reduced to specific terms with ReviGO, listed on the left.

WGCNA & Cytoscape: 3-6 Hours, 109 Nodes

P Value	GO Term
5.40E-12	cellular macromolecule metabolic process
5.40E-12	gene expression
1.13E-11	macromolecule metabolic process
9.63E-09	primary metabolic process
1.93E-08	ncRNA metabolic process
4.79E-08	cellular metabolic process
8.67E-08	RNA metabolic process
1.36E-07	cellular process
1.89E-07	translation
5.85E-07	cellular macromolecule biosynthetic process
5.97E-07	nucleic acid metabolic process
5.97E-07	macromolecule biosynthetic process
4.77E-06	nucleobase, nucleoside, nucleotide and nucleic acid metabolic process
1.09E-05	ncRNA processing
1.09E-05	cellular protein metabolic process
1.74E-05	ribosome biogenesis
3.59E-05	metabolic process
4.04E-05	protein metabolic process
4.04E-05	cellular nitrogen compound metabolic process
4.04E-05	tRNA metabolic process
5.90E-05	cellular biosynthetic process

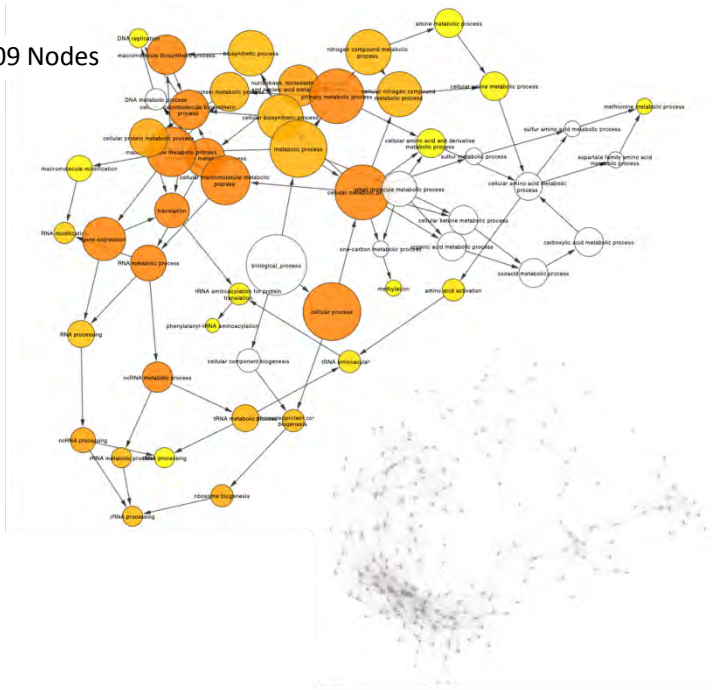


Figure 10. Co-transcriptional network of genes (grey) and network enrichment visualisation (yellow) expressed between 3 and 6 hours post germination. Yellow nodes represent enriched predicted functions (GO term, P < 0.05), reduced to specific terms with ReviGO, listed on the left.

WGCNA & Cytoscape: 12-24 Hours, 1677 Nodes

P Value	GO Term
8.30E-08	translation
1.66E-05	protein metabolic process
1.66E-05	cellular protein metabolic process
2.00E-05	gene expression
3.16E-04	macromolecule biosynthetic process
3.38E-04	primary metabolic process
3.38E-04	cellular biosynthetic process
3.38E-04	cellular macromolecule biosynthetic process
3.38E-04	biosynthetic process
6.36E-03	macromolecule metabolic process
6.95E-03	metabolic process
7.99E-03	cellular metabolic process
1.03E-02	cellular macromolecule metabolic process
1.03E-02	tRNA metabolic process
2.26E-02	amino acid activation
2.26E-02	tRNA aminoacylation
3.77E-02	tRNA aminoacylation for protein translation
3.77E-02	ncRNA metabolic process

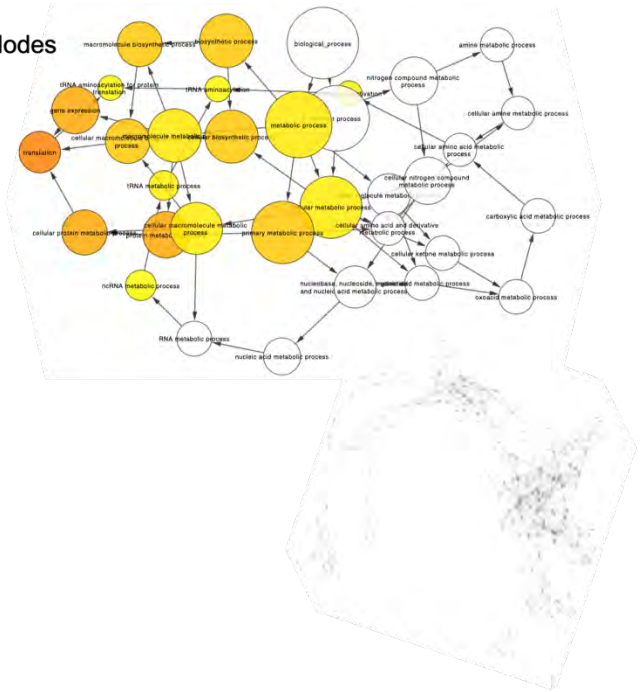


Figure 11. Co-transcriptional network of genes (grey) and network enrichment visualisation (yellow) expressed between 12 and 24 hours post germination. Yellow nodes represent enriched predicted functions (GO term, $P < 0.05$), reduced to specific terms with ReviGO, listed on the left.

Duplicated Gene Pair Expression

As the genome of *R. delemar* is likely to have undergone a whole genome duplication event (Ma et al. 2009), duplicate gene pairs were identified (Ma et al. 2009) and the expression profiles were analysed to identify pairs which displayed alternate expression profiles over the course of germination. As these duplicates have been maintained within the genome, it was hypothesised that some duplicates may have diverged in function. Evidence of divergence may be visible via alternate expression profiles of the duplicates. In total 70 pairs, which were differentially expressed over the course of germination to a significant level (FDR < 0.001), were identified to have alternate expression profiles (LogFC between each gene within the pair > 2) (Figure 12). Alternate expression profiles support the hypothesis that these duplicated genes may have diverged in function or process regulation. Of these genes, pairs with the most highly divergent expression patterns over the course of germination had the following predicted functions: Glycosyltransferase, Nucleoporin, SMR domain, Ribosomal protein S2, Protein Kinase, Metallopeptidase, Phosphatidyl inositol 4-phosphate-5-kinase and a conserved fungal protein of unknown function (Figure 13).

Functions of these potential growth regulators should be explored further, to determine whether these processes would be good targets for germination or growth inhibition, in order to treat mucormycosis.

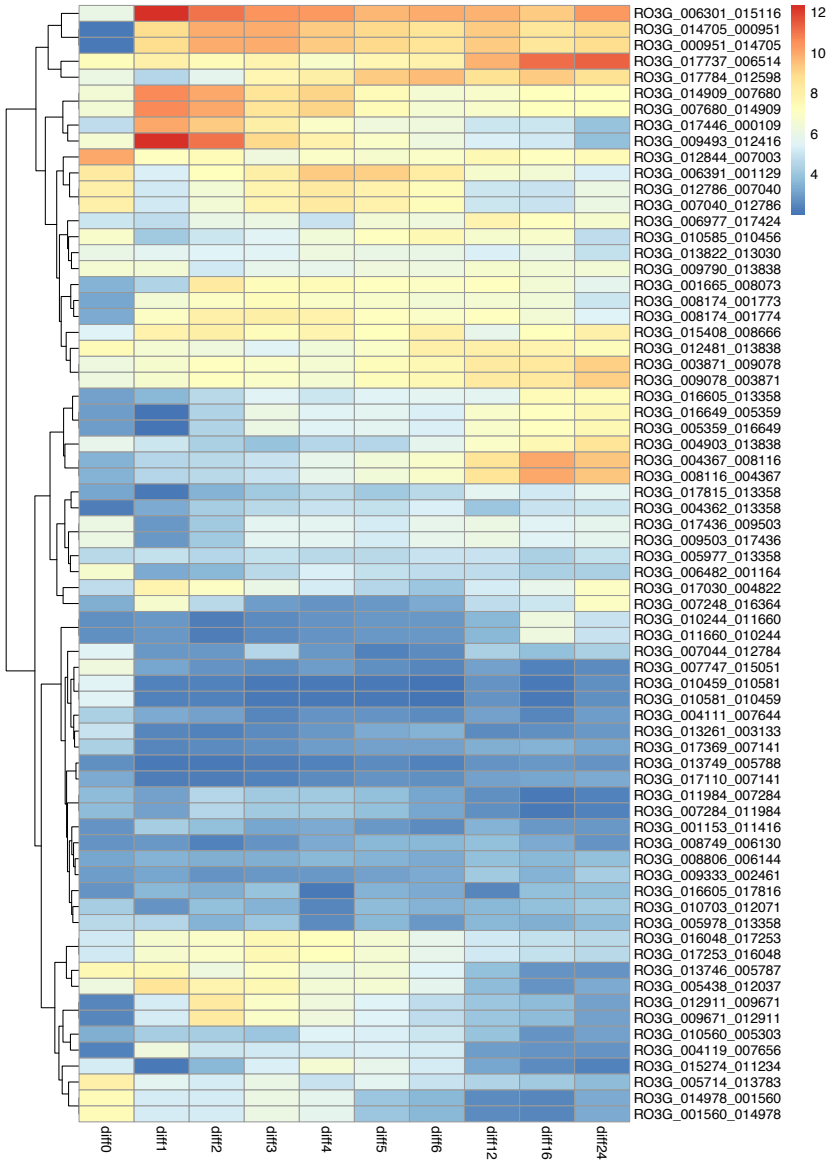
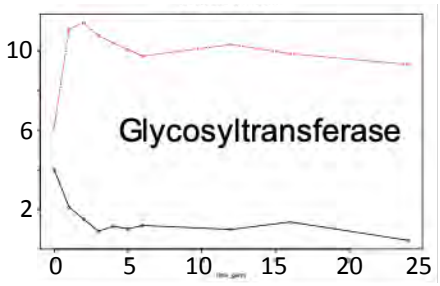
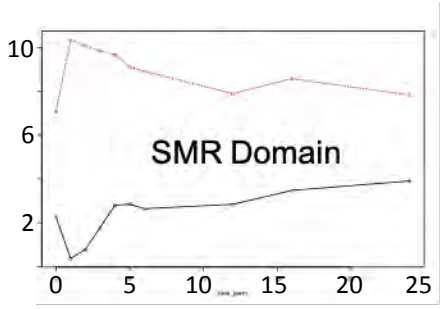


Figure 12. Heatmap displaying expression difference (LogFC) between duplicated gene pairs (gene pairs listed on the Y axis, e.g. *RO3G_001560_014978* represents pair *RO3G_001560* and *RO3G_014978*).

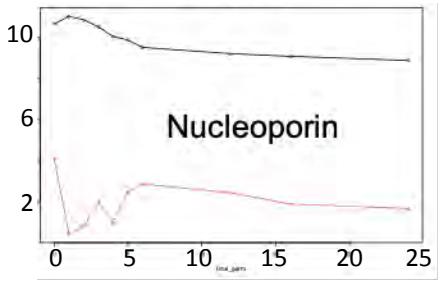
Best Hit: *P. blakesleeanus* glycosyltransferase family 2 protein
% Identity: 77%
E Value: 8e-151

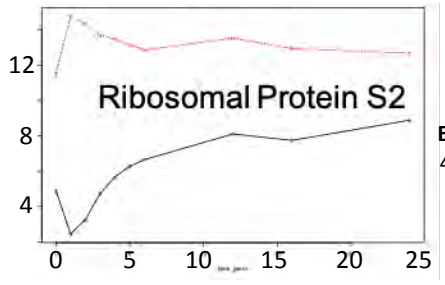




Best Hit: *R. microsporus* DUF1771-domain-containing protein
% Identity: 74%
E Value: 5e-77

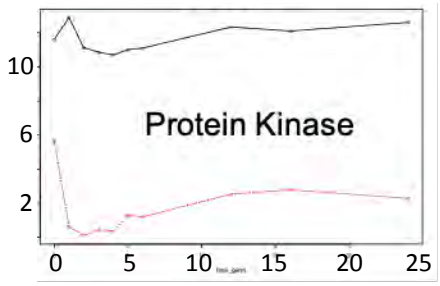
Best Hit: *R. microsporus* hypothetical protein (no well characterised homologues available)
% Identity: 74%
E Value: 9e-162

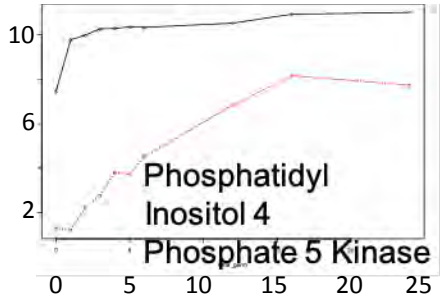




Best Hit: *R. microsporus* 40S ribosomal protein S0
% Identity: 80%
E Value: 7e-165
Best Studied Hit: *S. pombe* 40S ribosomal protein S0B
% Identity: 71%
E Value: 9e-56

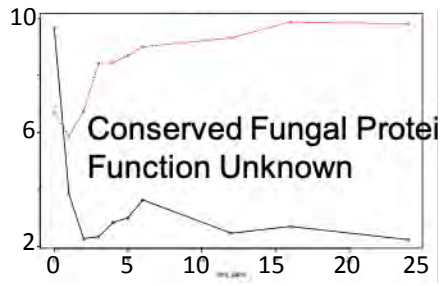
Best Hit: *R. microsporus* kinase-like protein
% Identity: 75%
E Value: 4e-114
Best Studied Hit: *P. lutzii Pb01* serine/threonine-protein kinase ppk8
% Identity: 67%
E Value: 5e-24

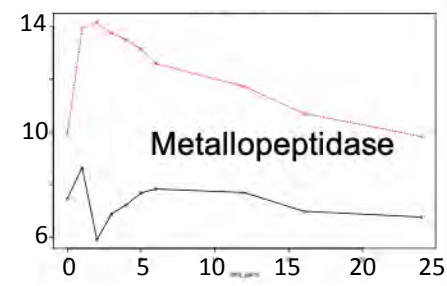




Best Hit: *R. microsporus* SAICAR synthase-like protein
% Identity: 80%
E Value: 0
Best Studied Hit: *A. flavus* multicopy suppressor of *stt4* mutation
% Identity: 66%
E Value: 2e-49

Best Hit: 'Hypothetical Protein' conserved across fungal species, but without any known function.





Best Hit: *R. microsporus* proliferation-associated protein 1
% Identity: 84%
E Value: 0
Best Studied Hit: *S.lignohabitans Map2p* (Microtubule Associated Protein) (DNA Binding)
% Identity: 65%
E Value: 2e-21

Figure 13. Expression profiles of individual gene pairs. The X axis corresponds to time post germination initiation (0-24h), whilst the y axis corresponds to expression (LogFC). Best hits determined with blastn.

Potential Roles of Plant and Fungal Hormones as Germination Regulators

Whilst the germination of human fungal pathogens may be triggered by nutritional or host factors, plant hormones are also capable of regulating germination of fungal spores. Auxins, sialic acid, gibberellic acids, abscisic acid, ethylene and jasmonic acid are all known to be capable of regulating the germination of fungal spores (Chanclud and Morel 2016). For this reason the genome of R. delemar was analysed to find homologues of plant and fungal hormone receptors and biosynthesis genes.

Expression of these homologues over germination will be discussed here. *bcABA* homologues (Izquierdo-Bueno et al. 2018) and genes predicted (GO) to play roles in abscisic acid biosynthesis were identified (Figure 14)(Appendix bcABA: gene homology trees). Of these, one gene (*R03G_015440*) shows upregulation (LogFC>15, significantly differentially expressed) within resting spores, followed by a decrease in expression upon germination initiation. Abscisic acid acts on plant seeds and spores as a germination inhibitor (Schopfer et al. 1979; Moody et al. 2016). Some phytopathogenic fungi have also been shown to produce abscisic acid (Takino et al. 2019; Izquierdo-Bueno et al. 2018), however it should be noted that *R. delemar* has not been shown to produce abscisic acid. If *R. delemar* is capable of producing ABA, this molecule may work in a similar way to maintain dormancy in resting spores through germination inhibition. However, until *R. delemar* has been shown to produce ABA, these results should be taken with caution, as fungal homologues displayed lower query covers, and other required ABA biosynthesis machinery was found to be lacking. Future work focusing on improving predictions of hormone biosynthesis pathways within the *R. delemar* genome would greatly benefit the field.

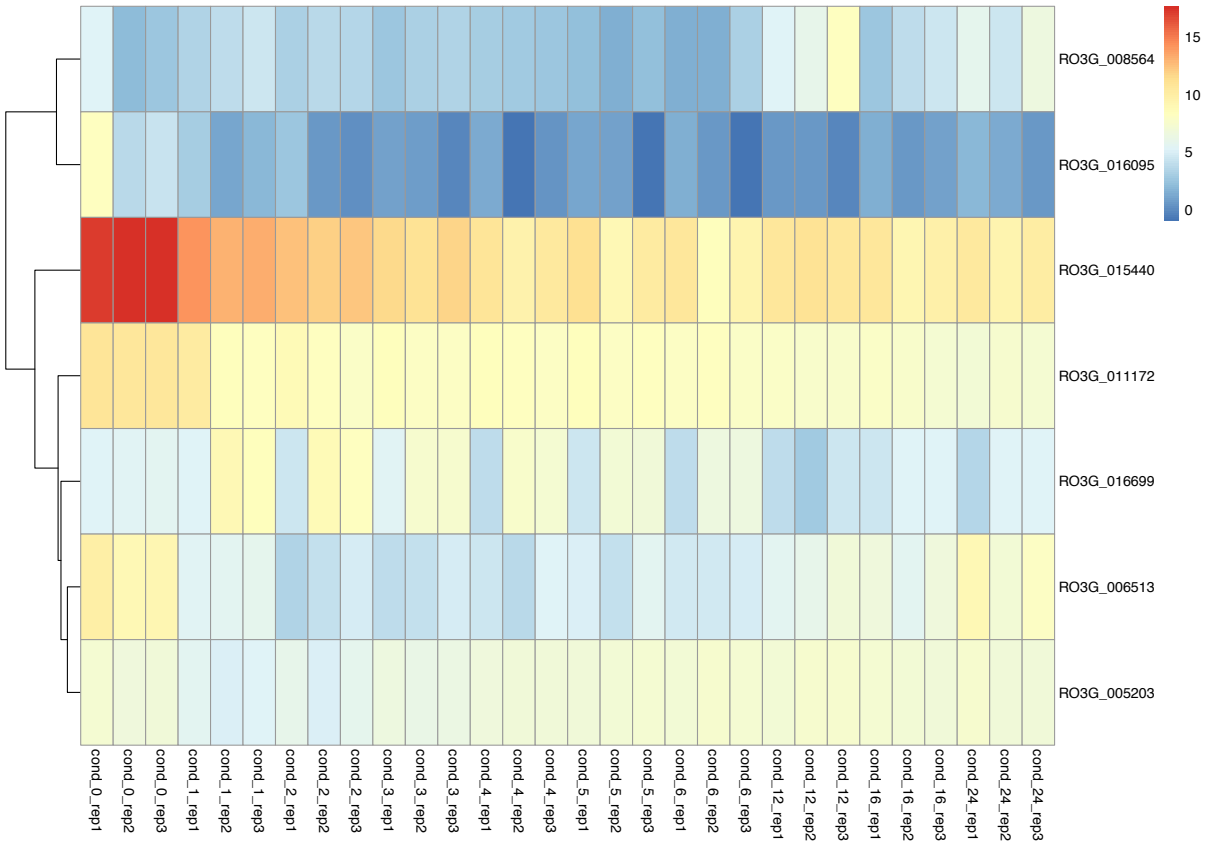


Figure 14. Expression (LogFC) of *bcABA1*, *2* and *4* homologues over germination.
RO3G_008564: GO prediction: abscisic acid homeostasis. No plant homologues detected through blastp.
RO3G_016095: GO prediction: abscisic acid homeostasis. No plant homologues detected through blastp.
RO3G_015440: blastp aligned to *bcABA2 Botrytis cinerea* with: E value: 0.009, % Identity: 92%. No plant homologues detected.
RO3G_011172: blastp aligned to *bcABA2 Botrytis cinerea* with: E value: 0.039, % Identity: 82%. No plant homologues detected.
RO3G_016699: blastp aligned to *bcABA1 Botrytis cinerea* with: E value: 0.029, % Identity: 85%. No plant homologues detected.
RO3G_006513: blastp aligned to *bcABA4 Botrytis cinerea* with: E value: 0.017, % Identity: 84%. No plant homologues detected.
RO3G_005203: GO prediction: abscisic acid metabolic process. No plant homologues detected through blastp

DELLA proteins are responsible for germination inhibition in plants (Dill et al. 2001). When gibberellins are released, they interact with the DELLA proteins to induce conformational changes which decreases the repressive effect of the DELLA proteins, and the inhibition is removed. Two DELLA homologues were identified in *R. delemar* (via Vesper custom HMMER annotation)(Figure 15)(Appendix DELLA: gene homology trees); of these, *R03G_010461* appears dynamically expressed over germination. This homologue appears highly expressed both within resting spores and those growing in a hyphal state. It may be possible that these homologues interact in a similar fashion, maintaining dormancy within resting spores and suppressing early germination cues in *R. delemar* which has entered its vegetative growth phase. These predicted functions were identified through the automated Vesper annotation pipeline (materials and methods), however a reciprocal BLAST against the plant database does not identify DELLA homology, highlighting annotation limitations. These results should therefore be taken with caution until experimental proof of *R. delemar* DELLA-like activity can be provided.

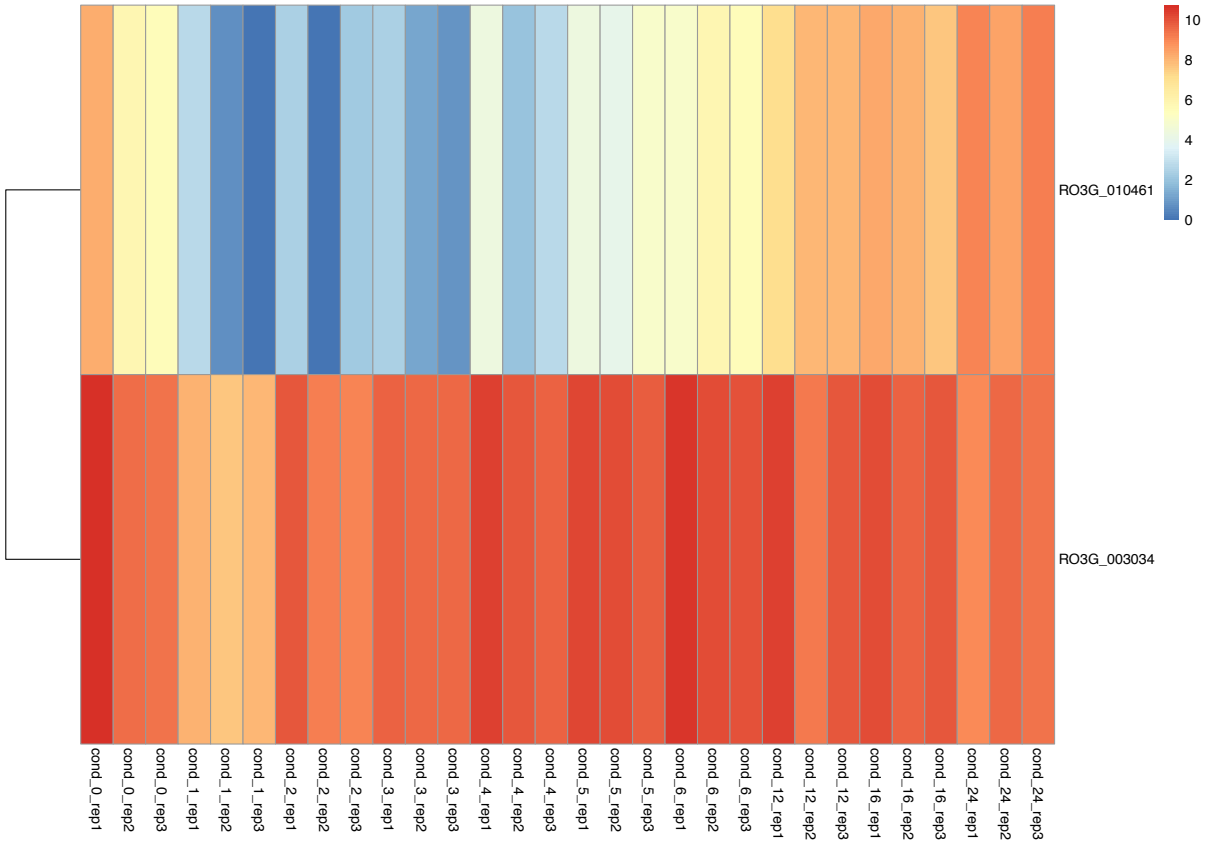


Figure 15. Expression (LogFC) of DELLA homologues over germination.
RO3G_010461 sequence homology to DELLA-like sequence identified through custom annotation, species unknown: E Value: 0.027, % Identity: 53%
RO3G_003034 sequence homology to DELLA-like sequence identified through custom annotation, species unknown: E Value: 0.07, % Identity: 46%

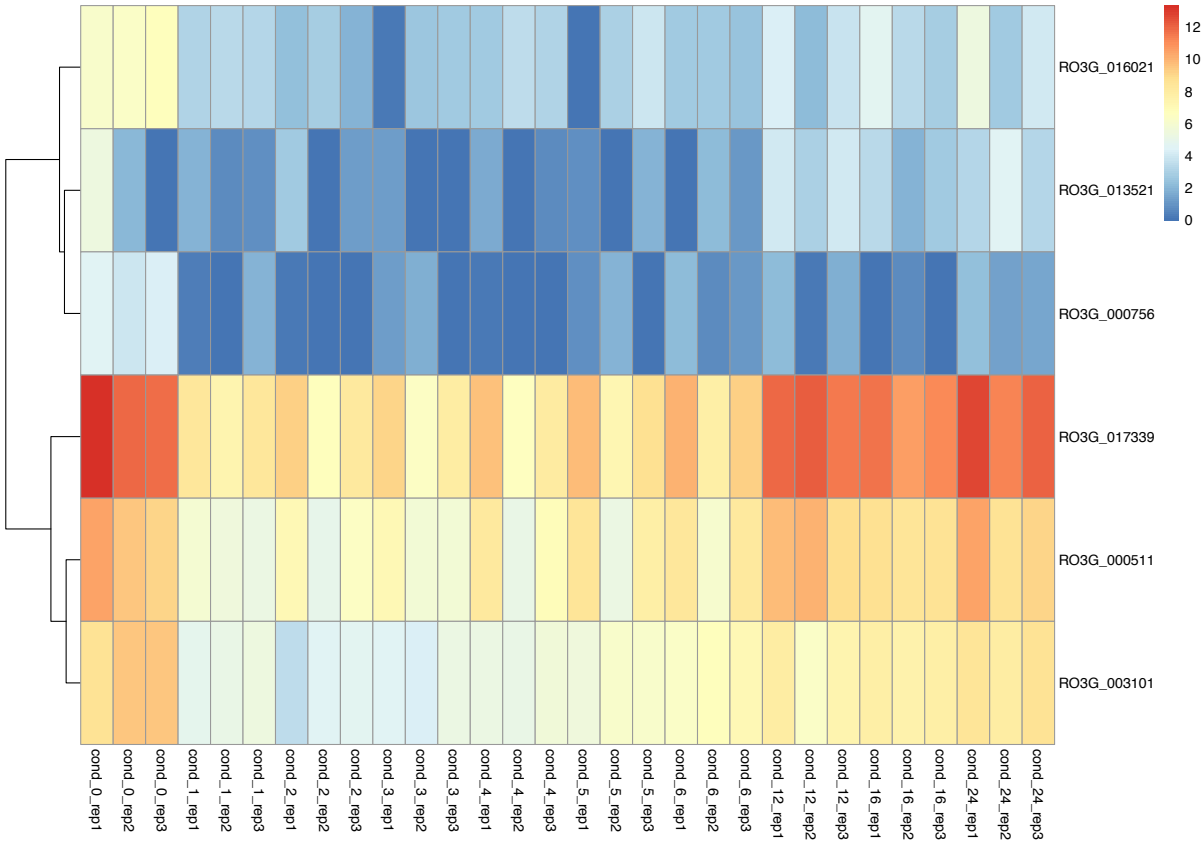


Figure 16. Expression (LogFC) of GLP homologues over germination. (Appendix GLP: gene homology trees)

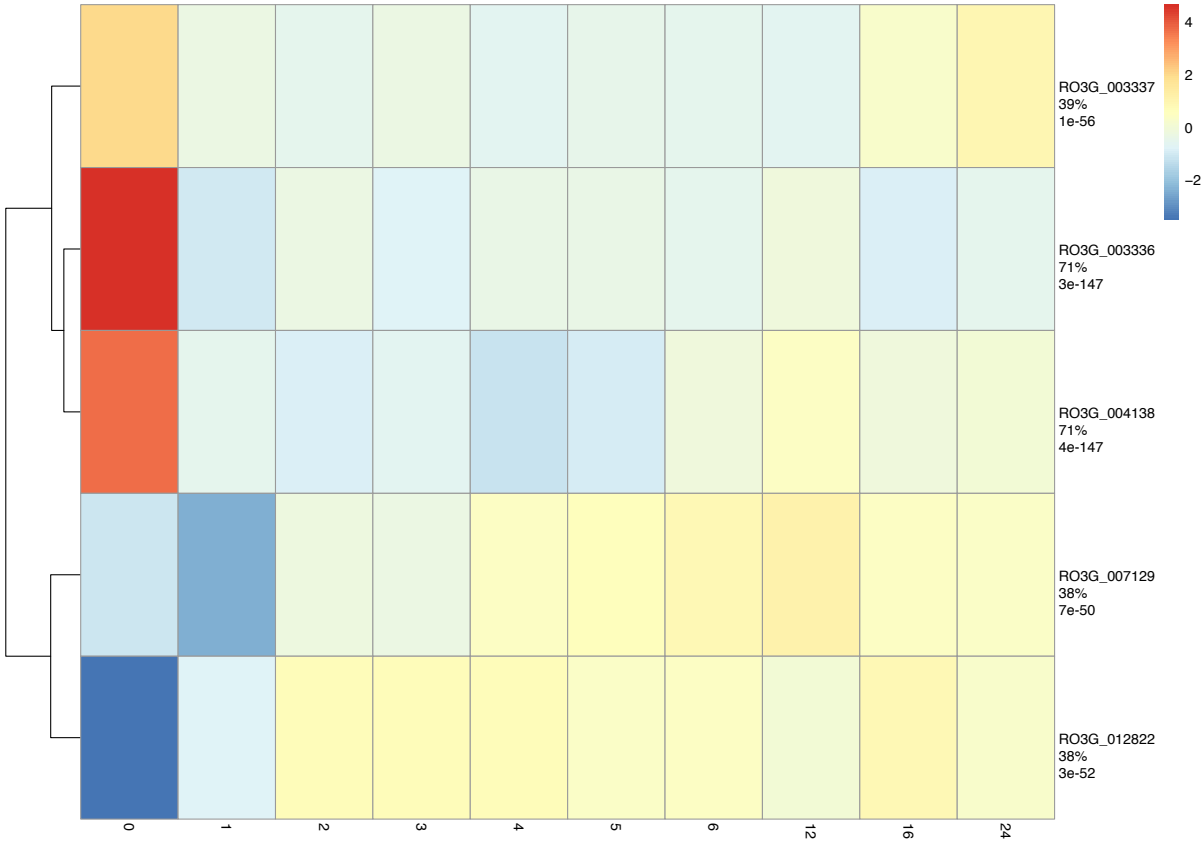
RO3G_016021 sequence homology to GLP1-like sequence identified through sequence identified through custom annotation, species unknown*:* E Value: 6e-04, % Identity: 29%*. RO3G_013521:* sequence homology to GLP4-like sequence identified through sequence identified through custom annotation, species unknown: E Value: 0.014, % Identity: 28%. *RO3G_000756:* sequence homology to GLP1-like sequence identified through sequence identified through custom annotation, species unknown: E Value: 2e-10, % Identity: 71%. *RO3G_017339:* sequence homology to GLP2-like sequence identified through sequence identified through custom annotation, species unknown: E Value: 0.01, % Identity: 32%. *RO3G_000511:* sequence homology to GLP4-like sequence identified through sequence identified through custom annotation, species unknown: E Value: 3e-12, % Identity: 32%. *RO3G_003101:* sequence homology to GLP1-like sequence identified through sequence identified through custom annotation, species unknown: E Value: 1e-05, % Identity: 30% These predicted functions were identified through the automated annotation pipeline (materials and methods), however a reciprocal BLAST against the plant database does not identify GLP homology. These results should therefore be taken with caution until experimental proof of *R. delemar* GLP-like activity can be provided.

Further bioinformatic studies with the putative *R. delemar* DELLA sequences such as alignments, modelling on known structures and detailed phylogeny with plant- and fungal protein families would confirm whether *R. delemar* has proteins that function similarly to DELLAs.

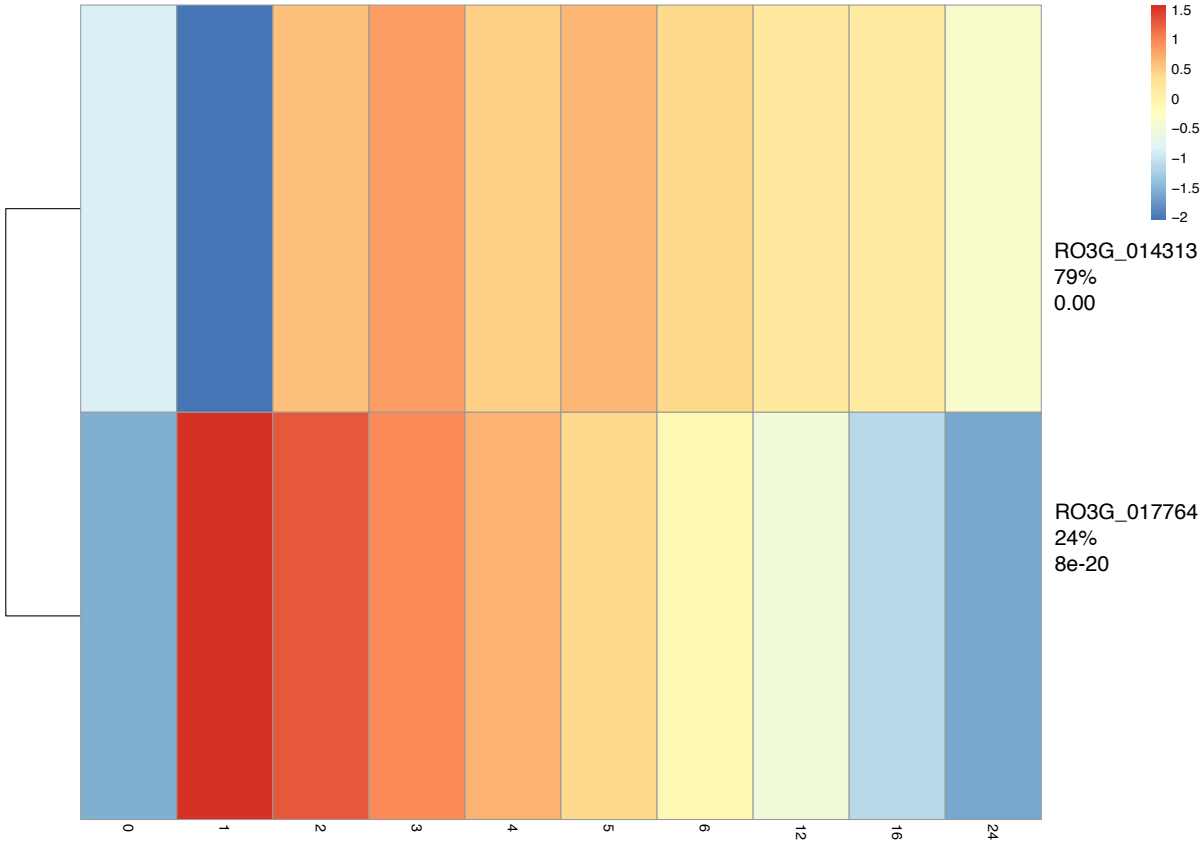
Potential Regulators With Known Functions In The Fungal Kingdom

Other known regulators of fungal germination and reproduction include trisporic acid and photoreceptors. Expression of genes predicted to have roles in these processes will be described here. Multiple genes predicted to have roles in 4dehydroxymethyl-trisporate-dehydrogenase production (Figure 17a) have increased expression in resting spores, whilst others exhibit upregulation at germination onset and throughout hyphal growth (Figure 17b).

A


RO3G_003337
39%
1e-56
RO3G_003336
71%
3e-147
RO3G_004138
71%
4e-147
RO3G_007129
38%
7e-50
RO3G_012822
38%
3e-52
4
2
0
−2
0
1
2
3
4
5
6
12
16
24

B


1.5
1
0.5
0
−0.5
−1
−1.5
−2
RO3G_014313
79%
0.00
RO3G_017764
24%
8e-20
0
1
2
3
4
5
6
12
16
24

C

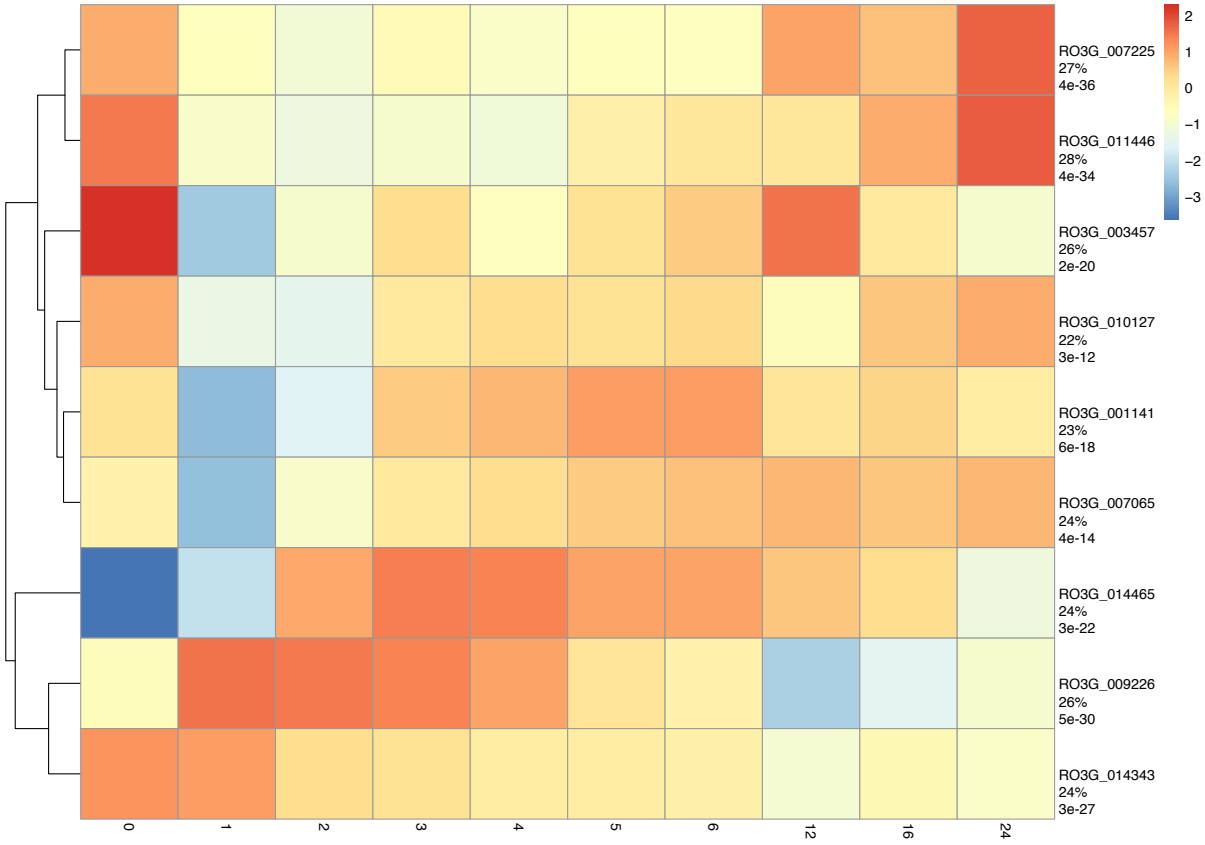


Figure 17. a) Expression (LogFC) of genes predicted to have roles in 4dehydroxymethyl-trisporate-dehydrogenase (homologues of 4dehydroxymethyl-trisporate-dehydrogenase from *Blakeslea trispora*) production over germination. Percentage identity and E value are shown below each homologue. b) Expression (LogFC) of genes predicted to have roles in trisporic acid production over germination (homologues of carotene oxygenase in *Blakeslea trispora*), percentage identity and E value are shown below each homologue. c) Expression (LogFC) of *A. nidulans FAR* homologues over germination, percentage identity and E value are shown below each homologue.

Light is sensed by fungi through photoreceptor proteins capable of sensing a range of wavelengths at varying intensities. Fungal photoreceptors include the phytochromes, known to sense red light in *Aspergillus nidulans*; the blue light sensitive white collar (WC) complex, best studied in *N. crassa;* and the Opsins, shown to sense green light in *N. crassa* (Idnurm and Heitman 2005b, 2005a). Homologues of genes known to encode

cryptochromes WC-1 & WC-2 show dynamic expression over germination (Figure 18); R03G_000967 and R03G_001009 appear highly expressed over hyphal growth, whilst *R03G_013578* is highly expressed in resting spores, and *R03G_003097* highly expressed at the onset of germination. This dynamic expression suggests the germination of *R. delemar* may also be regulated by light sensing receptors, in a similar way to other ancient fungi.

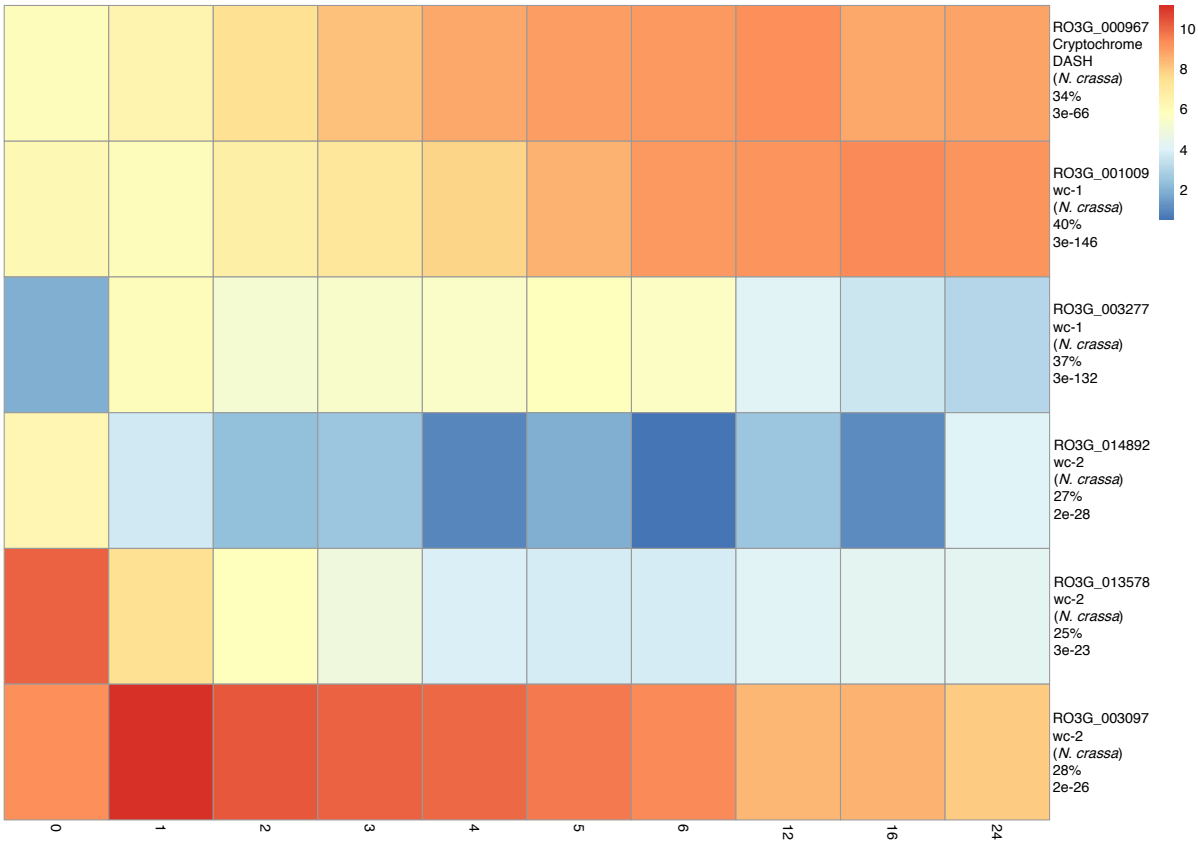


Figure 18. Expression (LogFC) of photoreceptor homologues over germination. Homologues and species are listed, along with percentage identity and E values, below the gene names.

RdAQP1 and RdAQP2 are thought to be transmembrane domains which show high homology to porins from other fungal families. It has been postulated that the porins are regulated by a His residue which may be protonated dependent upon pH and therefore regulates water uptake and swelling of spores, prior to hyphal emergence (Turgeman et al. 2016; Verma et al. 2014).

We see that both porins also appear dynamically regulated over germination (Figure 19), concordant with swelling phases, thus supporting their proposed mechanism of regulation.

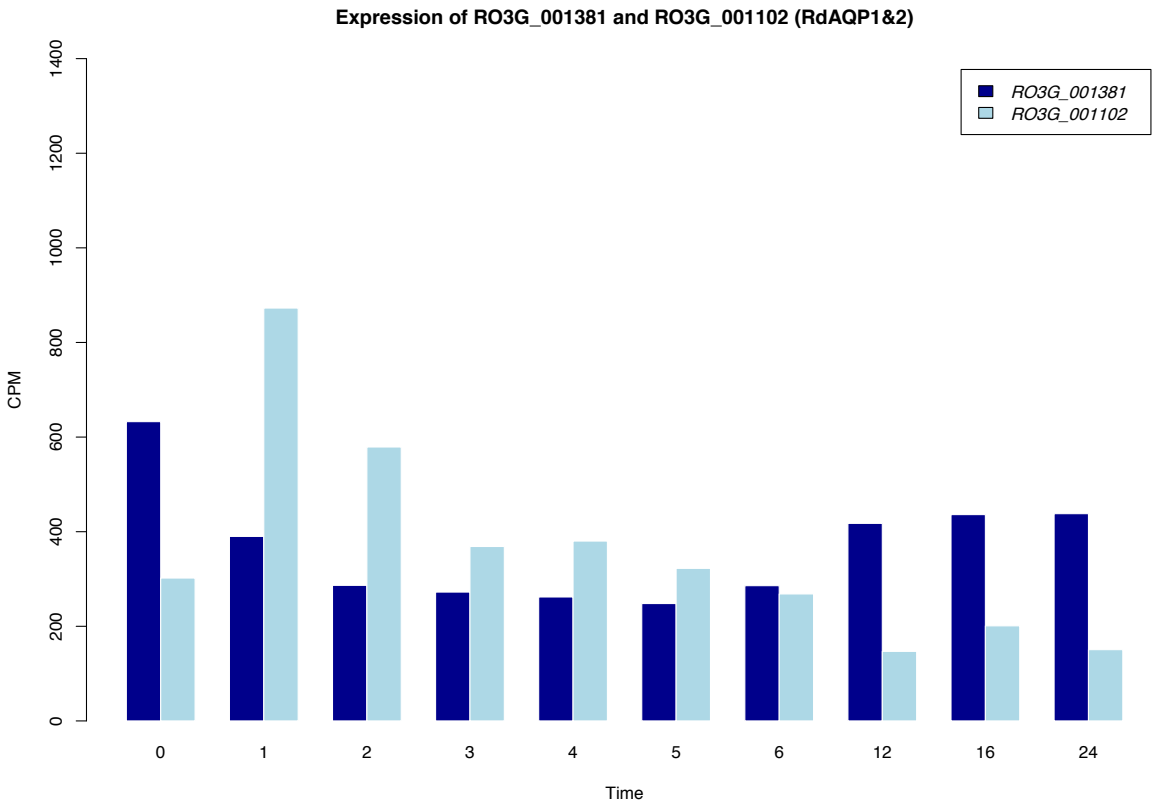


Figure 19. Expression (CPM) of *RO3G_001381* and *RO3G_001102* (RdAQP1&2) over germination.

Comparisons of Transcription Throughout Germination

It is unclear whether the mechanisms that underpin germination are conserved throughout the diverse fungal kingdom. To explore the extent of conservation, I compared my transcriptional data set to other available transcriptional data sets for *Aspergillus niger,* generated by Van Leeuwen et al and Novodvorska et al (van Leeuwen et al. 2013; Novodvorska et al. 2013). When expression profiles of homologous genes from *A. niger* and *R. delemar* are compared over the course of germination, genes with common or unique functions specific to that time point can be identified. The largest shift in the transcriptional landscape of *A. niger* can be seen at the initial stage of germination; we also observed this shift in *R. delemar*. Transcripts with predicted functions involved in transport and localization, proteolysis, and glucose, hexose, and carbohydrate metabolism increase at the initial stages of germination in both *A. niger* and *R. delemar*, while transcripts with predicted functions in translation, tRNA and rRNA processing, and amine carboxylic acid and organic acid metabolism decrease (Figure 20). We also observe differences between the two data sets: over isotropic and hyphal growth, homologous genes with predicted functions in valine and branched-chain amino acid metabolism were upregulated only in *R. delemar*, while homologous genes with predicted roles in noncoding RNA (ncRNA) metabolism, translation, amino acid activation, and ribosome biogenesis were downregulated exclusively in *R. delemar*. A 5% increase in genes that are uniquely up- or downregulated in *R. delemar* is found in high-synteny regions of the genome, compared to genes that are up or downregulated in both *R. delemar* and *A. niger*. The duplicated nature of the *R. delemar* genome may allow for specific and tight regulation of the germination process, a feature unique to *R. delemar*.

It should be noted that *A. niger* and *R. delemar* were cultivated under conditions with different media. *Aspergillus* complete medium (ACM), used to cultivate *A. niger*, and sabouraud dextrose broth (SAB), used to cultivate *R. delemar*, both contain a complex mix of salts, inorganic nutrients, and organic components. Peptides are provided in SAB by mycological peptone, whereas peptides are provided by bacto peptone in ACM. The main carbon source is the same for both ACM and SAB. Both media have a relatively low pH (ACM, pH 6.5; SAB, pH 5.6), and it is known that pH is important for regulating germination in both *R. delemar* and *A. nidulans*. There are currently limited studies that address differences in gene expression, when germination is initiated in filamentous fungi, under different growth media. Growth characteristics of *A. nidulans* have been shown to vary when contents of media differ, while various growth cultivation methods also alter gene expression in *Aspergillus oryzae.* The effect of adding or removing specific organic and inorganic nutrients from media on the growth of filamentous fungi is also better understood. When comparing data sets or designing experiments to address these issues, the effects of using distinctly different media should be considered. This is an area that would benefit from further work aimed at exploring these effects.

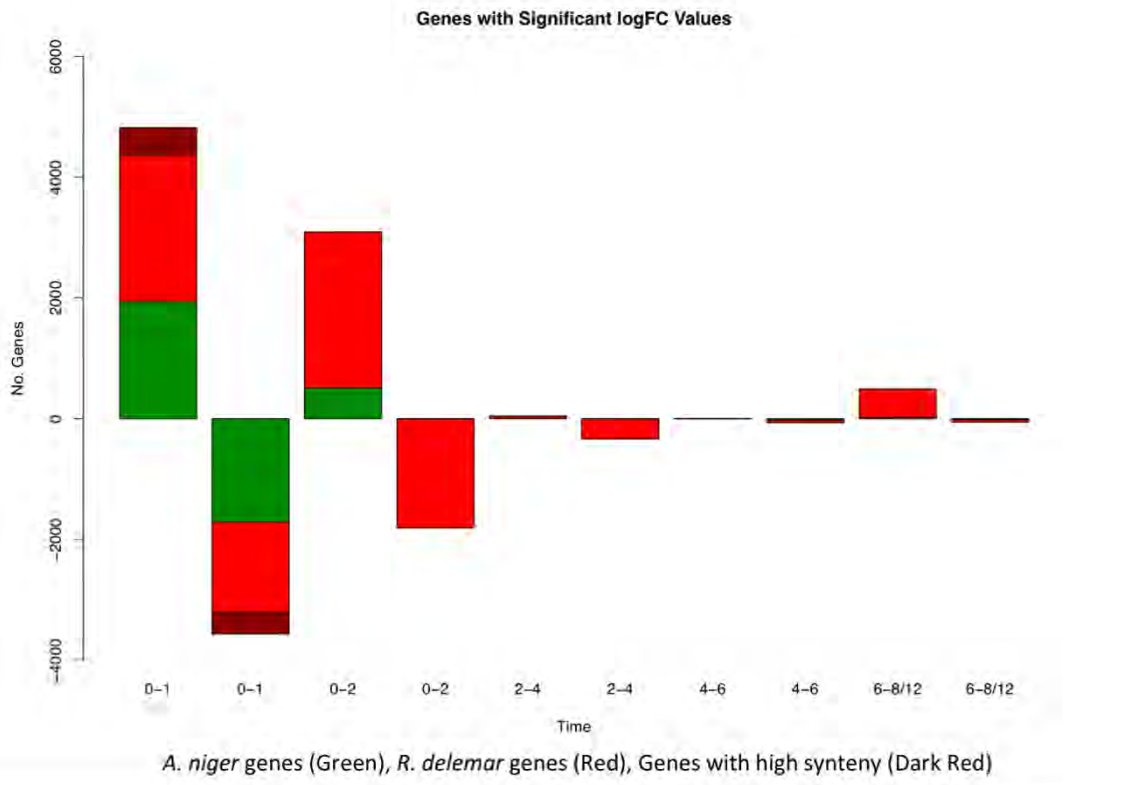


Figure 20. Number of homologous genes significantly differentially expressed (multiple comparisons corrected P value of < 0.05, Benjamini-Hochberg method) between time points, shown over time. Green represents the number of *A. niger* genes, red represents the number of *R. delemar* genes, and dark red represents the number of *R. delemar* genes found in high-synteny regions of the *R. delemar* genome.

Conclusions

This transcriptional analysis reveals that a temporal transcriptional pattern governs germination of *R. delemar* spores (Figure 1). Phenotype-specific (resting spore, swelling spore, hyphal fungi) transcript clusters (Figure 2-5) are identified, and along with pairwise differential expression analysis (Figure 6), it is shown that resting spores appear transcriptionally unique. In comparison, swelling spores appear transcriptionally consistent, whilst the switch to hyphal growth is underpinned by a significant change in the transcriptional landscape. Paired with this, analysis of metabolic transcripts (Figure 7), predicted pathways (Figure 8) and co-transcriptional networks (Figure 9-11) reveal a reliance of germinating spores on ROS resistance, cell wall remodelling components, protein synthesis apparatus, and iron acquisition and transport components. Analysis of duplicated gene pairs highlight individual genes which may be important for germination control. Homology searching reveals the dynamic regulation of germination and growth regulators, with homologues in well-studied fungi and plants. Finally, a comparison of germinating *A. niger* and *R. delemar* transcription demonstrates the presence of a 'core set' of genes conserved throughout germination, however genes unique to each species are also abundant.

Discussion

The above results increase our understanding of the molecular mechanisms controlling germination in *R. delemar*. They show that the initiation of germination entails a huge transcriptional shift; ROS resistance and respiration are required for germination to occur, while actin, chitin, and cytoskeletal components appear to play key

roles in initiating isotropic swelling and hyphal growth. Iron acquisition, Fe-S cluster regulation and sulphur metabolism were also key features of the germination transcriptome, confirming that *R. delemar* relies upon iron not only for pathogenesis (Ibrahim et al. 2010), but also for germination (Kousser et al. 2019). Transcripts co-expressed at the defined stages of germination (and unique to these stages) share functions in protein synthesis. This may offer regulation of protein processes, whilst maintaining specificity to morphological stage. *R. delemar* shares many transcriptional traits with *A. niger* at germination initiation; however, transcriptional features unique to *R. delemar* indicate that the duplicated nature of the genome may allow for alternative regulation of this process. Transcriptional results demonstrate the potential of light, fungal hormones and plant hormones to regulate germination. Specifically, resting spores show increased expression of bcABA2 homologues (Figure 14), which may inhibit fungal germination, similarly to *P. patens* spore germination inhibition via ABA (Moody et al. 2016). It should be noted that the custom annotation which predicted DELLA-like and GLP-like activity did not provide specific species homologues. As plant homologues were not detected through a traditional blastP approach, these results should be taken with caution. The hyphal form shows increased expression of both cryptochrome and white collar homologues, indicating that light sensing may play a role in germination regulation and hyphal development, as it does in *A. fumigatus* and the phototrophic *P. blakesleeanus* (Fuller et al. 2013; Idnurm et al. 2005). It should be noted that other homologues of the genes discussed here may exist within the *R. delemar* genome (Appendix Homology Trees), however only those which were differentially expressed (FDR < 0.001) over all time points have been investigated here. As a result, constituent expression is not discussed here, but should be investigated in further studies. These results have provided a significant overview of the transcriptome of germinating spores and expanded current knowledge in the Mucorales field.

Further Work

Further work focusing on the elucidation of genes essential to germination would benefit the field. Whilst co-expression network analysis aimed to reveal these, the networks (Figure 9-11) were ultimately too large to provide a practical list of candidate genes. To determine germination essential genes, transcriptional studies focusing on the times of phenotypic change (0-2hr, 4-6hr, 6-12hr), sampling at smaller intervals (every 10 minutes), would likely provide better information about germination specific genes. Further analysis of duplicated gene pairs might also aid in the identification of germination specific genes. Producing knockout mutants of the genes proposed here (Figure 13), which could be screened for germination defects, would confirm whether these candidate genes have diverged in function, and whether they play specific roles in germination. A full transcription factor annotation of the *R. delemar* genome, combined with an integrated ChIP-Seq RNA-Seq experimental approach, would also aid in determining germination regulation. This approach would generate germination-essential gene candidates via analysis of differentially regulated gene networks. Generation of a CRISPR-Cas9 knock out library in *R. delemar* might also identify germination specific genes. Germination specific genes would be attractive drug targets, as mucormycete germination within the host contributes to pathogenesis. Use of a metabolomics approach would aid in the validation of the PathwayTools pathway predictions, and may result in identification of natural compounds with therapeutic activities, as is common for secondary metabolites of fungi.

Chapter 5: Transcriptional Regulation of *Rhizopus*-Macrophage Interactions

The following text and figures have been previously published, or are adapted from the article "Host-pathogen transcriptomics of macrophages, Mucorales and their endosymbionts: a polymicrobial pas de trois" (Sephton-Clark et al. 2019). For this article, I conceived, designed and performed experiments, collected the data, performed the analysis and interpretation, wrote the manuscript, completed revisions and prepared the figures.

This chapter will introduce the host-pathogen interactions which govern mucormycosis and immunity. An introduction to how bacterial endosymbionts impact pathogenicity of mucorales species will also be given. Results will describe the differential interactions of two Mucorales species (*Rhizopus delemar RA 99-880* and the UoB strain of *Rhizopus microsporus*) with the innate immune system, and their interactions with environmental bacterial endosymbionts. The transcriptional responses of *R. delemar* and *R. microsporus* to innate immune cells, and the corresponding immune cell response will be examined. The variation in these responses will be assessed, given the presence or absence of bacterial endosymbionts within the fungi. It can be seen that the fungal response is driven by interaction with innate immune cells. The effect of the bacterial endosymbiont on the fungus is species specific, with a minimal effect in the absence of stress, but strongly influencing fungal expression during spore interactions with innate immune cells. In contrast, the macrophage response varies depending on the infecting fungal species, but also depending on endosymbiont status. The most successful macrophages elicit a pro-inflammatory response, and through germination inhibition macrophage survival is enhanced. This reveals

species-specific host responses to related Mucorales species and shows that bacterial endosymbionts impact the innate immune cell response.

Host-Pathogen Interactions in Mucormycosis

Mucormycosis has been studied in the context of mucorales-host interactions using model organisms and *in vitro* tissue culture methods. Mouse models have shown that spore interactions with endothelial cells are vital to causing diseases in immunocompromised individuals, as pathogenicity is linked to the variable copy number of the gene encoding CotH, a family of cell surface proteins important for the spores' adherence to the GRP (glucose regulated protein) receptors on endothelial cells (Liu et al. 2010; Gebremariam et al. 2014; Watkins et al. 2018). Mouse studies have also revealed the reliance of mucorales spores on free iron to cause invasive mucormycosis. The DKA (Diabetic Ketoacidic) mouse model is more susceptible to developing mucormycosis (Gebremariam et al. 2016). DKA and other acidosis disorders result in pH-mediated dissociation of iron from protein transporters, resulting in elevated concentrations of free iron within individuals. This is clearly seen in patient data, as individuals with elevated iron concentrations (within serum) are highly susceptible to mucormycosis (Ibrahim, 2014).

This reliance on iron is also seen *in vitro*. Iron limitation has been reported as a mechanism by which macrophages control mucorales spore germination, whilst iron-scavenging pathways have been reported to confer pathogenicity to *Rhizopus spp.* (Andrianaki et al. 2018; Chibucos et al. 2016). The reliance on innate immune cells such as macrophages to control spores has been demonstrated in the established zebra fish model (Voelz et al. 2015). Within the zebra fish model, innate immune cells form granuloma-like structures around infecting mucorales spores. In doing so they inhibit spore germination, dissemination and delay invasive infection (Inglesfield et al. 2018). In an immunocompromised zebra fish model,

these innate granulomas are less likely to form, and infecting spores rapidly disseminate, leading to uncontrolled infection and increased mortality rates (Inglesfield et al. 2018).

The interaction between host and mucorales spore can be further regulated by the presence of an endosymbiont (Itabangi et al. 2019; Partida-Martinez & Hertweck 2005). Many Mucorales species have been shown to harbour bacterial endosymbionts whose species can vary between Mucorales isolates (Ibrahim et al. 2008; Kobayashi & Crouch 2009; Mondo et al. 2017; Itabangi et al. 2019). Itabangi et al. recently explored the effect of an endosymbiont on the interaction between *R. microsporus* and innate immune cells. This study has shown that a bacterial endosymbiont influences the outcome of *Rhizopus microsporus* infections in both zebrafish and murine models, through modulation of both fungal and phagocyte phenotypes (Itabangi et al. 2019).

It is clear that understanding the interaction between Mucorales spores, their endosymbionts and innate immune cells is key to understanding mucormycosis. Studies frequently focus upon the interaction of a single species of the Mucorales order with innate immune cells (Warris et al. 2005; Chamilos et al. 2008; Schmidt et al. 2013; Kraibooj et al. 2014; Inglesfield et al. 2018), despite numerous phenotypic and genomic differences existing between Mucorales species and infecting isolates (Hoffmann et al. 2013; Mendoza et al. 2014; Schwartze et al. 2014). Several works comparing *Aspergillus spp*. to *Rhizopus spp*. have revealed similar immunostimulatory capacities, but differences in their responses to host stress (Warris et al. 2005; Chamilos et al. 2008; Schmidt et al. 2013; Kraibooj et al. 2014). Exploring and understanding fungal responses to the host is essential to improving our understanding of mucormycosis, yet it remains unclear how Mucorales species respond to,

and interact with, the innate immune system, and to what extent this varies by species and endosymbiont presence.

This work explores the interplay between phagocytes, *R. delemar*, and *R. microsporus* using fungal isolates found to harbour bacterial endosymbionts (Itabangi et al. 2019). This work investigates the differences between these two fungal species, how they respond transcriptionally to innate immune cells, and how their respective bacterial endosymbionts affect this interaction. This work also explores the transcriptional response of innate immune cells to these infectious spores, and determines how their response is influenced by the presence of an endosymbiont.

Results

To better understand the interactions between Mucorales spores and innate immune cells, I performed a paired transcriptional study to identify responses of macrophages and fungal spores, whilst exploring the influence of the endosymbiont on this interaction. *Rhizopus delemar* and *Rhizopus microsporus* spores were either cured via ciprofloxacin treatment to remove the bacterial endosymbiont (cured) or maintained in media permissive to bacterial endosymbiosis (wt). Cured spores were passaged twice in the absence of ciprofloxacin to limit the impact of the drug on transcriptional responses. The cured and wt spores of both *R. delemar* and *R. microsporus* were allowed to swell in sabouraud broth (SAB) until 95% of the population had reached mid isotropic phase (Sephton-Clark et al. 2018). Due to the differences in germination rates between the species (Chapter 2), this occurred at 2 hours for *R. delemar* and 4 hours for *R. microsporus*. Swollen spores were then used to infect the J774.1 murine macrophage-like cell line. Swollen fungal spores were co-cultured with macrophages for one hour, after which unengulfed spores were removed, and phagocytosed spores were incubated within the macrophages for a further two hours. In all cases 95% phagocytosis was achieved. Macrophages and spores from the resulting infection were processed to explore their transcriptional response to this infection scenario. Macrophages that had phagocytosed swollen fungal spores (cured and wt) were isolated and sequenced via the 10X Genomics Chromium Single Cell Sequencing platform. Macrophages left unexposed to the fungi were used as a negative control. RNA was also isolated from fungal spores (cured and wt) which had been engulfed by macrophages, and this was sequenced with a bulk RNA-Seq approach. Unexposed fungi (cured and wt) were incubated in macrophage media (serum-free Dulbeccos Modified Eagle Medium) for a matched time and used as a negative control.

The Fungal Response

The overall response to phagocytosis from *R. delemar* and *R. microsporus*, obtained through the bulk RNA-Seq approach, was examined with principle component analysis (PCA) (Figure 1). Large differences between the transcriptomes of both fungal species can be observed, when exposed or unexposed to macrophages, while the presence or absence of their respective endosymbionts has a weak but differential effect on PCA (Figure 1). The presence or absence of the endosymbiont appears to have very little bearing on the transcriptional patterns displayed by *R. delemar*, as samples fell into two distinct clusters, most strongly influenced by macrophage status (Figure 1a). *R. microsporus* exhibits a similar trend upon exposure to macrophages, however the presence of the endosymbiont influenced clustering more so (Figure 1b). Itabangi et al., show that the presence of the endosymbiont *Ralstonia pickettii* impacts fungal cell wall organization, resistance to host-relevant stress, spore germination efficiency, and pathogenesis of *R. microsporus* (Itabangi et al. 2019).

It should be noted that both sample groups contain an outlier. Given the smaller range of the *R. microsporus* PCA axis (Figure 1b), the outlier for this group appears to effect grouping less, compared to the *R. delemar* outlier. The outliers were both processed in separate biological experiments, and both samples obtained a good read level and alignment level when sequenced and processed with the bioinformatics pipeline. As the cause could not be determined in either instance, and it is not recommended to calculate significant differential expression with two biological repeats, the outliers were kept in for analysis. It should therefore be noted that the results may be skewed due to the outliers presence. This will be extenuated due to the strict statistical thresholds imposed. To improve the results, a fourth biological repeat should be performed in the future, time and cost permitting.

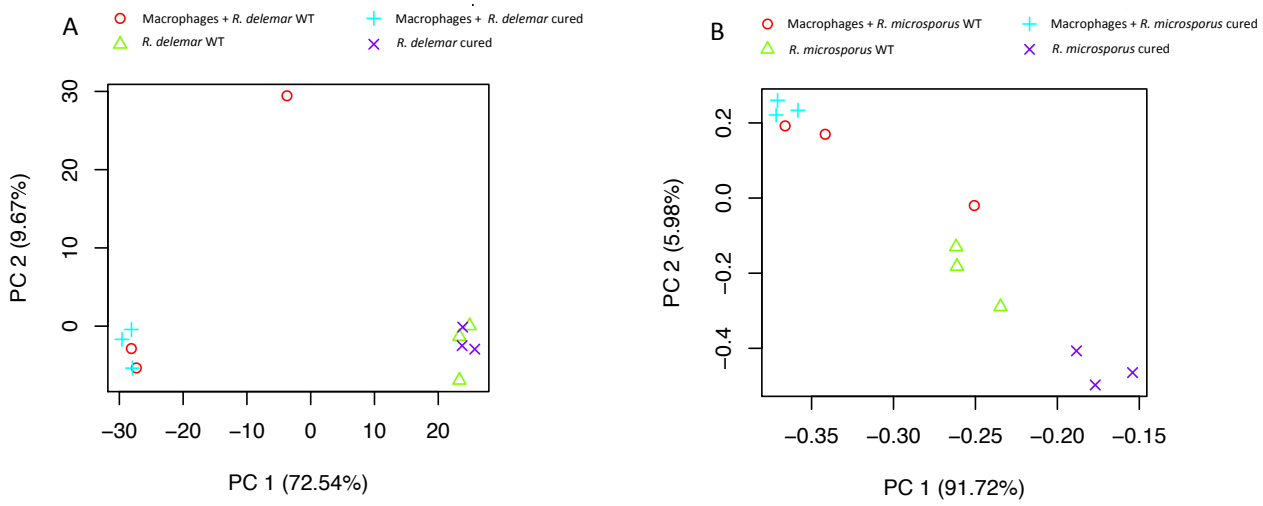


Figure 1. Principal component analysis of fungal genes differentially expressed across all samples. A) *R. delemar* wt and cured, macrophage engulfed or serum free DMEM (dulbecco's modified eagle medium) control. B) *R. microsporus* wt and cured, macrophage engulfed or serum free DMEM control. Biological replicates (n=3) are shown for each sample.

There are 2,493 genes that are significantly differentially expressed (Log fold change > 2; false discovery rate < 0.05) in *R. delemar* across all conditions (Figure 2a), while *R. microsporus* only exhibits 40 genes significantly differentially expressed across all conditions (Log fold change > 2; false discovery rate < 0.05) (Figure 2b). The theme of a muted transcriptional response from *R. microsporus* is also seen within pairwise comparisons of conditions. Pairwise comparisons of differential expression across each experimental condition show similar trends in responses between *R. delemar* and *R. microsporus*, however *R. microsporus* responds with a reduced gene set (Figure 3). The limited response of *R. microsporus* may be due to a differential growth stage. As *R. microsporus* germinates slower than *R. delemar*, after 3 hours within the phagosome *R. microsporus* may not have reached the same morphological growth stage as *R. delemar,* contributing to an apparent muted response. Pairwise comparisons showed the biggest shift in transcriptional response when comparing phagocytosed fungal spores to those unexposed

to macrophages, regardless of endosymbiont status. When phagocytosed (Figure 4), *R. microsporus* upregulates genes enriched in GO categories corresponding to thiamine metabolism, sulfur metabolism, glycerol metabolism, alcohol dehydrogenase activity and transmembrane transporter activity (hypergeometric test, corrected P value < 0.05). This is consistent with the fungal response seen to macrophage stress (Parente-Rocha et al. 2015), and the micronutrient scavenging response to nutritional immunity (Ballou and Wilson 2016; Shen et al. 2018; Andrianaki et al. 2018). Phagocytosed *R. microsporus* downregulated genes enriched in GO categories corresponding to rRNA processing, ribosome biogenesis and ribosome localization (hypergeometric test, corrected P value < 0.05) (Figure 4), consistent with growth arrest within the phagolysosome (Inglesfield et al. 2018; Andrianaki et al. 2018)

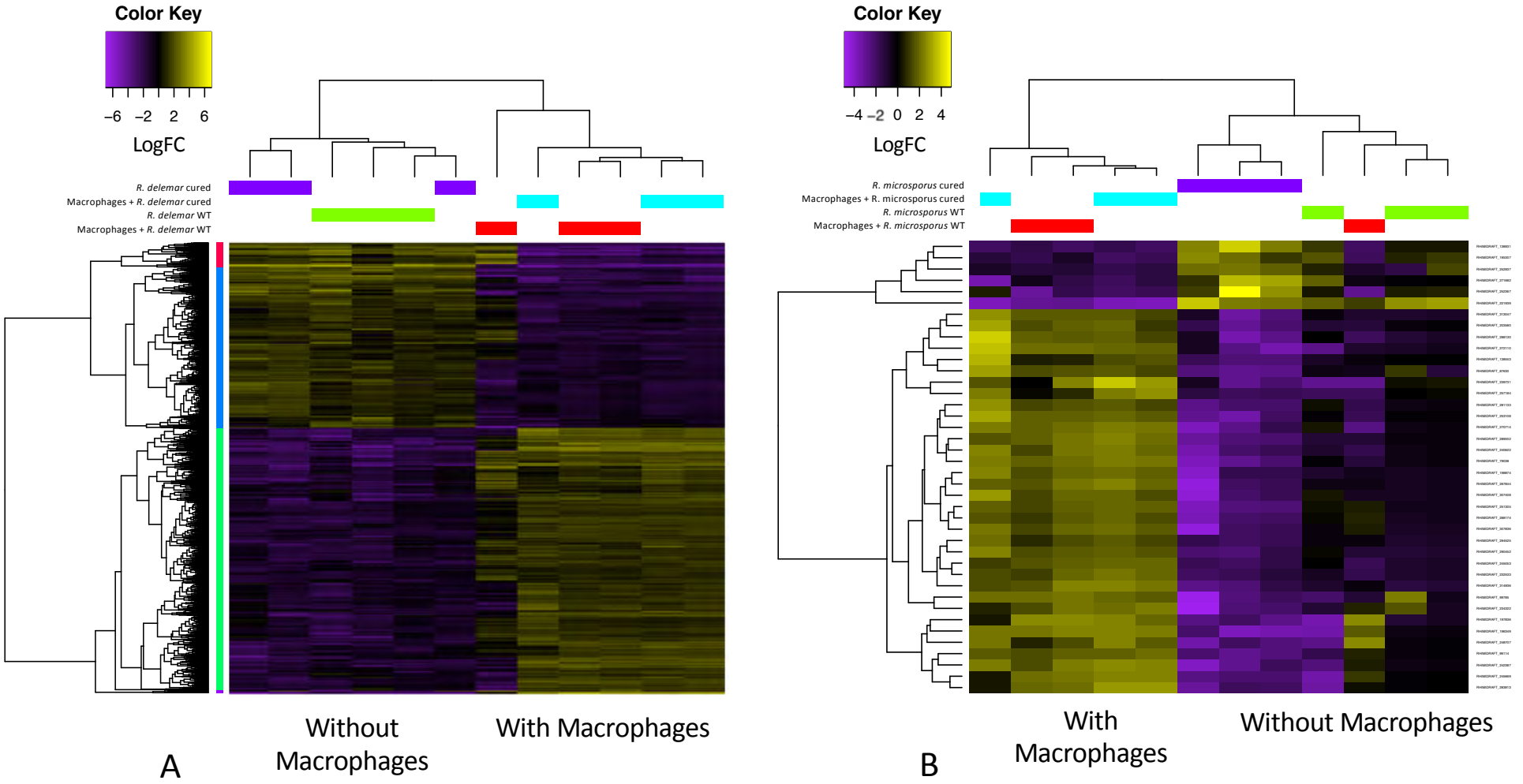


Figure 2. Clustering of fungal transcriptional changes. A) Heatmap displaying significantly differentially expressed genes in *R. delemar*. Expression levels are plotted in Log2, space and mean-centered (FDR < 0.001) B) Heatmap displaying significantly differentially expressed genes in *R. microsporus*. Expression levels are plotted in Log2, space and mean-centered (FDR < 0.001). Biological replicates (n=3) are shown for each sample.

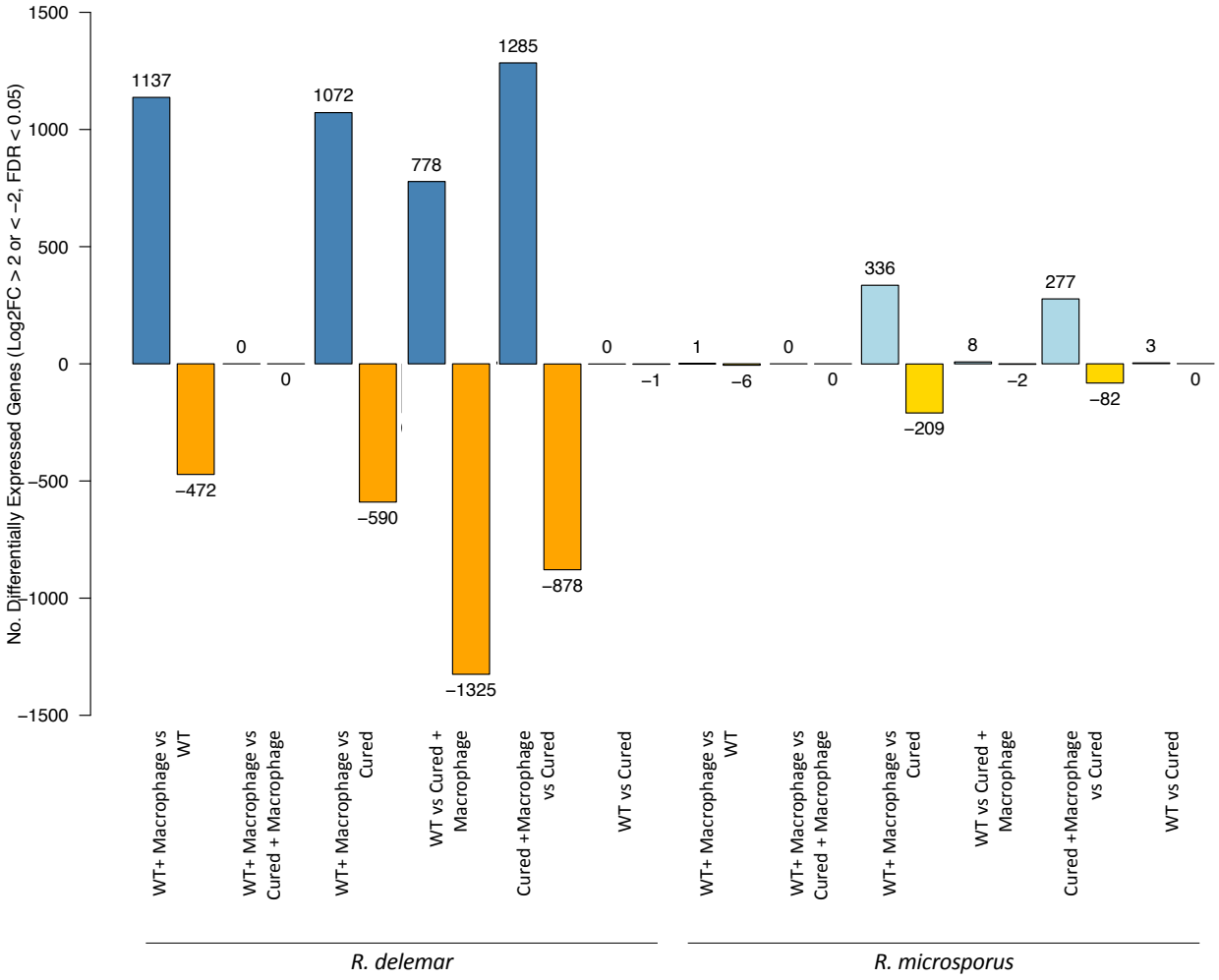


Figure 3. Differential expression of fungal genes in compared conditions. The graph displays the number of genes significantly differentially expressed (multiple comparisons corrected P value < 0.05) between samples listed on the X axis. Blue bars indicate genes with an increase in expression (LogFC > 2), whilst orange bars indicate genes with a decrease in expression (LogFC < −2). Averages are taken from data produced with 3 biological replicates (n=3) for each sample.

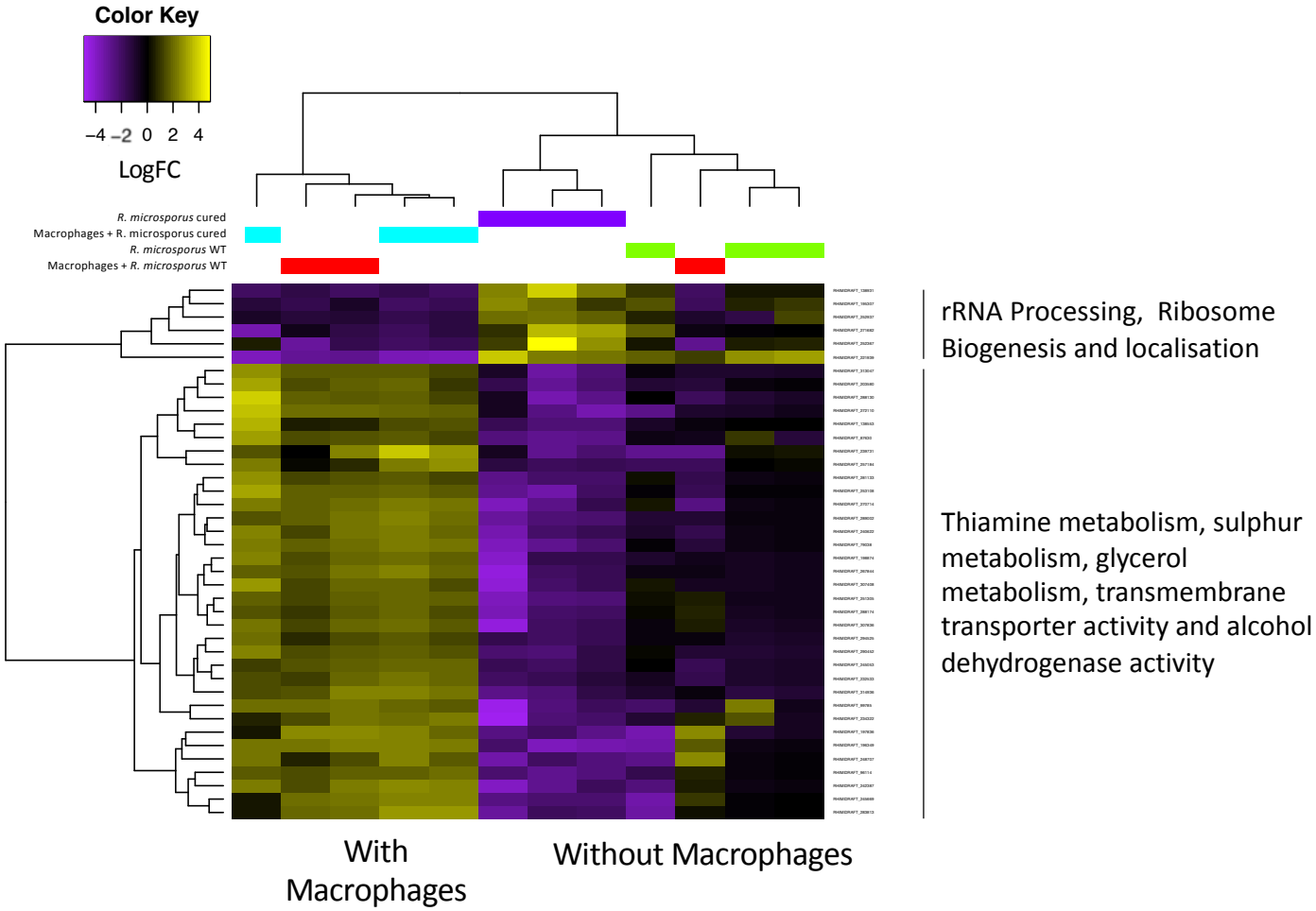


Figure 4. Clustering of fungal transcription with functional annotation. Heatmap displaying significantly differentially expressed genes in *R. microsporus* . Expression levels are plotted in Log2, space and mean-centered (FDR < 0.001). Biological replicates (n=3) are shown for each sample. Gene names and predicted functions can be found in the appendix (*R.microsporus*_SigDE).

Comparisons of *R. delemar* conditions (Figure 5) reveal that, upon phagocytosis, spores upregulate genes enriched in KEGG classifications corresponding to MAPK signalling, phenylalanine metabolism, tyrosine metabolism, glutathione metabolism and fatty acid synthesis (hypergeometric test, corrected P value < 0.05). Upregulation of these processes is consistent with intra-phagosomal survival (Yadav et al. 2011; Lorenz and Fink, 2005; Eisenman et al. 2011; Andrianaki et al. 2018).

Unexposed *R. delemar* spores upregulate genes enriched in KEGG classifications corresponding to ketone body synthesis, protein processing via the endoplasmic reticulum, amino sugar and nucleotide sugar metabolism (hypergeometric test, corrected P value < 0.05). This is consistent with metabolic activation and cell wall biogenesis (Figure 5).

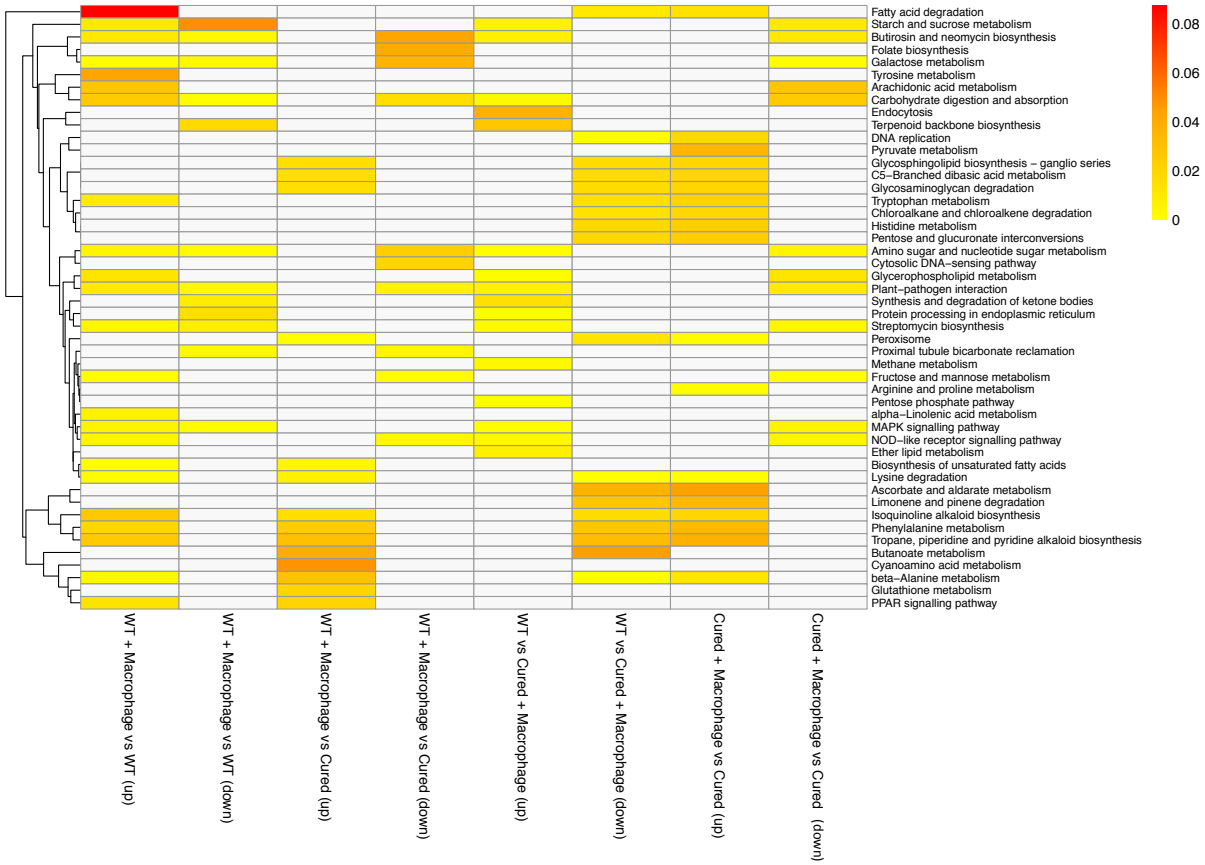


Figure 5. Functions of genes differentially expressed in *R. delemar*. Enriched KEGG categories for the up/down regulated genes over sample comparisons (listed on the X axis). The enrichment of the category is indicated by the colour bar. White corresponds to no enrichment, and yellow to red corresponds to the given P value of the enrichment. Data produced with 3 biological replicates (n=3) for each sample.

Previously identified duplicate gene pairs of *R. delemar* (Chapter 3, 4) were analysed for significant alternate expression (LogFC between genes within a pair > 2), dependent on endosymbiont status and macrophage exposure. Whilst all *R. delemar* spores appeared to alternately regulate oxygen, ROS and superoxide metabolism, only cured spores exposed to macrophages also differentially regulated glycolysis (Figure 6). This indicates that although the role of the endosymbiont appears lesser when the overall transcriptional response is viewed (Figure 1), the presence of the endosymbiont may aid fungal resistance to external stress by permitting metabolic flexibility, perhaps through regulation of metabolic pathways. A similar mechanism by which *Burkholderia* regulates sexual reproduction in *R. microsporus* has been observed, showing that endosymbionts have the ability to regulate host processes (Mondo et al, 2017).

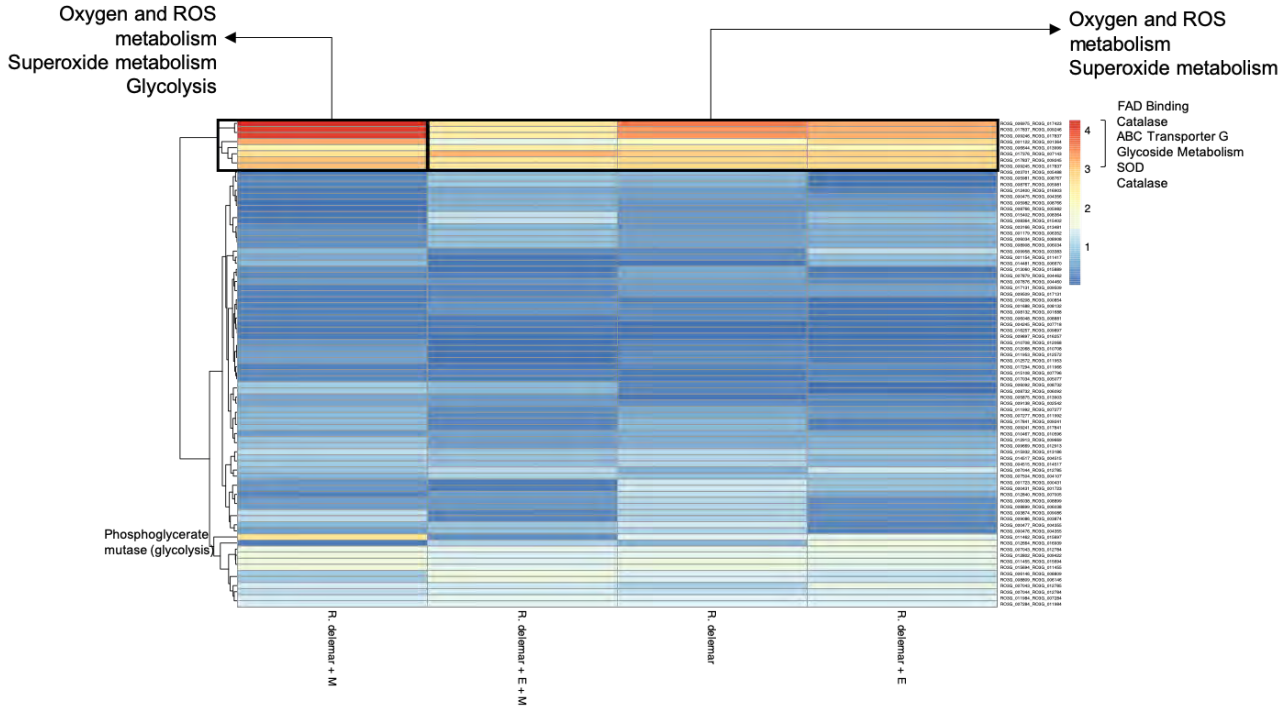


Figure 6. Heatmap displaying the difference in expression (LogFC) between duplicated gene pairs in the spores of *R. delemar*, wt (+E) or cured, which have been phagocytosed (+M) or remain unexposed to macrophages.

When comparing transcriptional profiles of wt and cured spores incubated in serum-free DMEM (sfDMEM) for 3 hours (time matched to phagocytosis assay), very few transcriptional changes in either species in response to curing were observed. In *R. delemar*, a single gene, predicted to be a putative protein phosphatase, was repressed. In *R. microsporus* , three genes were induced: an autophagy-related protein, a C2H2 zinc finger transcription factor, and ribosomal protein L2.

Despite these small changes, loss of the endosymbiont appears to impact the size of transcriptional responses of both fungal species to macrophages (Figure 3), and Itabangi et al have shown the importance of the endosymbiont for virulence (Itabangi et al, 2019). Specifically, an overall increase in the number of fungal genes differentially regulated upon phagocytosis for both species can be observed. Exposure of wt *R. microsporus* to macrophages induced the expression of one gene with no known function and repressed 6 genes. The repressed genes include an autophagy-related protein and a zinc finger transcription factor, as well as 4 unannotated genes (Figure 3). In contrast, when cured *R. microsporus* spores were exposed to macrophages, 277 genes were significantly induced and 82 repressed, compared to unexposed cured spores (Figure 3). Induced genes were enriched (hypergeometric test, corrected P value < 0.05) for the following GO categories: organelle organisation, pre-ribosome and ribosome activity, ATPase activity, hydrolase activity, pyrophosphatase activity, helicase activity, nucleic acid binding, RNA metabolism, nitrogen metabolism, chromatin silencing. Repressed genes were enriched (hypergeometric test, corrected P value < 0.05) for the following GO categories: oxidoreductase activity, hydrogen sulphide metabolism, glycolysis, sulphur metabolism, hexose catabolism, siderophore

activity, iron assimilation, nitrogen metabolism, carboxylic acid metabolism. This suggests an overall failure to properly respond to host stresses such as iron starvation in the absence of the bacterial endosymbiont.

A similar impact of the endosymbiont was observed for *R. delemar*. While the overall fungal response to phagocytosis is characterized by a robust transcriptional response, induced genes in wt samples (1137 genes, Figure 3) were enriched for KEGG classifications corresponding to: alanine metabolism, PPAR signalling, aromatic compound biosynthesis and degradation, lysine metabolism, lipid metabolism, MAPK signalling, sugar metabolism, tyrosine metabolism, secondary metabolite biosynthesis (Figure 5). Repressed genes in wt samples (472 genes, Figure 3) were enriched for KEGG classifications corresponding to: carbohydrate metabolism, secondary metabolite biosynthesis, ketone body processing, protein processes, MAPK signalling (Figure 5). In contrast, induced genes in cured samples (1285 genes, Figure 3) were enriched for KEGG classifications corresponding to: fatty acid metabolism, DNA replication, amino acid metabolism, glycan metabolism, pyruvate metabolism and secondary metabolite processing (Figure 5). Repressed genes in cured samples (878 genes, Figure 3) were enriched for KEGG classifications corresponding to: sugar metabolism, amino acid metabolism, lipid metabolism, MAPK signalling, NOD-like receptor signalling. Again, this suggests that the endosymbiont has an overall suppressive impact on fungal transcription in response to macrophage challenge.

When comparing the transcriptional responses of ortholog genes shared by *R. delemar* and *R. microsporus*, we see only a small proportion behave similarly (213 genes, detailed in the online appendix, Ortho_Genes). When phagocytosed, wt spores from both species upregulate ortholog genes involved in fatty acid

catabolism, transcription, regulation via polymerase II, and organelle organization. Phagocytosed cured spores from both species upregulate ortholog genes involved in RNA processing, chromosome organization and condensed chromosome pathways. When unexposed, we see wt spores upregulate ortholog genes involved in translocation, protein binding, siderophore activity, cobalmin processing, and post-translational protein targeting. Cured unexposed spores upregulate ortholog genes with roles in siderophore activity and transferase activity. A list of the 213 orthologs and their predicted functions can be found in online the appendix (Ortho_Genes). Overall, *R. delemar* and *R. microsporus* both respond transcriptionally to the presence of macrophages, however the size and composition of this response differs between species.

Due to work which has linked iron scavenging to survival within the phagolysosome (Andrianaki et al. 2018), expression of genes predicted to be involved in ferrous iron transport was analysed. There are 12 genes in the *R. delemar* genome with predicted ferrous iron roles. While 8 showed no significant change over the tested conditions, 3 (*R0G3_006623*, *R0G3_007727*, *R0G3_011864*) appeared highly expressed in wt and cured phagocytosed spores, compared to unexposed spores. The last gene, *ROG3_009943*, is highly expressed in wt spores unexposed to macrophages. Together, this suggests there may be condition dependent specialization in the expression of ferrous iron transport in *R. delemar*.

The Macrophage Response

To investigate the innate immune response when challenged with *R. delemar* and *R. microsporus*, I conducted single cell RNA-Sequencing of J774.A1 murine macrophages, unexposed and exposed for 3 hours, to the four types of pre-swollen spores. Transcription of both challenged and unchallenged macrophages displayed underlying population heterogeneity (Figure 7). The functions of genes highly expressed (Figure 8) and displaying reduced expressed (Figure 9) within the mixed clusters span broad categories including metabolic processes, nucleotide processing, cell cycle processes and stress response pathways. The heterogeneity displayed may be due to alternate cell cycle states of the macrophages sampled. Synchronisation of the macrophages prior to infection might reduce the heterogeneity seen here, and allow for better detection of the transcriptional response to infection, at a single cell level.

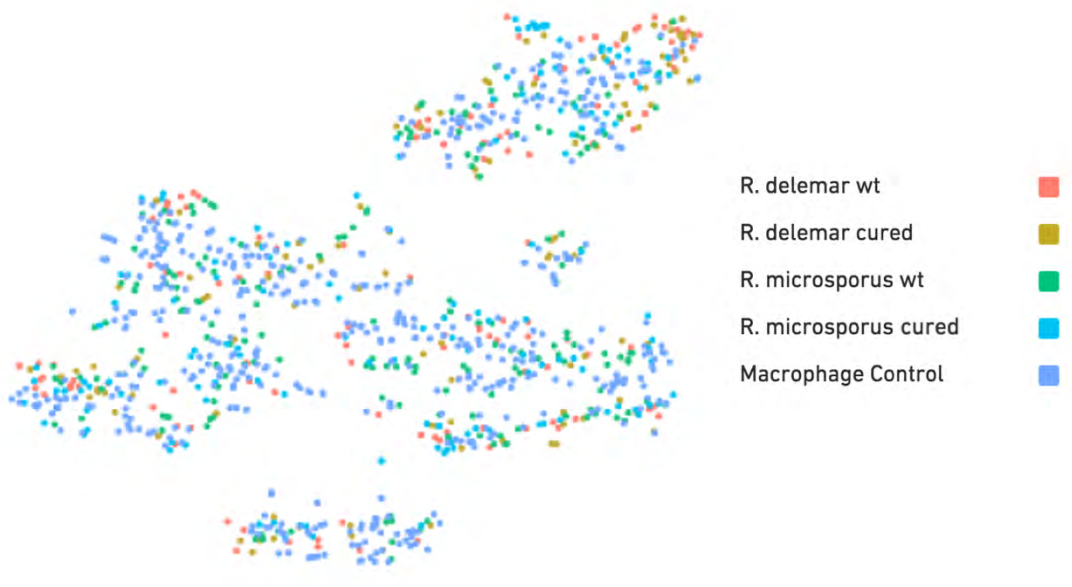


Figure 7. Single cell plot generated by Loupe Cell Browser (K-means clustering). The 10X single cell genomics pipeline requires use of the 10X genomics specific software to visualize single cell data. Unfortunately the software does not provide information on PCA axis units, although it is likely to represent semantic space.

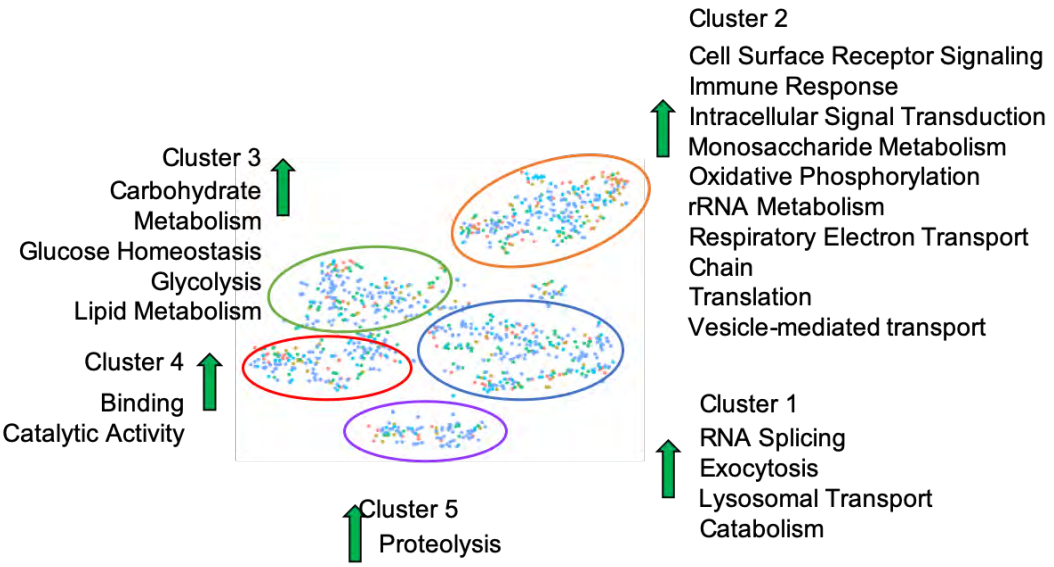


Figure 8. Functions classifications (GO terms) corresponding to genes which appear highly expressed (Log2FC > 2) within each cluster of single cells (cluster determined with K means clustering). Whilst cluster 3 (carbohydrate metabolism), 4 (binding activity) and 5 (proteolysis) appear functionally distinct, clusters 1 and 2 highly express genes with a range of broad functions, which offer inconclusive results about the state of the macrophages within these clusters.

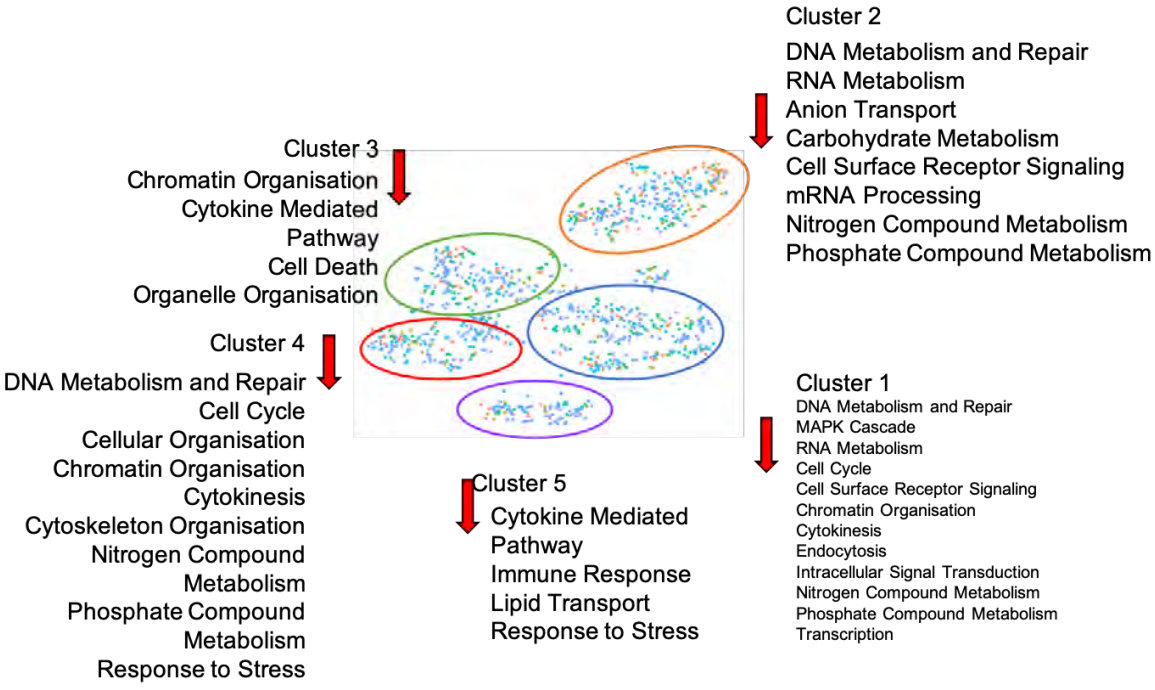


Figure 9. Functions classifications (GO terms) corresponding to genes which exhibit reduced expression (Log2FC < -2) within each cluster of single cells (cluster determined with K means clustering). None of the clusters appear functionally distinct, there are however functions which appear to be consistently down regulated between clusters, these include: cytokine signalling (cluster 1, 3, 5), chromatin organization (cluster 1, 3, 4), nucleotide metabolism (cluster 1, 2, 4), phosphate metabolism (cluster 1, 2, 4) and response to stress (cluster 4, 5).

Due to lack of access to individual single cell data for further autonomous manipulation (other than in the Loupe cell browser GUI), principal component analysis of the aggregated transcriptional data was performed. Despite the heterogeneity displayed (Figure 7), aggregated analysis reveals there is a clear difference in transcription between macrophages that have and have not been exposed to the fungi, which appears to drive the PCA clustering (Figure 10). To therefore identify the transcriptional patterns of genes responding to the spores, the expression of a subset of genes previously identified as immune response genes (Muñoz et al. 2018) was focused upon for further analysis (Figure 11).

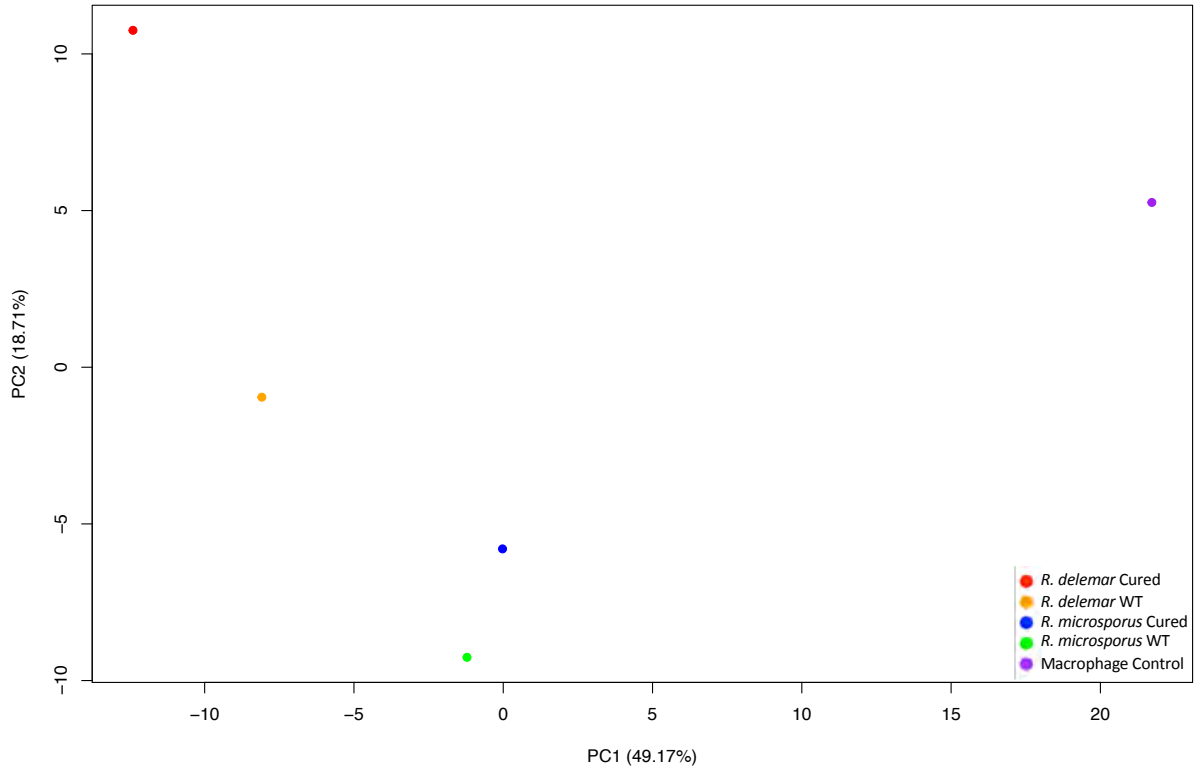


Figure 10. Principal component analysis of macrophage genes differentially expressed across all samples. Single cell sequencing was performed on uninfected and infected macrophages. Transcriptional data from the experiment was analysed with the 10X genomics analysis pipeline, and aggregated prior to principle component analysis.

Across all exposed conditions, relative to unexposed macrophages, there was a profile consistent with cytokine activation, response to stimulus, and activation of the NF-Kb pathway. This was accompanied by repression of CCL5, which is involved in T-cell recruitment (Figure 11). However, different macrophage profiles can be seen in response to the two fungal species, and these are further influenced by the presence of the endosymbiont. While the response to wt *R. delemar* shows the most deviation from the macrophage-only control, exposure to cured *R. delemar* also elicited a strong and distinct macrophage response (Figure 11). Exposure to wt *R. delemar* elicits increased expression of general markers of activation, including GTPase activity and MHC class II protein binding (LAG3 repressor of T-cell activation, H2-M2, IFN-gamma induced IIGP1, MX1, KCTD14, PNP2), growth factor binding, IL1 receptor agonist activity and endocytosis (SERPINE1, ENG, FGFBP3, GM8898, GCNT2, IL1F6). Specifically, we observe modest increases in the expression of IFN-γ responsive CXCL10 (3.1 fold) and IRG1/IRG11 (5.9 fold), pro-inflammatory SAA3 (3.5 fold), and ENPP4 (2.8 fold), but also induction of the M2 polarizing PSTPIP2/21 (6.7 fold), the IL-4 responsive signalling modulator CISH (5.4 fold), and the vascular damage responsive F3/F31 (5.9 fold) (Martinez et al. 2013). These latter genes are not as strongly induced during exposure to *R. microsporus*, which may reflect the aggressive nature of infection by *R. delemar* relative to *R. microsporus*. In contrast, infection with cured *R. delemar* showed a decrease in the induction of these M2-polarisation markers (PSTPIP2, 3.5 fold relative to uninduced). The transcriptional profile is instead shifted to include increased transcription of genes involved in G protein signalling and phosphoinositide binding (PDE7B, CCL1, SCARF1, RGS16, PLEKHA4) (Figure 11).

A similar change in macrophage polarization was observed during exposure to wt and cured *R. microsporus*. For both wt and cured *R. microsporus*, expression of IFN-γ responsive CXCL10, SAA3, and ENPP4 was comparable to unexposed macrophages. Compared to *R. delemar*, exposure to wt *R. microsporus* induced a more limited expression of genes with roles in cytokine activation, ERK1 and ERK2 regulation, and regulation of NF-kB cascade (Figure 11). There was also a weaker induction of the vascular damage responsive F3/F31 genes, and a relatively stronger upregulation of the PLA2G16 phospholipase, TRIM30D, SLC1A2 and the M2 polarizing IL-6. However, other key polarizing genes, particularly PSTPIP2/21, SAA3, and ENPP4 were only weakly induced. Overall, this profile suggests a weak M1-like activation consistent with poor phagocytosis and reduced overall antifungal activity that can be observed in macrophages interacting with endosymbiont-harbouring *R.microsporus* spores (Itabangi et al. 2019).

Finally, cured *R. microsporus* induced a strong pro-inflammatory response, which included upregulation of CXCL3, the neutrophil chemoattractant, consistent with observations of differences in phagocyte recruitment in zebrafish upon infection with wt vs. cured spores (Itabangi et al. 2019). Markers of NF-kB activation were also strongly induced in this population. Cured *R. microsporus* also strongly induced the expression of TNFRSF8 (CD30), a marker of lymphocyte activation occasionally associated with subcutaneous fungal infections. Overall, this is suggestive of a shift to a more pro-inflammatory profile. Itabangi et al. observe that cured *R. microsporus* is more sensitive to phagocyte-mediated killing and phagocyte recruitment compared to wt (Itabangi et al. 2019), to test whether a more successful response to the spores could be mounted via the induction of a pro-inflammatory response, further *in vitro* experiments were carried out (Figure 12).

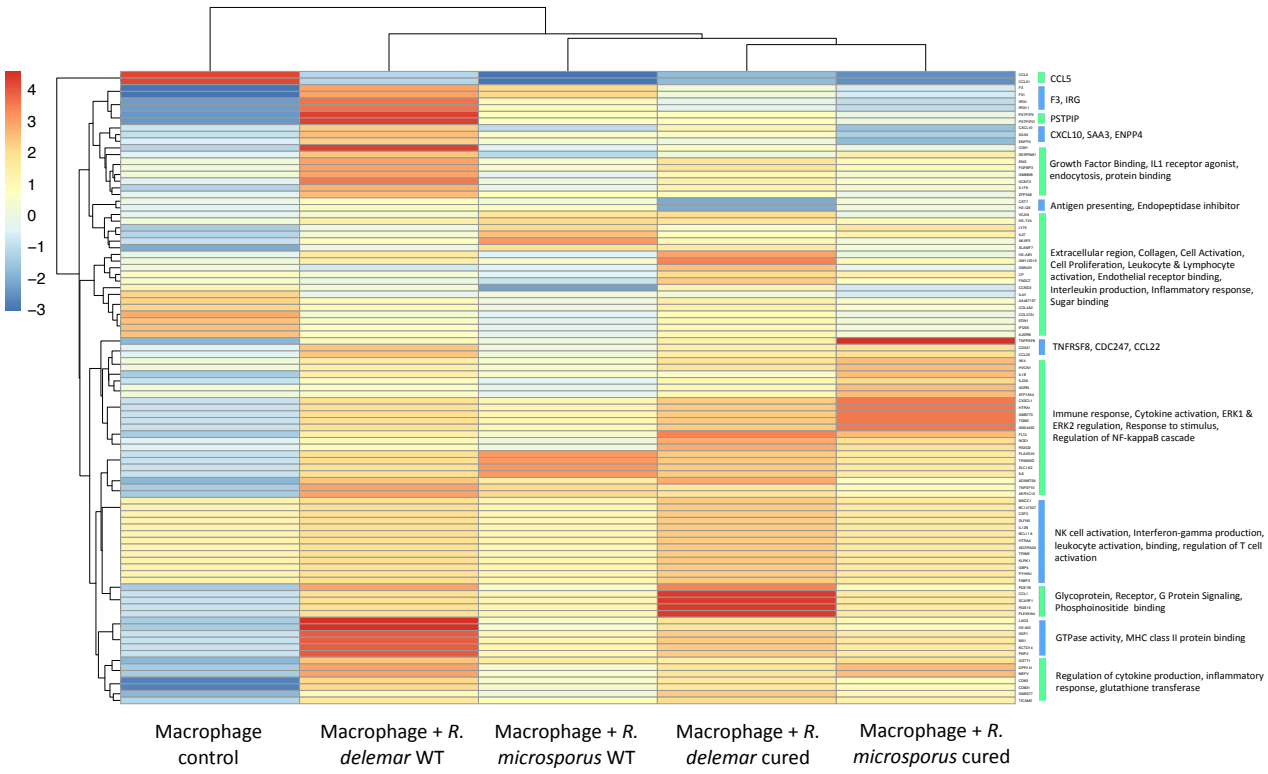


Figure 11. Clustering of macrophage transcription. Heatmap displaying immune response genes significantly differentially expressed between macrophage populations. Expression levels of aggregated data are plotted in Log2 (FDR < 0.001).

Modulating the Infection Outcome

R. delemar exhibits rapid germination followed by hyphal extension (Sephton-Clark et al. 2018). The transcriptional profile observed in macrophages exposed to *R. delemar* is consistent with a strong damage response, likely prompted by the germination of *R. delemar* spores. I took the following approach to determine whether macrophages would be better able to control the infection if the fungi were slowed in their developmental progress. The necessity of genes involved in chitin synthesis and regulation appeared important for both *R. delemar* and *R. microsporus* in response to phagocytosis (Chapter 6). When fungal spores

were pre-treated with the chitin synthesis inhibitor Nikkomycin Z (24 μg/ml) they remained viable but exhibited reduced swelling. By challenging macrophages with spores pre-treated with Nikkomycin Z (24 μg/ml), macrophage survival was increased at 7.5 hours post infection (Figure 12). As the macrophages are better able to control these spores, this suggests that spores undergoing the initial stages of germination may offer less of a challenge for the macrophages. This is consistent by data shown by Itabangi et al., who examined macrophage response to both resting and swelling *R. microsporus* spores (Itabangi et al. 2019).

The transcriptional data show a strong M2 alternative activation signal during *R. delemar*-macrophage interaction, but a weaker M2 polarisation during *R. microsporus*-macrophage interaction that was further shifted towards NF-kB-mediated M1 upon endosymbiont cure. To shift the macrophage polarization towards M1 classical activation, macrophages were pre-treated with NF-kB activating lipopolysaccharide (LPS). This offered a protective effect upon Mucorales infection, significantly improved the ability of macrophages to control *R. microsporus*. At 7.5 hours post infection, 59.7% of macrophages survived when pre-treated with LPS, compared to 24.6% without (Figure 12).

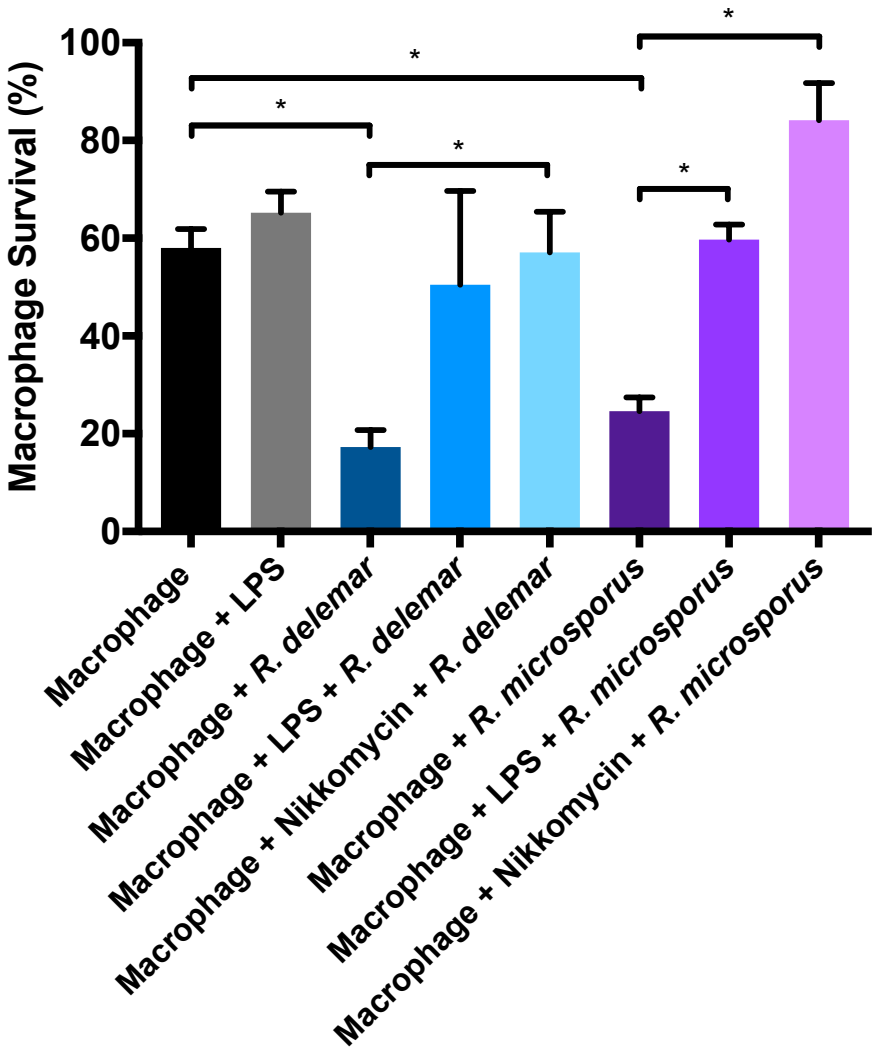


Figure 12. Macrophage survival following exposure to *R. delemar* (wt) and *R. microsporus* spores (wt). Macrophages +/- LPS pre-treatment were infected with fungal spores (Multiplicity of infection (MOI) 5:1), pre-swollen in SAB consistent with single cell experiments (biological n=3). Macrophages were infected with fungal spores that were pre-treated with +/- Nikkomycin Z (24 µg/ml; biological n=3 for each sample). Macrophage survival was determined 7.5 hours post infection with time course live cell imaging. Asterisk denote significant differences between samples (*= $p < 0.01$), determined with the Wilcoxon-Mann-Whitney test, corrected for multiplicity with the Bonferroni method.

Conclusions

These results show that the fungal response to innate immune cells differs by species in the Rhizopodaceae family. Although *R. delemar* and *R. microsporus* share a small conserved response to macrophage exposure, the majority of their response differs. Results show that the fungal transcriptional response appears largely unperturbed by the presence or lack of an endosymbiont, in the absence of stress. However, the presence of an endosymbiont greatly effects the response of the host. Activation of the host and inhibition of spore germination successfully modulates the infection outcome.

Discussion

Surprisingly, the scale of the fungal response varied appreciably between species. As previously discussed, this may be an artifact of the differential growth rates these species display. This variation is also observed in other relatively closely related species; *C. albicans* and *C. glabrata* differ substantially in their responses to the host (Brunke and Hube, 2012).

Unsurprisingly, the macrophage response (Figure 11) reflected in vitro observations; the more 'M1-like' the transcriptional response, the better the macrophages control the spores in vitro. Unfortunately, the macrophage single cell sequencing approach taken here (10X genomics single cell) resulted in limited meaningful single cell data. This was due to the unavailability of single cell data to the user, outside of their specified user pipeline (which provided inflexible and limited analysis options). To improve on the single cell analysis, further experiments utilising an alternative system such as Drop-Seq should be carried out.

As anticipated, activation of pro-inflammatory pathways increased macrophage survival in response to the spores. This consolidates several studies which demonstrate improved innate immune cell response to fungal pathogens when primed (Rogers et al. 2013; Municio et al. 2013; Blasi et al. 1995). In addition, this analysis reveals profound differences in the host response to two related *Rhizopus* species. In particular, results show a M2/damage-associated response during infection with wt *R. delemar* spores that is shifted towards an M1 protective response upon infection with cured *R. microsporus* spores. The ability to germinate prior to phagocyte control also appears key to virulence, as blocking spore germination with the chitin synthase inhibitor Nikkomycin Z improves macrophage survival, and highlights the requirement for chitin synthase for spore development in these species.

Further Work

Future work focusing on modulation of the innate immune system in both in vitro and in vivo models would allow a better understanding of the innate immune response to Mucorales species. Modulation of pathways which include the genes identified here (Figure 11) with exogenous cytokines, signalling molecules and inhibitors would allow for further elucidation of the pathways involved in the macrophage response. Clinical modulation or stimulation of the immune system could provide protection against infection in high-risk patients. Topical or systemic germination inhibitors might also be explored as options for a prophylactic treatment in high risk patients. Antimycin A isomers and derivatives would be good candidates for this, however the cytotoxicity of Antimycin A itself makes it unsuitable for use in humans.

Chapter 6: Germination Inhibition

This chapter will introduce approaches which are capable of inhibiting and modulating fungal germination. My results demonstrate several compounds capable of inhibiting germination of *Rhizopus* spores. These compounds successfully inhibit germination through chitin synthase inhibition, electron transport chain inhibition and reactive oxygen species pathway regulation. Further to this, my results demonstrate inhibition of *Rhizopus* spore germination through treatment with natural compounds extracted from plants and lichen. Both compound screening and target based approaches have yielded compounds capable of germination inhibition, shedding light on the mechanisms which underpin germination.

Manipulation and Inhibition of Germination

This work has been adapted from the book chapter "Spore Germination of Pathogenic Filamentous Fungi" (Sephton-Clark and Voelz 2017), for which I performed the literature search, wrote the manuscript, completed revisions, and prepared the figures.

Germination is the developmental process underlying initiation of many fungal diseases. Thus, strategies that inhibit germination have been of much interest as a means to treat both human and plant fungal infections. Inhibition of Mucorales spore germination by the host is likely key to preventing infection in healthy individuals, however germination inhibition often fails in immunocompromised infection models (Voelz et al. 2015; Rosowski et al. 2018). The process of germination is therefore an attractive therapeutic target, which has been explored for *Aspergillus* species previously. Germination inhibition may be reversible, as seen for multiple *Aspergillus* species when inhibited (Nogueira et al. 2019). There are multiple fungistatic and fungicidal drugs currently available to treat fungal infections, however most remain innefective against *Rhizopus* species. Given the impact of *Rhizopus* germination on the host, and limited treatments for mucormycosis, I attempted to identify germination inhibitors.

Manipulation of physical parameters is a common strategy employed to ensure food safety. Decreasing water potential can aid with fungicidal action, as the germination of *Aspergillus niger* species is inhibited by reduced water availability (Long, 2017; Ni, 2005). Lowering temperature is also a common method for inhibiting germination of food spoiling fungi (Eckert, 1967; Barth, 2009). The common food spoilage agent *Rhizopus delemar* will not germinate below 5 °C (Eckert, 1988), whilst the pathogen *M. gypseum* will only tolerate temperatures over 35 °C (Leighton and Stock 1969) demonstrating temperature is clearly a strong mechanism for regulating germination.

Non-steroidal anti-inflammatory drugs (NSAIDs) such as Ibuprofen, have been shown to inhibit the germination of several fungal species capable of causing respiratory diseases, including *Aspergillus niger* (Dalmont, 2017). The use of aerosolized NSAIDs as a preventative measure has been suggested as a way to tackle these common afflictions, however one should be careful to note the appropriate controls for this study are absent, so conclusions which can be drawn remain limited. If effective, they could be especially useful in damp 172 housing where pathogens such as *Aspergillus niger* thrive (Dalmont, 2017).

Furthermore, statins have been shown to decrease germination of the human pathogen *Rhizopus oryzae*, as well as increasing susceptibility to oxidative stress (Bellanger et al. 2016).

The topical administration of cheap and accessible substances such as acetic acid to open wounds has been suggested as another treatment which is effective due to its inhibition of germination (Trzaska et al. 2015). Acetic acid has been shown to inhibit germination of mucormycosis causing species. The administration of acetic acid might provide effective means for preventing this hard-to-treat invasive disease in patients with deep tissue wounds which may have been exposed to contaminated soils (e.g. blast wounds) (Trzaska et al. 2015). Furthermore, the germination of several pathogenic fungi can be inhibited by substances such as ethanol (Plumridge et al. 2004; Trzaska et al. 2015; Dao & Dantigny 2011).

Nanoparticles, an excellent alternative to traditional chemical treatments, are capable of inhibiting germination in the tobacco leaf pathogen *P. tabacina*. Administration of such Zinc nanoparticles to tobacco leaves has been suggested as a cheap and efficient way to reduce pathogenesis and crop losses, as the nanoparticles show effectivity at very low doses (Wagner et al. 2016).

In addition, a range of biological strategies has been investigated. Several biomolecules can be used effectively to inhibit the germination of fungi. Chitosan derivatives which incorporate a pyridine were found to exhibit inhibition of germination on the plant pathogen *B. cinerea*, posing yet another method for the treatment of food or plants with a safe molecule, which would decrease plant disease by fungal infection (Jia et al. 2016). Similarly, the inhibition of germination of plant pathogens through biocontrol is a currently expanding field. Colonising crops with plant 'safe' bacteria or fungi, which produce molecules damaging to plant pathogens, can effectively control plant disease.

For example, organic compounds released by *Streptomyces albulu*s have been shown to be capable of inhibiting germination of the plant pathogen *Fusarium oxysporum* (Wu, 2015). *Pseudomonas antimicrobica* produces a molecule capable of decreasing germination rates of the prolific fungal plant pathogen *B. cinerea* (Wu, 2015; Walker, 2001).

Whilst many of these recently suggested approaches have great potential to offer new methods in combating fungal growth and disease, our current understanding of the underlying mechanisms of inhibition is limited and thus requires further study.

Results

Results have been previously published or adapted from the following articles: "Pathways of Pathogenicity: Transcriptional Stages of Germination in the Fatal Fungal Pathogen Rhizopus delemar"(Sephton-Clark et al. 2018) and "Host-pathogen transcriptomics of macrophages, Mucorales and their endosymbionts: a polymicrobial pas de trois" (Sephton-clark et al. 2019). For both I conceived, designed and performed the experiments, collected the data, performed the analysis and interpretation, wrote the manuscript, completed revisions, and prepared the figures.

Germination Inhibitors Identified Through Transcriptional Studies

Transcriptional and phenotypic results identified cell wall remodelling, respiration, REDOX and stress response to be key pathways differentially regulated during the transition from resting spore to swelling spore. To determine whether modulation of these pathways would lead to inhibition of germination, spores were treated with a chitin synthase inhibitor (Nikkomycin Z), a cytochrome c reductase inhibitor (Antimycin A) and exogenous reactive oxygen species (ROS) (Hydrogen peroxide).

An increase in intracellular ROS can be observed in *R. delemar* over the course of germination (Figure 1a). I investigated the significance of ROS detoxification during germination by testing for resistance to exogenous (H_2O_2) and endogenous (mitochondrial-derived) ROS (Figure 1b). Treatment with 5 mM but not 1 mM H_2O_2 was sufficient to inhibit spore germination. In contrast, spores were highly sensitive to treatment with 1.5 or 10nM antimycin A. Inhibition of the mitochondrial cytochrome c reductase leads to an accumulation of superoxide radicals

within the cell. Furthermore, antimycin A may exert a dual effect, as the expression of storage molecule transcripts appears high in both ungerminated spores and the hyphal form. High sensitivity to inhibition of oxidative phosphorylation with antimycin A is consistent with reports that utilization of these storage molecules as energy reserves is important for the initiation and maintenance of growth (Elbein 1974; Novodvorska et al. 2016; Svanström et al. 2014).

When spores of *R. delemar* and *R. microsporus* were treated with the chitin synthesis inhibitor Nikkomycin Z (24-120 µg/ml), they failed to germinate (Figure 1c) and displayed less chitin/chitosan in their outer cell wall (Figure 1c: *R. delemar* 120 µg/ml, *R. microsporus* 24-120 µg/ml). At Nikkomycin Z concentrations lower than 24 µg/ml, we see the spores are able to swell, however development appears halted after swelling.

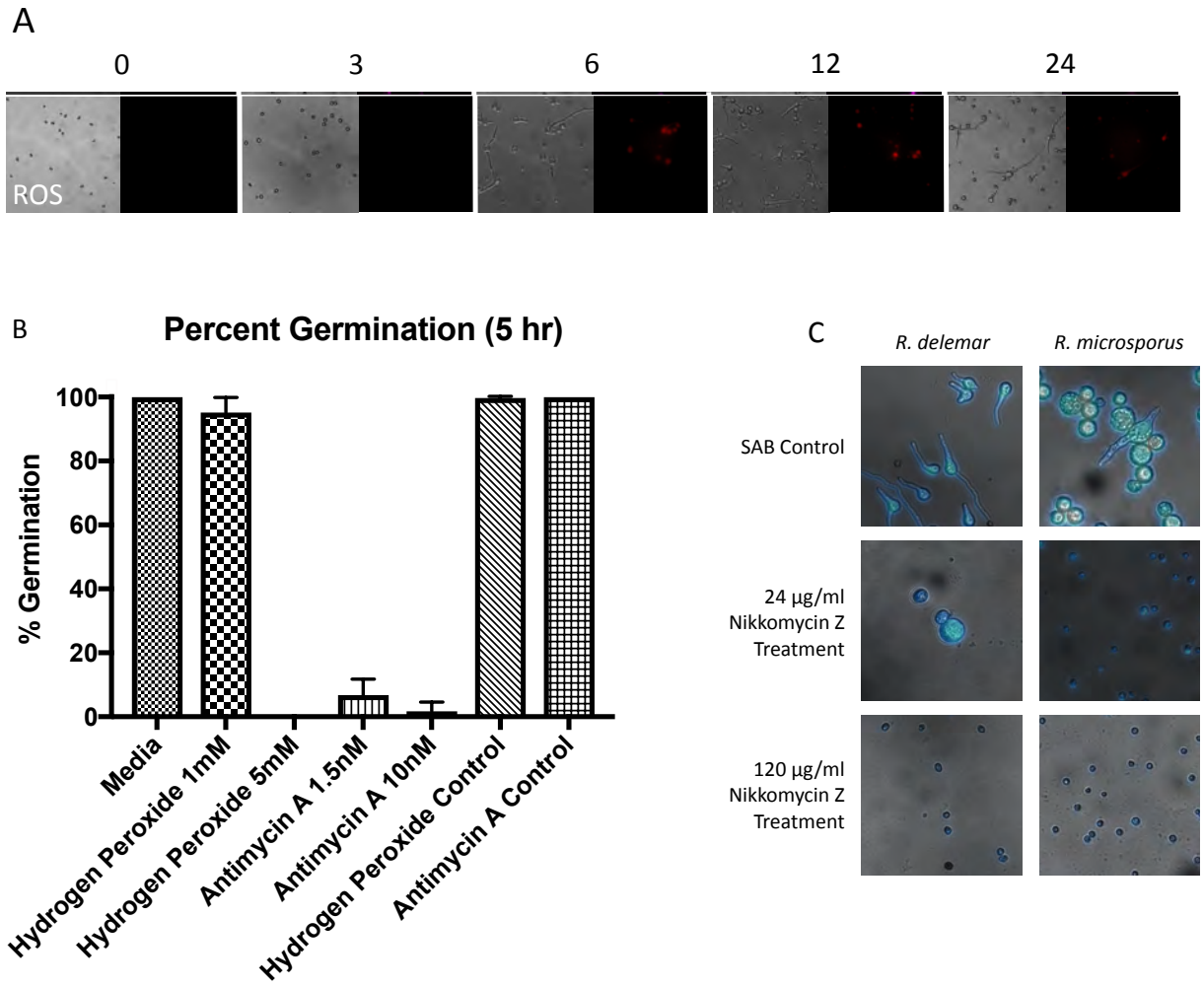


Figure 1. Targeted germination inhibition of *Rhizopus* species a) Spores germinated for 0, 3, 6, 12, and 24 h, stained to show ROS with carboxy-H2DCFDA. b) Germination is inhibited by 5 mM hydrogen peroxide and over 1.5 nM antimycin A, as determined by live-cell imaging, after 5 h of germination in SAB. The hydrogen peroxide control consists of an equivalent volume of H_2O, and the antimycin A control consists of an equivalent volume of 100% ethanol. c) Chitin synthase inhibition of *R. delemar* and *R. microsporus* germination. *R. delemar* and *R. microsporus* treated with Nikkomycin Z in SAB, labels indicate concentration of inhibitor. Fluorescence indicates calcofluor white staining, and thus the availability of chitin/chitosan in the cell wall.

Germination Inhibitors Identified Through Natural Compound Screening

A screen identified compounds from a natural compound library, provided by Strathclyde University, capable of inhibiting germination of Mucorales species. The candidate identified to inhibit germination, A10, is an extract from either plant, lichen or fungi. A10 inhibits the metabolic activity of *R. microsporus* in a dose dependent manner (Figure 2), with high doses inhibiting as potently as the known inhibitor acetic acid (Trzaska et al. 2015). The flavonoid chrysin, a known antifungal (Shimura et al. 2007), was identified from a list of compounds (provided by Strathclyde University) as a potential active molecule within A10.

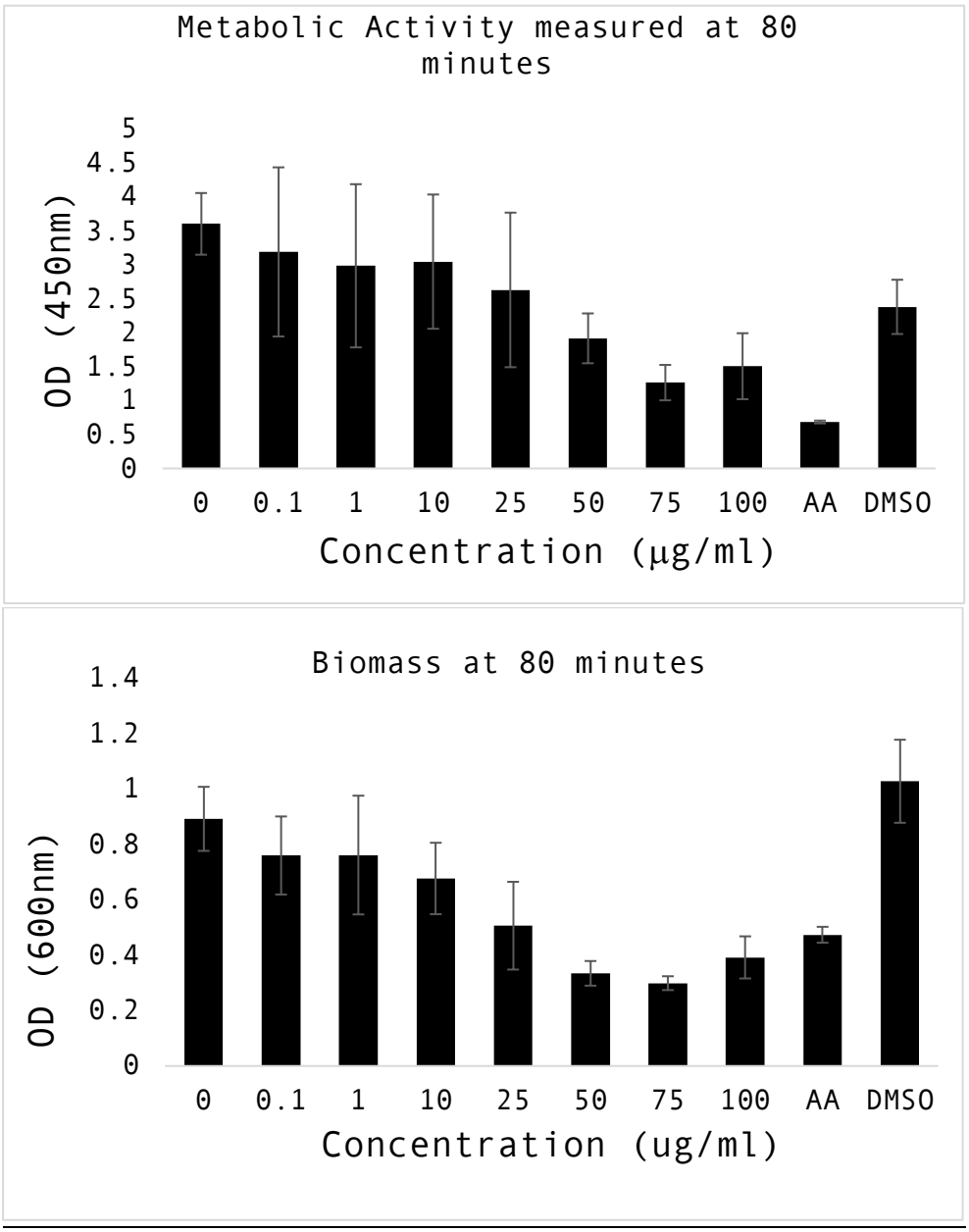


Figure 2

Metabolic activity and Biomass of *Rhizopus microsporus* spores when treated with increasing dosages of a plant extract supplied by Strathclyde, compared to acetic acid (AA) and DMSO controls. Metabolic activity correlates positively with readings at OD 450nm, Biomass to OD 600nm.

Discussion

The inhibitors identified in this work highlight the roles of chitin synthase and ROS signalling in regulating germination in *Rhizopus* species. Hydrogen peroxide has the potential to be used as a topical prophylactic treatment for high-risk patients, as solutions of 3-6% H_2O_2 (3% = 0.88M) are well tolerated by the skin (Mahran et al. 2019). H_2O_2 appears fungicidal to *Rhizopus* species at 5mM, a concentration which is unlikely to cause skin irritation. Multiple pharmaceutical companies have attempted to develop Nikkomycin Z for clinical use as an antifungal (Galgiani, 2016), however it has not yet made it to market as an approved antifungal treatment. Nikkomycin Z serves as a good candidate for the treatment of mucormycosis, and should be explored further as a treatment option with in vivo models. The potential for germination inhibition with natural compounds should be further explored with high resolution mass spectrometry of the fungicidal A10, to determine the structures of compounds within A10. A germination screen with purified forms of these compounds would offer candidates for testing with mammalian cell lines, to determine toxicity and drug development suitability.

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Appendix

ComPfam

	R. delemar	*R. microsporus*	q-value
PF01498	873	1	6.96E-127
PF07714	418	0	4.69E-60
PF00385	499	8	7.87E-60
PF13358	532	32	3.27E-42
PF03372	373	10	1.17E-39
PF08238	284	2	1.47E-36
PF13551	258	3	1.62E-31
PF00400	429	31	1.59E-30
PF00078	330	14	2.18E-30
PF13893	293	9	7.33E-30
PF07282	10	78	1.09E-28
PF00077	177	0	1.58E-24
PF13516	194	2	7.20E-24
PF13606	179	1	4.45E-23
PF12895	164	0	1.15E-22
PF13894	374	36	1.49E-21
PF08284	140	1	2.36E-17
PF07653	153	3	9.95E-17
PF00271	124	1	4.77E-15
PF12894	2	33	1.74E-13
PF13833	94	0	3.81E-12
PF02992	126	4	3.94E-12
PF13191	4	33	8.65E-12
PF09668	99	1	1.51E-11
PF13857	99	1	1.51E-11
PF14259	87	0	3.94E-11
PF00096	61	85	6.25E-11
PF03732	129	6	6.41E-11
PF07690	151	10	7.81E-11
PF13041	0	23	8.70E-11
PF13812	92	1	1.45E-10
PF12937	42	69	1.56E-10
PF13504	80	0	2.17E-10
PF12762	95	2	6.02E-10
PF13637	120	6	7.56E-10
PF00153	177	17	8.42E-10
PF00512	76	0	8.48E-10
PF08662	93	2	8.84E-10
PF04670	89	2	3.54E-09
PF01209	82	1	3.55E-09
PF00025	115	6	3.82E-09
PF13374	70	0	5.60E-09
PF13650	86	2	8.34E-09
PF00665	87	2	8.97E-09
PF05148	77	1	1.28E-08
PF08659	66	0	2.28E-08
PF12773	0	18	2.66E-08
PF03184	64	0	3.66E-08
PF09011	65	0	3.66E-08
PF00071	18	40	4.49E-08

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RHIMIDRAFT_197836

R. microsporus genes significantly (FDR<0.001) differentially expressed (40 genes). The predicted functions for each gene can be found in the GO file, within the online appendix (R_micro_GO).

hypothetical protein HMPREF1544_02453 [Mucor circinelloides f. circinelloides 1006PhL]
hypothetical protein A0J61_00230 [Choanephora cucurbitarum]
cytochrome P450 [Syncephalastrum racemosum]
cytochrome P450 [Syncephalastrum racemosum]
cytochrome p450 [Lichtheimia corymbifera JMRC:FSU:9682]
hypothetical protein LRAMOSA00373 [Lichtheimia ramosa]
hypothetical protein LRAMOSA09367 [Lichtheimia ramosa]
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cytochrome P450 CYP5313 [Mucor circinelloides f. lusitanicus CBS 277.49]
hypothetical protein HMPREF1544_05316 [Mucor circinelloides f. circinelloides 1006PhL]
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cytochrome P450 CYP5313 [Mucor circinelloides f. lusitanicus CBS 277.49]
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cytochrome p450 monooxygenase [Mucor ambiguus]
cytochrome P450 CYP5313 [Mucor circinelloides f. lusitanicus CBS 277.49]
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cytochrome P450 CYP5313 [Phycomyces blakesleeanus NRRL 1555(-)]
cytochrome P450 CYP5313 [Phycomyces blakesleeanus NRRL 1555(-)]
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p450 monooxygenase [Lichtheimia corymbifera JMRC:FSU:9682]
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cytochrome P450 [Syncephalastrum racemosum]
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cytochrome P450 [Absidia repens]
cytochrome P450 [Absidia repens]
cytochrome P450 [Hesseltinella vesiculosa]
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lanosterol 14-alpha demethylase [Hesseltinella vesiculosa]
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cytochrome P450 [Mucor ambiguus]
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hypothetical protein [Parasitella parasitica]
hypothetical protein [Parasitella parasitica]
hypothetical protein HMPREF1544_11256 [Mucor circinelloides f. circinelloides 1006PhL]
cytochrome p450 superfamily protein [Mucor ambiguus]
hypothetical protein MUCCIDRAFT_162569 [Mucor circinelloides f. lusitanicus CBS 277.49]
hypothetical protein HMPREF1544_02140 [Mucor circinelloides f. circinelloides 1006PhL]
CYP5312 protein [Mucor circinelloides f. lusitanicus CBS 277.49]
cytochrome P450 [Mucor ambiguus]
cytochrome p450 monooxygenase [Lichtheimia corymbifera JMRC:FSU:9682]
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cytochrome p450 4v2 [Lichtheimia corymbifera JMRC:FSU:9682]
cytochrome p450 monooxygenase [Lichtheimia corymbifera JMRC:FSU:9682]
hypothetical protein LRAMOSA00755 [Lichtheimia ramosa]
cytochrome p450 704c1-like [Lichtheimia corymbifera JMRC:FSU:9682]
hypothetical protein LRAMOSA08735 [Lichtheimia ramosa]
cytochrome P450 [Absidia repens]
cytochrome P450 CYP5203 [Phycomyces blakesleeanus NRRL 1555(-)]
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hypothetical protein RMATCC62417_12643 [Rhizopus microsporus]
cytochrome P450 [Rhizopus microsporus var. microsporus]
hypothetical protein RMCBS344292_04064 [Rhizopus microsporus]
cytochrome P450 [Rhizopus microsporus]
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hypothetical protein RMATCC62417_08225 [Rhizopus microsporus]
cytochrome P450 [Rhizopus microsporus var. microsporus]
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hypothetical protein RMCBS344292_03990 [Rhizopus microsporus]

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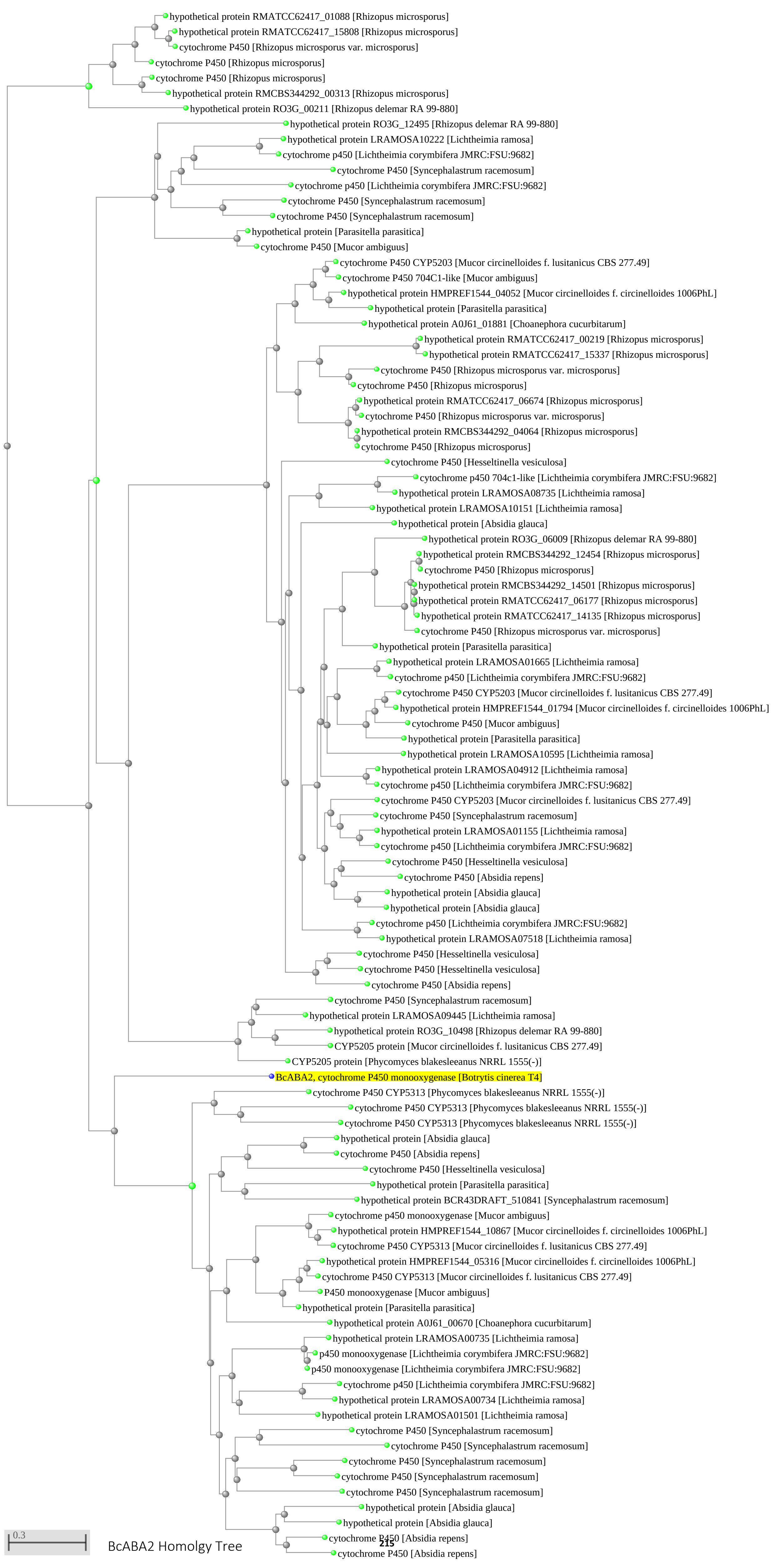
BcABA1 Homolgy Tree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cytochrome P450 [Hesseltinella vesiculosa]
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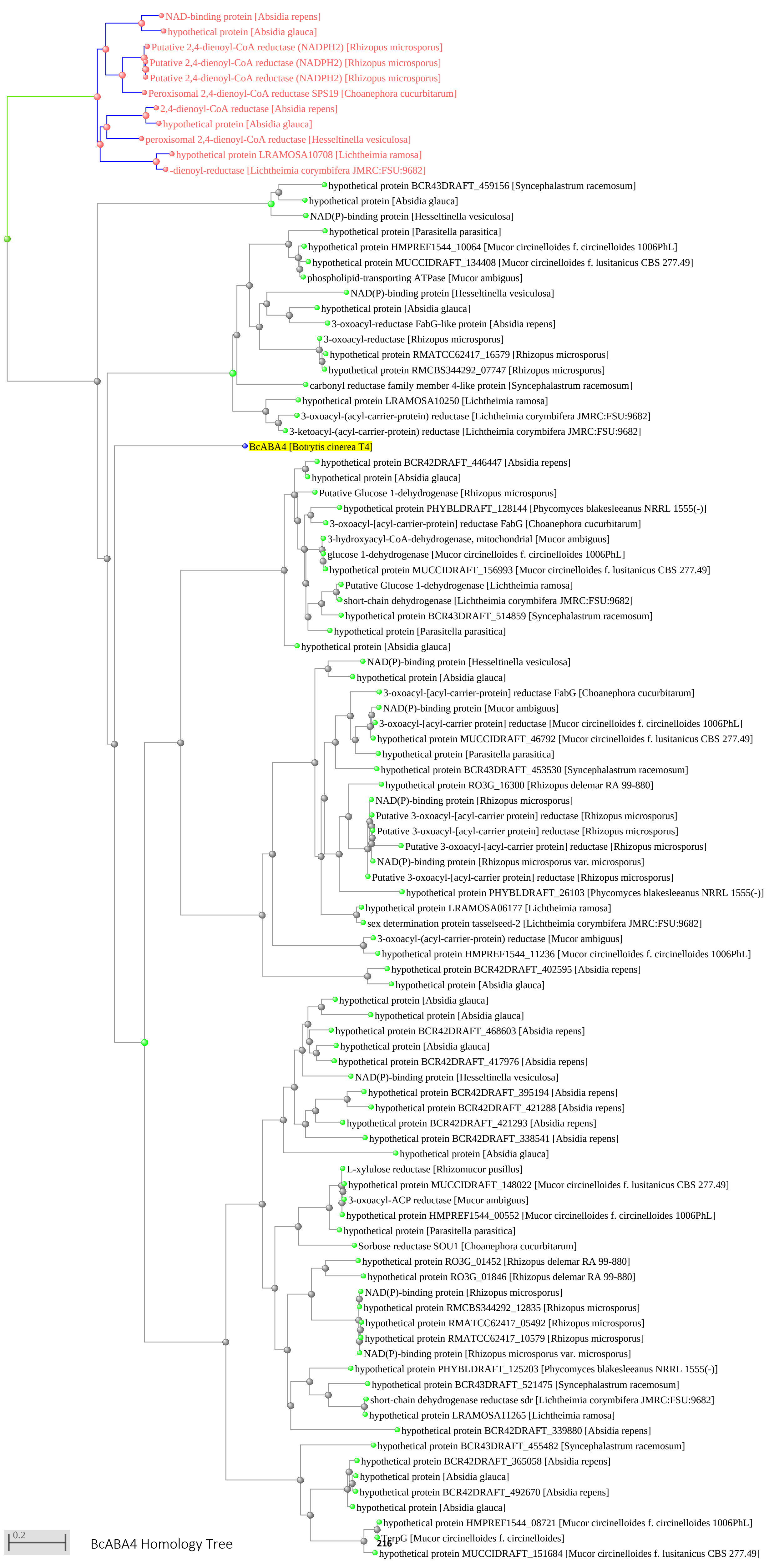
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NAD(P)-binding protein [Hesseltinella vesiculosa]
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3-oxoacyl-reductase FabG-like protein [Absidia repens]
3-oxoacyl-reductase [Rhizopus microsporus]
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hypothetical protein RMCBS344292_07747 [Rhizopus microsporus]
carbonyl reductase family member 4-like protein [Syncephalastrum racemosum]
hypothetical protein LRAMOSA10250 [Lichtheimia ramosa]
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short-chain dehydrogenase [Lichtheimia corymbifera JMRC:FSU:9682]
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hypothetical protein [Absidia glauca]
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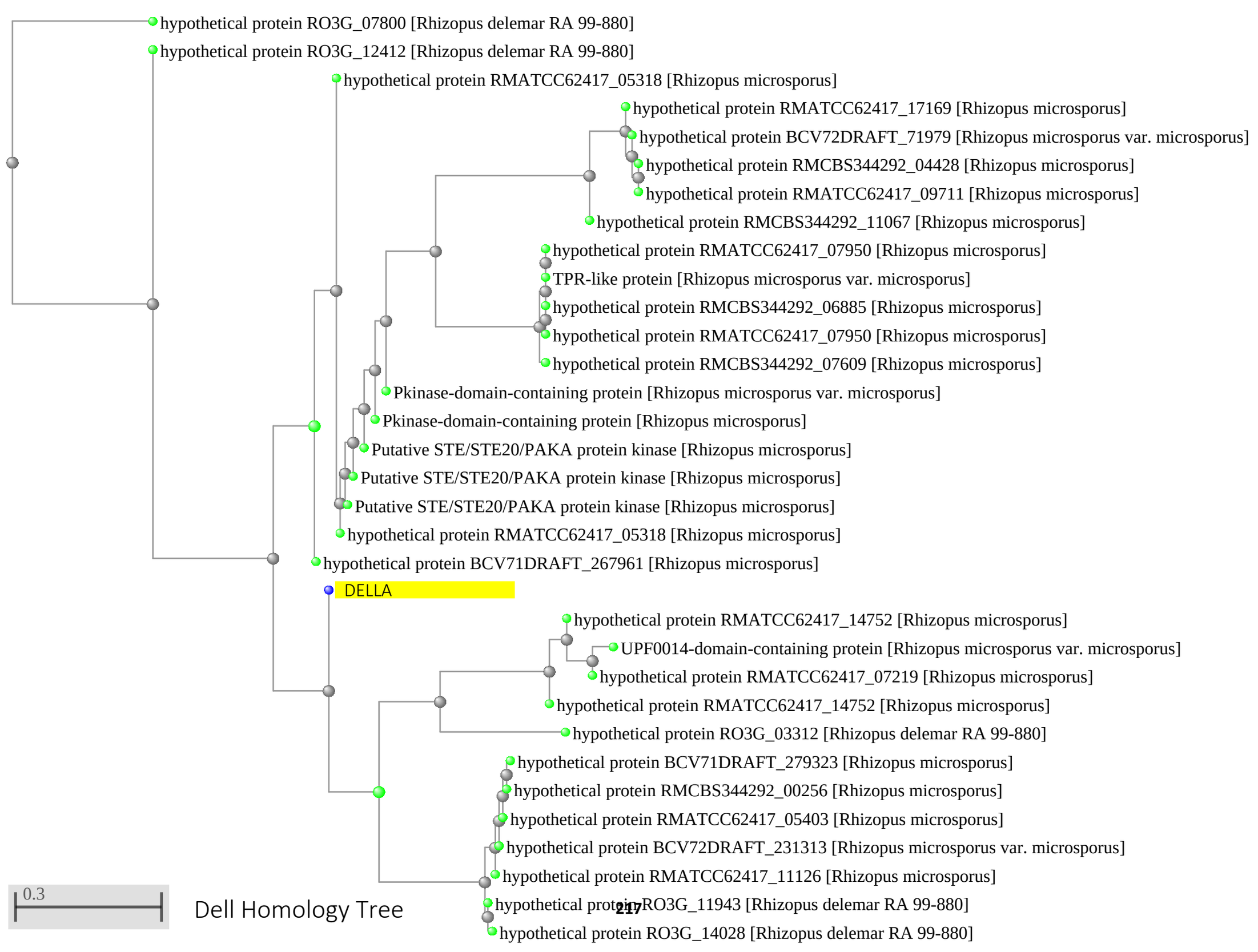
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hypothetical protein RMATCC62417_05318 [Rhizopus microsporus]
hypothetical protein RMATCC62417_17169 [Rhizopus microsporus]
hypothetical protein BCV72DRAFT_71979 [Rhizopus microsporus var. microsporus]
hypothetical protein RMCBS344292_04428 [Rhizopus microsporus]
hypothetical protein RMATCC62417_09711 [Rhizopus microsporus]
hypothetical protein RMCBS344292_11067 [Rhizopus microsporus]
hypothetical protein RMATCC62417_07950 [Rhizopus microsporus]
TPR-like protein [Rhizopus microsporus var. microsporus]
hypothetical protein RMCBS344292_06885 [Rhizopus microsporus]
hypothetical protein RMATCC62417_07950 [Rhizopus microsporus]
hypothetical protein RMCBS344292_07609 [Rhizopus microsporus]
Pkinase-domain-containing protein [Rhizopus microsporus var. microsporus]
Pkinase-domain-containing protein [Rhizopus microsporus]
Putative STE/STE20/PAKA protein kinase [Rhizopus microsporus]
Putative STE/STE20/PAKA protein kinase [Rhizopus microsporus]
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hypothetical protein BCV71DRAFT_267961 [Rhizopus microsporus]
DELLA
hypothetical protein RMATCC62417_14752 [Rhizopus microsporus]
UPF0014-domain-containing protein [Rhizopus microsporus var. microsporus]
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hypothetical protein RMATCC62417_14752 [Rhizopus microsporus]
hypothetical protein RO3G_03312 [Rhizopus delemar RA 99-880]
hypothetical protein BCV71DRAFT_279323 [Rhizopus microsporus]
hypothetical protein RMCBS344292_00256 [Rhizopus microsporus]
hypothetical protein RMATCC62417_05403 [Rhizopus microsporus]
hypothetical protein BCV72DRAFT_231313 [Rhizopus microsporus var. microsporus]
hypothetical protein RMATCC62417_11126 [Rhizopus microsporus]
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hypothetical protein RO3G_14028 [Rhizopus delemar RA 99-880]
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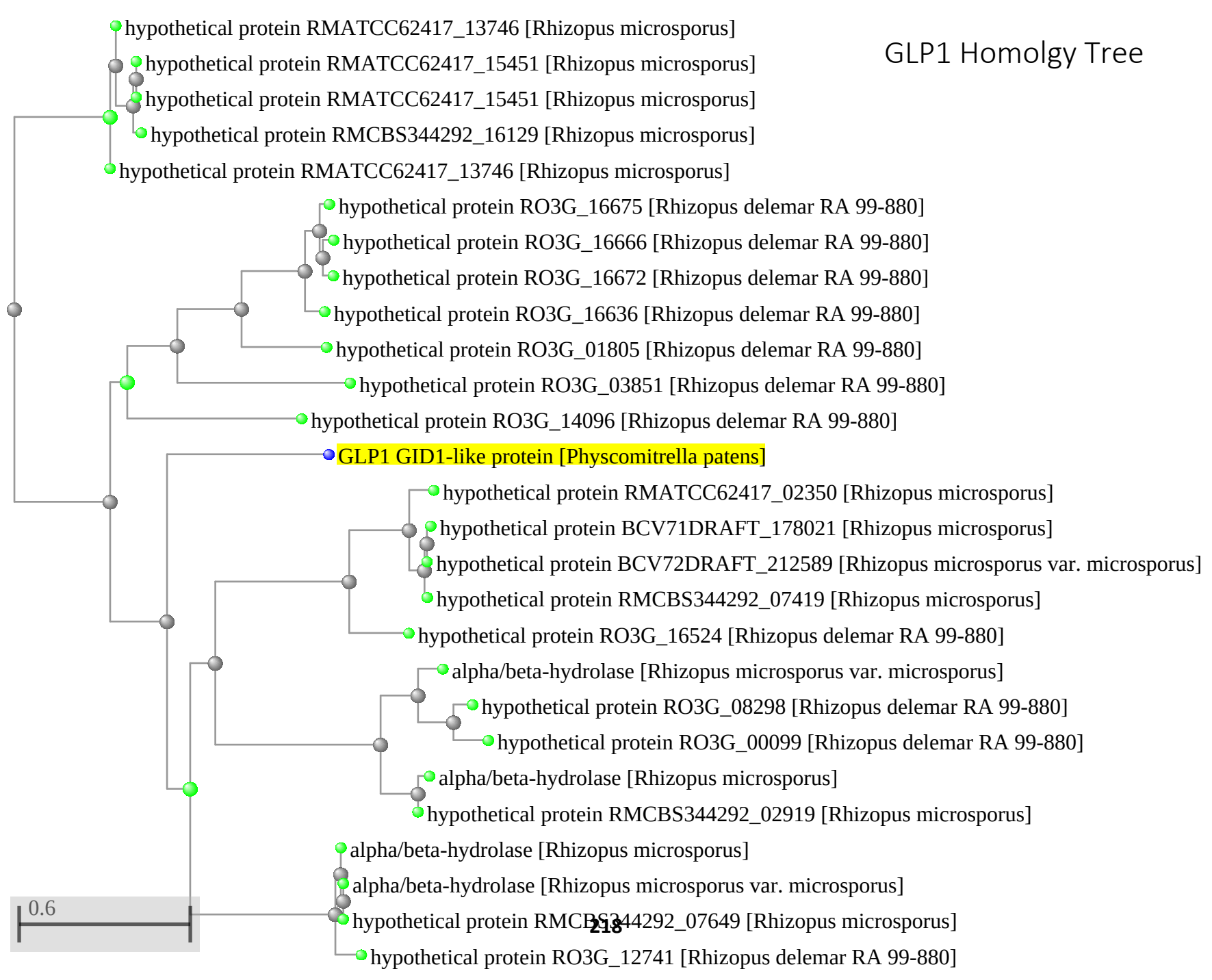
Dell Homology Tree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hypothetical protein RMCBS344292_16129 [Rhizopus microsporus]
hypothetical protein RMATCC62417_13746 [Rhizopus microsporus]
hypothetical protein RO3G_16675 [Rhizopus delemar RA 99-880]
hypothetical protein RO3G_16666 [Rhizopus delemar RA 99-880]
hypothetical protein RO3G_16672 [Rhizopus delemar RA 99-880]
hypothetical protein RO3G_16636 [Rhizopus delemar RA 99-880]
hypothetical protein RO3G_01805 [Rhizopus delemar RA 99-880]
hypothetical protein RO3G_03851 [Rhizopus delemar RA 99-880]
hypothetical protein RO3G_14096 [Rhizopus delemar RA 99-880]
GLP1 GID1-like protein [Physcomitrella patens]
hypothetical protein RMATCC62417_02350 [Rhizopus microsporus]
hypothetical protein BCV71DRAFT_178021 [Rhizopus microsporus]
hypothetical protein BCV72DRAFT_212589 [Rhizopus microsporus var. microsporus]
hypothetical protein RMCBS344292_07419 [Rhizopus microsporus]
hypothetical protein RO3G_16524 [Rhizopus delemar RA 99-880]
alpha/beta-hydrolase [Rhizopus microsporus var. microsporus]
hypothetical protein RO3G_08298 [Rhizopus delemar RA 99-880]
hypothetical protein RO3G_00099 [Rhizopus delemar RA 99-880]
alpha/beta-hydrolase [Rhizopus microsporus]
hypothetical protein RMCBS344292_02919 [Rhizopus microsporus]
alpha/beta-hydrolase [Rhizopus microsporus]
alpha/beta-hydrolase [Rhizopus microsporus var. microsporus]
hypothetical protein RMCBS344292_07649 [Rhizopus microsporus]
hypothetical protein RO3G_12741 [Rhizopus delemar RA 99-880]
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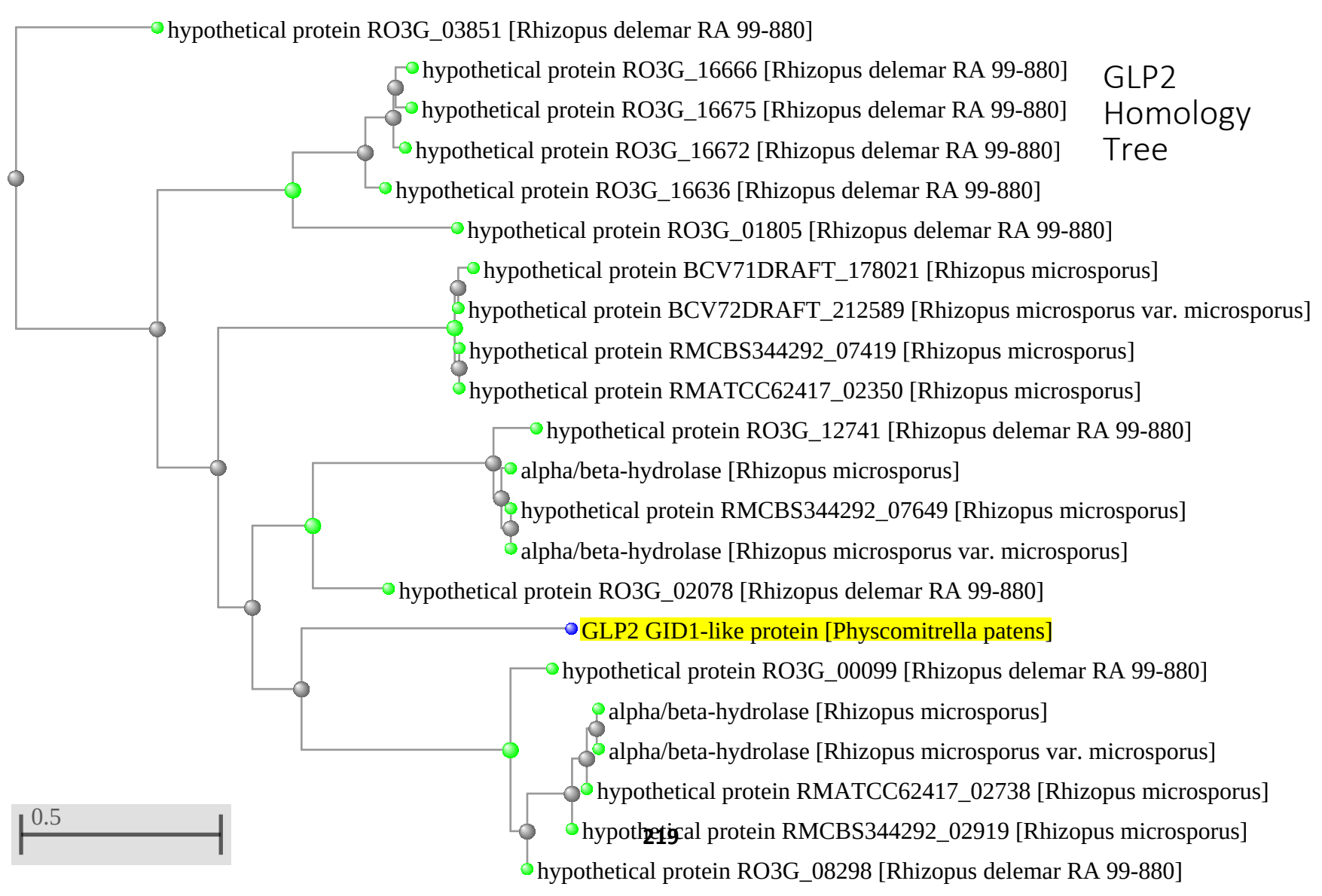
GLP2 Homology Tree
hypothetical protein RO3G_03851 [Rhizopus delemar RA 99-880]
hypothetical protein RO3G_16666 [Rhizopus delemar RA 99-880]
hypothetical protein RO3G_16675 [Rhizopus delemar RA 99-880]
hypothetical protein RO3G_16672 [Rhizopus delemar RA 99-880]
hypothetical protein RO3G_16636 [Rhizopus delemar RA 99-880]
hypothetical protein RO3G_01805 [Rhizopus delemar RA 99-880]
hypothetical protein BCV71DRAFT_178021 [Rhizopus microsporus]
hypothetical protein BCV72DRAFT_212589 [Rhizopus microsporus var. microsporus]
hypothetical protein RMCBS344292_07419 [Rhizopus microsporus]
hypothetical protein RMATCC62417_02350 [Rhizopus microsporus]
hypothetical protein RO3G_12741 [Rhizopus delemar RA 99-880]
alpha/beta-hydrolase [Rhizopus microsporus]
hypothetical protein RMCBS344292_07649 [Rhizopus microsporus]
alpha/beta-hydrolase [Rhizopus microsporus var. microsporus]
hypothetical protein RO3G_02078 [Rhizopus delemar RA 99-880]
GLP2 GID1-like protein [Physcomitrella patens]
hypothetical protein RO3G_00099 [Rhizopus delemar RA 99-880]
alpha/beta-hydrolase [Rhizopus microsporus]
alpha/beta-hydrolase [Rhizopus microsporus var. microsporus]
hypothetical protein RMATCC62417_02738 [Rhizopus microsporus]
hypothetical protein RMCBS344292_02919 [Rhizopus microsporus]
hypothetical protein RO3G_08298 [Rhizopus delemar RA 99-880]
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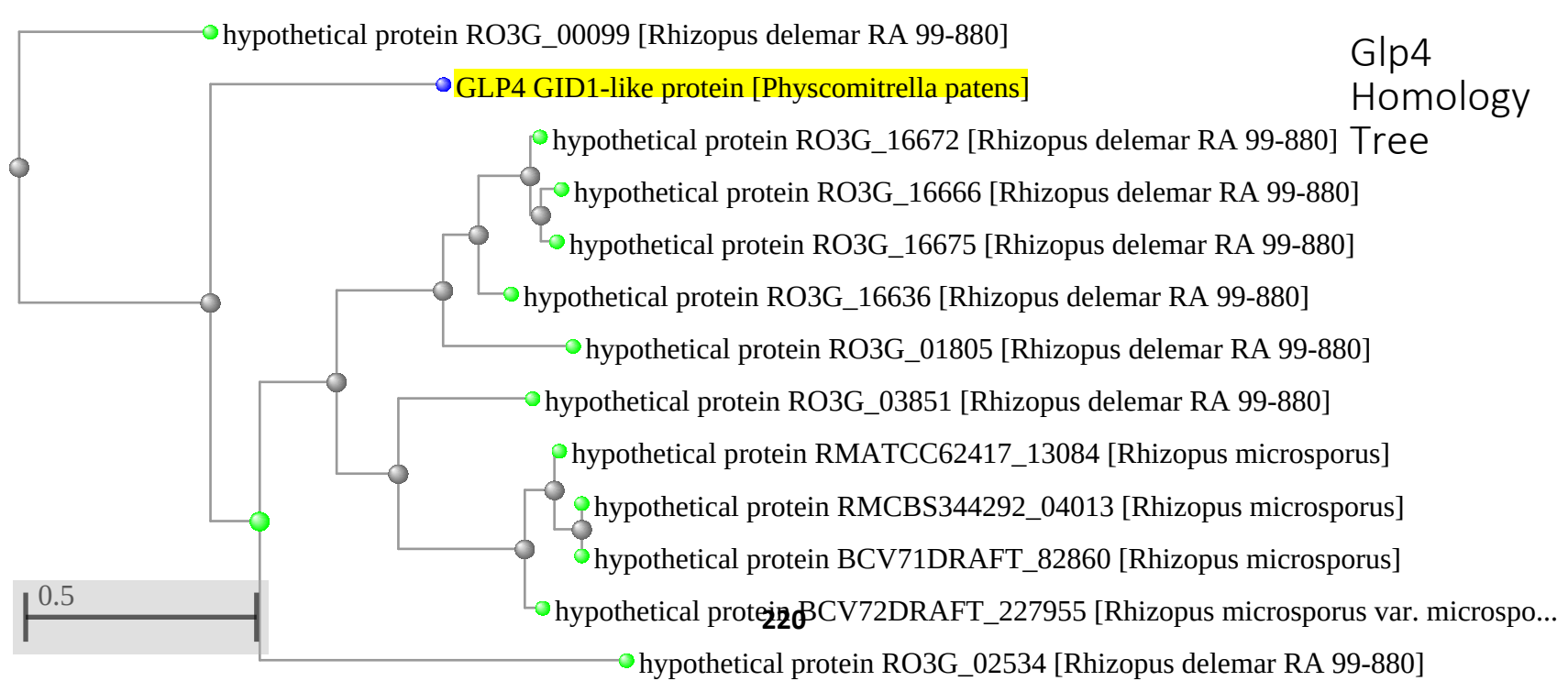
Glp4
Homology
Tree
hypothetical protein RO3G_00099 [Rhizopus delemar RA 99-880]
GLP4 GID1-like protein [Physcomitrella patens]
hypothetical protein RO3G_16672 [Rhizopus delemar RA 99-880]
hypothetical protein RO3G_16666 [Rhizopus delemar RA 99-880]
hypothetical protein RO3G_16675 [Rhizopus delemar RA 99-880]
hypothetical protein RO3G_16636 [Rhizopus delemar RA 99-880]
hypothetical protein RO3G_01805 [Rhizopus delemar RA 99-880]
hypothetical protein RO3G_03851 [Rhizopus delemar RA 99-880]
hypothetical protein RMATCC62417_13084 [Rhizopus microsporus]
hypothetical protein RMCBS344292_04013 [Rhizopus microsporus]
hypothetical protein BCV71DRAFT_82860 [Rhizopus microsporus]
hypothetical protein BCV72DRAFT_227955 [Rhizopus microsporus var. microspo...
hypothetical protein RO3G_02534 [Rhizopus delemar RA 99-880]
0.5