Intercompartmental streaming in *Aspergillus*

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Intercompartmental streaming in *Aspergillus*

Intercompartimentale stroming in *Aspergillus*
(met een samenvatting in het Nederlands)

Proefschrift

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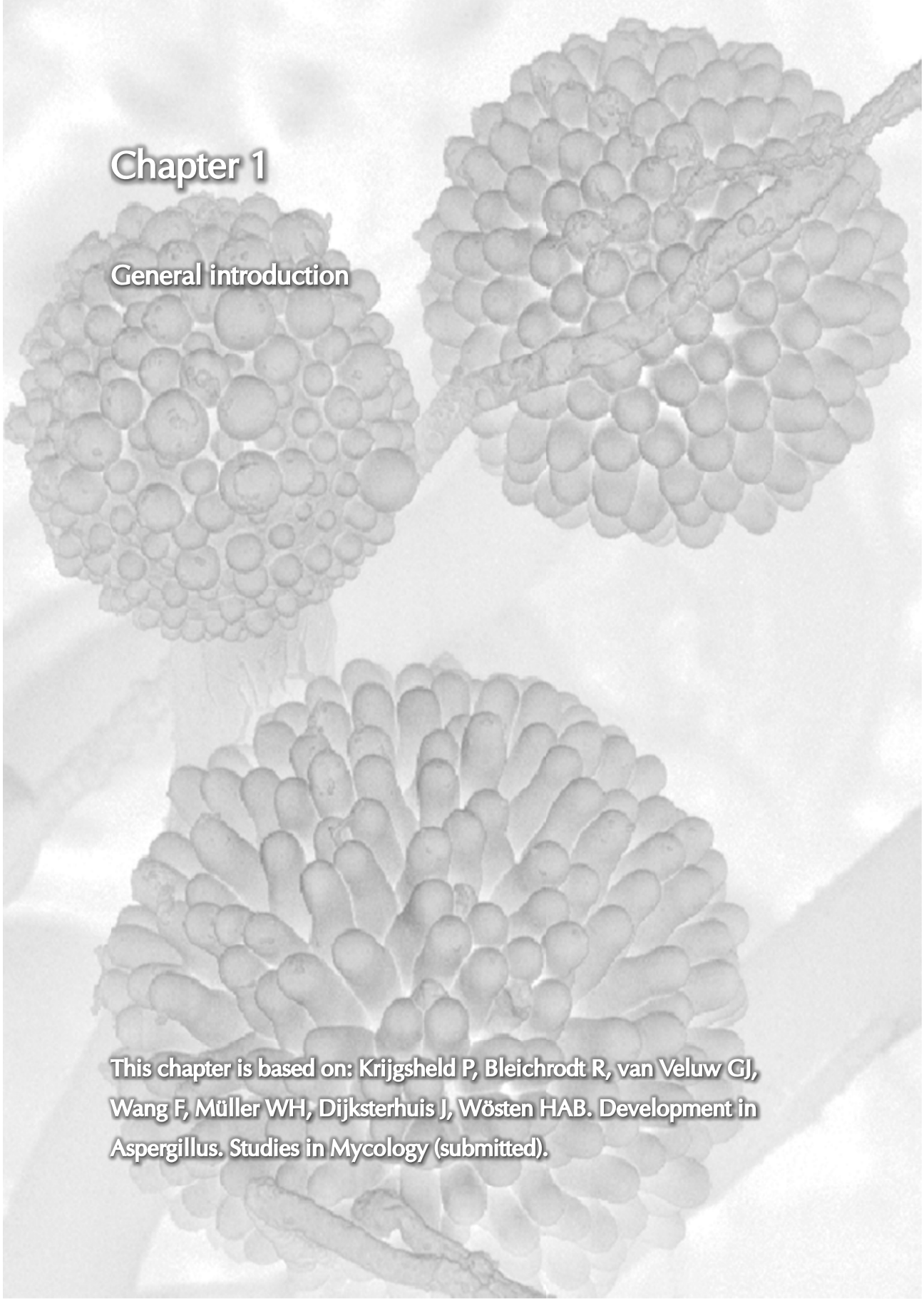
Voor mijn familie

Contents



Chapter 1

General introduction

This chapter is based on: Krijgsheld P, Bleichrodt R, van Veluw GJ, Wang F, Müller WH, Dijksterhuis J, Wösten HAB. Development in Aspergillus. Studies in Mycology (submitted).

INTRODUCTION

Fungi are widespread in nature. More than 100.000 fungal species have been identified but many more have still to be described since it has been estimated that there are about 1.5 million species on earth (Hawksworth, 1991, 2001). The fungi form a kingdom within the eukaryotic domain that consists of the phyla Chytridiomycota, Zygomycota, Glomeromycota, Ascomycota and Basidiomycota. Fungi form yeast-like cells or grow by means of filaments known as hyphae. Yeasts form colonies of individual cells. In contrast, filamentous fungi form a mycelium, which consists of a network of interconnected hyphae. Hyphae formed by the lower fungi (Chytridiomycota, Zygomycota, and Glomeromycota) are not septated. As a result, a mycelium is formed that shares a common cytoplasm. The higher fungi (Ascomycota and Basidiomycota) form hyphae that are septated. These septa have pores that allow streaming of nutrients, macromolecules and even organelles (Shatkin and Tatum, 1959; Moore and McAlear, 1962; Gull, 1978; Lew, 2005). Therefore, the cytoplasm within mycelia of the ascomycetes and the basidiomycetes is also considered to be continuous.

Aspergilli

Aspergillus is an anamorph genus, which belongs to the Ascomycota. It comprises between 260 (Geiser et al., 2007; Samson and Varga, 2009) and 837 species (Hawksworth, 2011). These species are classified in approximately ten different teleomorph genera (Geiser, 2009). For instance, *Aspergillus nidulans* is part of the teleomorph genus *Emericella*, while *Aspergillus fumigatus* and *Aspergillus niger* belong to the genera *Neosartorya* and *Petromyces*, respectively. This shows that *Aspergillus* is a diverse group of fungi. Indeed, comparison of the genomic sequences of *A. nidulans* and *A. fumigatus* (Galagan et al., 2005) showed that these fungi are as related to each other as fish to humans. These animals separated about 450 million years ago but diversification in the genus *Aspergillus* is assumed to have occurred within 200 million years (Galagan et al., 2005). The large differences in genomic sequence have been proposed to be caused by an accelerated evolutionary rate (Cai et al., 2006).

Aspergillus species are among the most abundant fungi worldwide. They are not very selective with respect to abiotic growth conditions (Table 1). For instance, they can grow over a wide range of temperature (6-55 °C) and at relatively low humidity. In fact, *Aspergillus penicilloides* is among the most xerophilic fungi (Williams and Hallsworth, 2009). Moreover, *Aspergillus* species feed on a large variety of substrates including animal faeces and human tissue. Nonetheless, they are predominantly found on complex plant polymers (Bennett, 2010) and are considered to be common food spoilage fungi. The success of *Aspergillus* is also explained by their effective dispersal. Spores of this genus are among the most dominant fungal structures in the air, dispersing themselves both short and long distances (Bennett, 2010). Aspergilli are not only known because of their saprobic life style. *A. niger* has been reported to be a pathogen of *Zingiber officinale* plants (Pawar et al., 2008). Moreover, a wide variety of aspergilli are opportunistic pathogens of animals and humans. They do not infect healthy individuals but do invade individuals with a

Table 1: Conditions for vegetative growth of selected aspergilli.

Species	Optimum Temp (°C)	Temp range (°C)	Optimum pH	pH range	Minimal Water activity	Optimum Water activity	Minimum Relative humidity (%)	Optimum Relative humidity (%)	References
A. niger	35-37	6-47	6.0	1.5-9.8	0.77	0.97	88-89	96-98	Astoreca et al., 2007; Ayerst, 1969; Leong et al., 2006; Mehra and Jaitly, 1995; Panasenko, 1967; Pitt, 1981
A. oryzae	30-37	7-47	6.0-7.5	4-8		0.99			Chipeta et al., 2008; Gibson et al., 1994; Nasseri et al., 2002; Panasenko, 1967
A. fumigatus	37	10-55	5.5-6.5	3.0-8.0	0.82	0.97	85	98-99	Al-Doory, 1984; Ayerst, 1969; Ogundero, 1981; Panasenko, 1967; Singh and Sandhu, 1982
A. clavatus	20-25	5-42			0.88		88	98	Panasenko, 1967
A. terreus	37	15-42	5.0		0.78				Al-Doory, 1984; Mehra and Jaitly, 1995; Singh and Sandhu, 1982
N. fischeri	26-45	11-52				0.98			Beuchat, 1986; Nielsen et al., 1988; Samson et al., 2000; Valik and Pieckova, 2001
A. nidulans	35-37	6-51	7.0	2-12	0.78		80	95	Agnihotri, 1964; Al-Doory, 1984; Lacey, 1980; Panasenko, 1967

compromised immune system (Pitt, 1994; Brakhage, 2005). Aspergilli (*i.e. A. fumigatus*, and to a lesser extent species such *Aspergillus flavus*, *A. niger*, *Aspergillus terreus*, and *A. nidulans*) cause invasive aspergillosis (involving several organ systems, particularly pulmonary disease), non-invasive pulmonary aspergilloma, and allergic broncho-pulmonary aspergillosis (Denning, 1998; Stevens et al., 2000).

Aspergillus species secrete a wide variety of enzymes that degrade polymers within the substrate into molecules that can be taken up to serve as nutrients. For instance, amylases are secreted to degrade starch, xylanases to degrade xylan and pectinases to degrade pectin within plant material. Similarly, elastase is secreted in the human lung to degrade elastin. The capacity to secrete large amounts of proteins (and other metabolites such as organic acids) in combination with established fermentation technology and molecular biology make aspergilli such as *A. niger, Aspergillus oryzae, Aspergillus awamori, Aspergillus sojae,* and *A. terreus* attractive cell factories for the production of homologous and heterologous proteins (Meyer et al., 2011). The potential of these fungi is exemplified by strains of *A. niger that* produce more than 30 grams per liter of glucoamylase (Finkelstein et al., 1989). Of concern, *Aspergillus* species can form mycotoxins that are toxic for animals and humans. *A. flavus* produces aflatoxin, which is one of the most carcinogenic natural molecules. In addition, different aspergilli, including *Aspergillus westerdijkiae*, can form ochratoxin on food products such as coffee and grapes (Leong et al., 2007).

This chapter describes the current understanding of the formation of a differentiated vegetative mycelium of *Aspergillus*, and the formation of the a-sexual conidiospores and their germination. *A. nidulans, A. fumigates, A. oryzae,* and *A. niger* have been chosen as the lead organisms for this chapter. Regulation of a-sexual development has been studied well. In this chapter, I focus on the role of FluG, BrlA, AbaA, WetA, MedA, StuA, VosA and FlbA-E. For other proteins that play a role in regulation of a-sexual development and for the role of environmental conditions I refer to Clutterbuck (1977), Etxebeste et al. (2010b), Penalva and Arst (2004), Skromne et al. (1995) and Krijgsheld et al. (2012).

DORMANCY AND GERMINATION

Spores should not germinate on the conidiophore but rather when they have been dispersed. Moreover, spores should only germinate when environmental conditions are favorable for fungal growth. This requires regulatory mechanisms to keep spores dormant. The volatile 1-octen-3-ol is produced by fungi as a degradation product of linoleic acid. It has been shown to inhibit germination of conidia of *Penicillium paneum* and *A. nidulans* (Chitarra et al., 2004; Herrero-Garcia et al., 2011). The critical concentration of 1-octen-3-ol needed to inhibit germination is obtained when spores are present at a high density. This situation would be met on a conidiophore and in this situation the volatile acts as a self-inhibitor. Upon spore dispersal the concentration of 1-octen-3-ol drops. As a result, outgrowth of the conidia is no longer self-inhibited but only depends on the environmental conditions.

During dormancy, spores may be exposed to various stress conditions such as UV-radiation, drought, and relatively high temperatures. Conidiospores of *Aspergillus* are moderately resistant to these stress situations due to a number of resistance mechanisms, involving melanin and compatible solutes such as trehalose (reviewed by Krijgsheld et al., 2012).

Three stages can be distinguished during germination of conidiospores. In the first phase of germination, dormancy is broken by environmental cues such as the presence of water and air either or not in combination with inorganic salts, amino acids or fermentable sugars (Osherov and May, 2001). The environmental cues are signaled via signal-transduction pathways. In the second phase of germination, spores grow isotropically. This process that is also known as swelling is observed between 2 and 6 h after inoculation of *A. niger* at 25 °C (van Leeuwen et al., 2012a; van Leeuwen et al., 2012b). During this stage, the diameter of the spore increases two fold or more due to water up-take. This is accompanied by a decrease in the microviscosity of the cytoplasm (Dijksterhuis et al., 2007). In the third phase of germination, a germ tube is formed by polarized growth. To this end, the morphogenetic machinery is re-directed to the site of polarization (d'Enfert, 1997; Momany, 2002; Harris and Momany, 2004; Harris, 2006). Polarized growth of *A. niger* can be observed 6 h after inoculation at 25 °C (van Leeuwen et al., 2012a; van Leeuwen et al., 2012b). At a later stage, the growth speed of the germ tube increases. By branching and inter-hyphal fusion a fungal mycelium is established.

Transcripts of about one third of the genes can be detected by micro-arrays in dormant conidiospores of *A. niger* (van Leeuwen et al., 2012a; van Leeuwen et al., 2012b). Transcripts representing the functional gene classes protein synthesis, protein fate, energy, and transcription are enriched in the RNA pool. A strong drop in the amount of RNA is observed in the first two hours of germination (van Leeuwen et al., 2012a; van Leeuwen et al., 2012b). Notably, transcripts belonging to the functional gene classes energy, protein synthesis, and rRNA and tRNA transcription (but not mRNA synthesis) are up-regulated when compared to dormant spores. In addition, some sub-categories of metabolism (nucleotide-, amino acid-, and phosphate metabolism) show increased RNA levels. Taken together, decay of mRNA and initiation of protein synthesis are characteristic for early germination. Up-regulation of genes involved in protein synthesis has also been shown in germinating conidia of *A. fumigatus* (Lamarre et al., 2008). The importance of protein synthesis in early stages of germination is also indicated by the fact that the protein synthesis inhibitor cycloheximide prevents isotropic growth, while inhibitors of the cytoskeleton and DNA- and RNA synthesis do not affect this process (Osherov and May, 2000). The total number of genes that are expressed in germinating conidia of *A. niger* gradually increase between 2 and 8 h after inoculation at 25 °C (van Leeuwen et al., 2012a). After 6 h of germination the functional gene classes ribosome synthesis and rRNA and tRNA transcription are down-regulated when compared to conidiospores that have germinated for 2 h.

VEGETATIVE GROWTH

In nature, aspergilli grow within and on solid substrates. Colonies can reach a diameter in the (sub-) millimetre (micro-colonies) to centimeter (macro-colonies) scale depending on the size and the composition of the substrate. For instance, micro-colonies are formed on a wheat kernel, whereas macro-colonies can be formed within the lobes of a lung. In the laboratory, aspergilli are routinely grown on agar media or in liquid media. On agar medium, aspergilli form radial symmetrical macro-colonies. The mycelium of *A. nidulan*s (Lee and Adams, 1994a) and *A. niger* extend their diameter with approximately 0.25 mm per h in excess of nutrients and at a temperature of 37 °C and 30 °C, respectively. Colonies can also be grown between porous polycarbonate membranes on an agar medium (Wösten et al., 1991). Oxygen, carbon dioxide, water, nutrients and excreted molecules can freely diffuse through the pores (0.1-1 μm) of the polycarbonate membranes but the pores are too small to enable hyphae to grow into the underlying medium or to grow into the air. Scanning electron microscopy shows that the periphery of a 7-day-old sandwiched *A. niger* colony consists of a single layer of hyphae (Figure 1A, D). A few millimeters behind the periphery this layer becomes thicker and comprises of up to six hyphae growing on top of each other. Notably, three distinct zones are observed another two millimeters towards the center (Figure 1B, E). The upper and lower zone of hyphae consists of a layer of up to five hyphae on top of each other, while the intermediate zone comprises a loose network of thin and thick hyphae, and some conidiophores that hardly, if at all, sporulate. Three distinct zones are also observed in the innermost center of the colony (Figure 1C, F). In this case, the upper and lower zones consist of up to twenty and six layers of hyphae, respectively. The intermediate zone comprises a dense network of both thin and thick hyphae, and a relatively high number of conidiophores that hardly, if at all, sporulate. An *A. niger* colony grows in a similar way when a 0.45 mm thin agarose layer is present in between the polycarbonate membranes. Taken together, *A. niger* grows near two-dimensionally when grown as a sandwiched colony.

Mycelium can grow dispersed, as clumps or as micro-colonies during submerged growth in liquid medium. Clumps are aggregated hyphae that are considered to be an intermediate state between dispersed and pelleted growth. The morphology of the mycelium has an enormous impact on the production of enzymes and primary or secondary metabolites. For instance, micro-colonies, also known as pellets, are required for the production of citric acid by *A. niger* (Vecht-Lifshitz et al., 1990). It is not clear how morphology exactly affects productivity. It has been proposed that this is due to the effect of the fungal morphology on the viscosity of the medium (Bhargava et al., 2003). Viscosity correlates with the extent of dispersed growth; large micro-colonies thus result in a low viscosity. The center of large pellets may experience oxygen starvation and other nutrients may also become limiting in this part of the mycelium. These conditions may also impact productivity of the pellets.

Pellet formation is caused by coagulation of the conidiospores in the culture. Parameters that affect coagulation of *A. niger* conidiospores are agitation, initial pH, and medium composition (Metz and Kossen, 1977). Agitation is one of the main

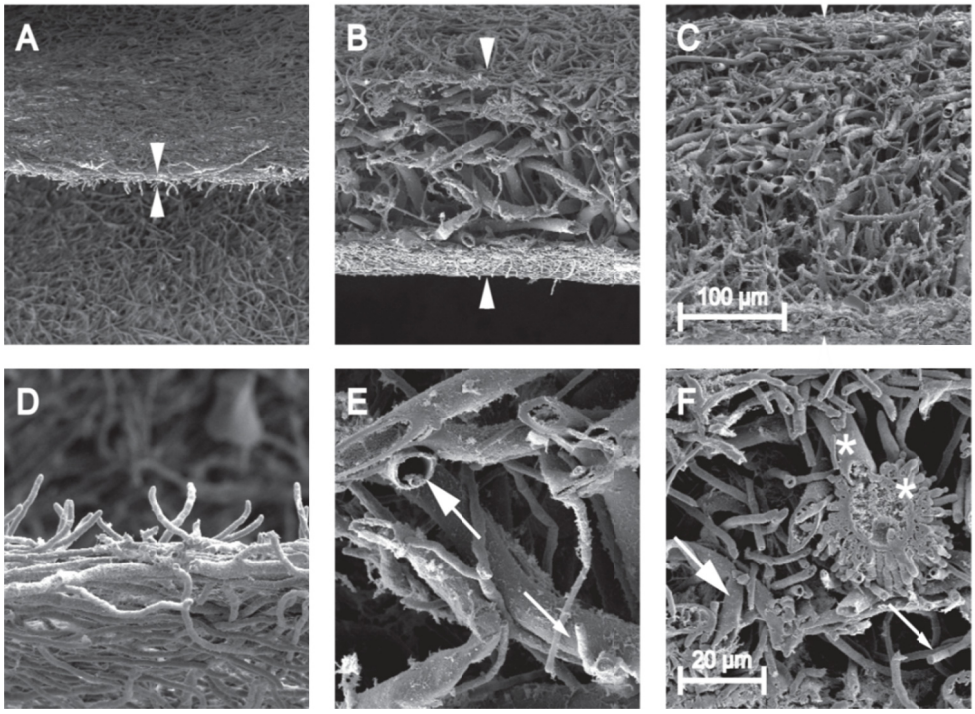


Figure 1: Scanning electron microscopy of cross sections of a 7-day-old sandwiched *A. niger* colony. Cross sections were made at the periphery (A, D), four millimeter behind the periphery (B, E) and at the innermost center (C, F). The thickness of the colony is indicated by the distance between the white triangles. Panels D-F represent higher magnifications of A-C, respectively. Thin and thick arrows point at thin and thick hyphae, respectively. In H asterisks mark a conidiophore that forms few spores. Bars in panel C, for A-C, and F, for D-F, represent 100 and 20 µm.

factors affecting the mycelial morphology. It decreases the chance that spores aggregate and therefore promotes dispersed growth. Trace elements can also influence the morphology of the mycelium. Dispersed growth is favored in the presence of ≥ 2 parts per billion manganese. Loose pellets are observed in an acidic environment (pH < 5). Similarly, *A. oryzae* grows completely dispersed at a pH < 3.5 (Carlsen et al., 1996). Coagulation of *A. niger* spores can be influenced by addition of 0.1% nonionic surface-active agents such as 'Span' or 'Tween'. Coagulation is reduced when the hydrophilic group of the non-ionic agent consists of a hydroxyl moiety (in the case of Span), and is promoted when the hydrophilic group is a polyoxyethylene (in the case of Tween). Pellet formation can also be manipulated by changing the surface composition of spores. Formation of micro-colonies was affected in strains of *A. nidulans* in which either or both *dewA* and *rodA* were inactivated (Dynesen and Nielsen, 2003). The effect was strongest when both hydrophobin genes were inactivated, which was accompanied by a huge drop in surface hydrophobicity of the conidiospores (see below). This and the effect of surface active molecules in the medium shows that spore surface hydrophobicity is an important determinant of pellet formation.

Research in the last two decades has shown that the mycelium of *Aspergillus* is heterogeneous with respect to gene expression, growth, and secretion. Genome wide expression analysis has shown that the RNA composition of central and peripheral zones of colonies of *A. niger* (Levin et al., 2007a) and *A.* oryzae (Masai et al., 2006) is different. In the case of 7-day-old colonies of *A. niger*, 25% of the active genes show a two-fold or more difference in RNA accumulation between the innermost and outermost zone of the mycelium (Levin et al., 2007a). For instance, RNA levels of the glucoamylase gene *glaA* are 3-fold higher at the periphery of maltose-grown colonies when compared to the center. Similarly, accumulation of transcripts of the ferulic acid esterase gene *faeA* is five-fold higher at the periphery of xylose grown colonies. Notably, 9% of the genes that are active in a 7-day-old colony are expressed in only one of five concentric zones. For instance, genes related to nitrate metabolism are specifically expressed in the outer zone of the colony, whereas mRNA of the hydrophobin *hfbD* is almost exclusively found in a central zone. Half the variation in RNA profiles is explained by differences in the composition of the medium underlying each zone of the colony, whereas the other half of the variation is caused by medium-independent mechanisms (Levin et al., 2007a). These findings imply that differentiation occurs within the vegetative mycelium of *Aspergillus.*

The heterogeneity of the mycelium of *A. niger* is also indicated by the fact that distinct zones of the colony grow and secrete proteins (Levin et al., 2007a; Levin et al., 2007b; Masai et al., 2006; Wösten et al., 1991). Proteins are formed throughout the *A. niger* mycelium (Levin et al., 2007a; Levin et al., 2007b; Wösten et al., 1991) (Figure 2) but they are mainly secreted at the periphery. Growth is observed in this outer zone but also in the innermost centre (Figure 2). Spatial growth and protein production is not affected when six-day-old colonies are transferred to fresh medium for 16 h. However, after transfer protein secretion is not only observed at the periphery of the colony but also in central parts of the mycelium (Figure 2). These data show that non-growing zones of the mycelium abundantly secrete proteins upon transfer to fresh medium (Levin et al., 2007a). This is a remarkable finding considering the fact that protein secretion is generally assumed to take place in growing hyphae only (Moukha et al., 1993; Wessels, 1989; Wessels, 1990; Wösten et al., 1991).

The finding that 7-day-old macro-colonies are heterogeneous with respect to RNA accumulation, growth and protein secretion raises the question whether heterogeneity is also observed between and within micro-colonies. Indeed, micro-colonies within liquid shaken cultures are heterogeneous with respect to size and gene expression (de Bekker et al., 2011b). Based on flow cytometry, a population of small and a population of large micro-colonies can be distinguished in such cultures. Moreover, expression of genes encoding secreted proteins is heterogeneous between the micro-colonies within a liquid culture. In fact, the population of lowly expressing micro-colonies is larger than the population of small pellets. This indicates that size is not the only determinant for expression of genes encoding secreted proteins. Heterogeneous gene expression can also be observed within micro-colonies (de Bekker et al., 2011b). At least, the total amount of RNA per hypha is about 50 times higher at the periphery of 1-mm-wide micro-colonies when compared to the center.

Heterogeneous gene expression is not only observed between micro-colonies or between zones of micro- or macro-colonies of *Aspergillus*, it is also observed between hyphae in a particular zone. It has been described that only part of the hyphae at the periphery of macro-colonies of *A. niger* secrete glucoamylase (Wösten et al., 1991). This observation is explained by heterogeneous expression of the glucoamylase gene *glaA* within this zone (Vinck et al., 2005). In fact, two populations of hyphae can be distinguished at the outer zone of the colony; those highly and those lowly expressing *glaA*. The hyphae highly expressing *glaA* also highly express other genes encoding secreted proteins (Vinck et al., 2011). Moreover, these hyphae highly express the glyceraldehyde-3-phosphate dehydrogenase gene and show a high 18S rRNA content. Thus, two populations of hyphae are present at the periphery of a colony; those that are lowly and those that are highly metabolically active. From the fact that the lowly active hyphae have a growth rate similar to that of the highly active hyphae it has been concluded that a "low" activity of hyphae is sufficient to support hyphal growth. However, a "high" metabolism would be needed to support secretion of large amounts of proteins (Vinck et al., 2011). Single hypha transcriptome analysis indicates that heterogeneity between neighboring hyphae goes beyond two types of hyphae. Individual hyphae each have their own composition of RNA (de Bekker et al., 2011a). So far, we can only guess why hyphae are heterogeneous at the colony periphery. The leading hyphae explore the substrate and they may be exposed to rapid changes in the environment. A heterogeneous hyphal population may contribute to the survival under such conditions. Notably, the transcription factor FlbB, which is involved in a-sexual development (see below), has been shown to accumulate at 60% of the tips of newly formed branches (Etxebeste et al., 2009). This is another example of heterogeneity within the *Aspergillus* mycelium.

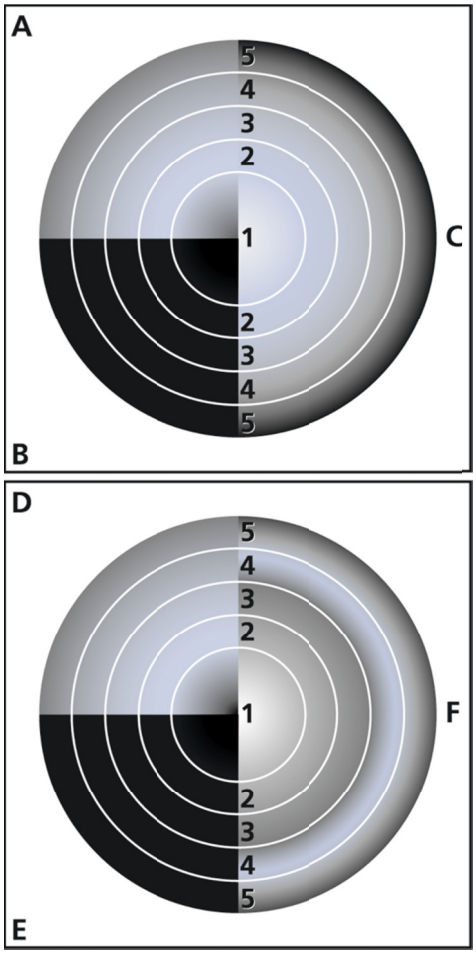


Figure 2: Growth (A, D), protein synthesis (B, E) and protein secretion (C, F) in a 7-day-old xylose grown sandwiched colony of *A. niger* before (A-C) and after transfer (D-F) to fresh medium (Adapted from Levin et al., 2007a).

Heterogeneity within the mycelium is surprising considering the fact that the cytoplasm of a fungal mycelium is assumed to be continuous. This is based on the fact that the septa

within and between hyphae are porous allowing streaming of water, (in)organic compounds and even organelles (Jennings, 1984, 1987). Heterogeneity between hyphae would require a certain extent of immobility of molecules. Closure of septa would be a mechanism to maintain differences in composition between hyphae. Septa of vegetative hyphae of the basidiomycete *Schizophyllum commune* can be in a closed or open state (van Peer et al., 2009a; van Peer et al., 2009b). The incidence of plugging of septa in this fungus depends on the environmental conditions. For instance, a higher number of septa close upon exposure to heat stress or antibiotics. Notably, plugging of septa is reversible, at least in the case of heat stress. In Chapters 3 and 4 it is described that septa of aspergilli can also reversibly plug. In the case of *A. niger* plugging was shown to depend on environmental conditions.

A-SEXUAL DEVELOPMENT

After a period of vegetative growth, air-exposed colonies of *A. nidulans* and *A. niger* form two types of aerial hyphae (Figure 3). One type is quite similar to vegetative hyphae of these aspergilli and has a diameter of about 2-3 µm. The second type of aerial hyphae has a diameter of about 4-5 and 6-7 µm in the case of *A. nidulans* and *A. niger,* respectively. These so-called stalks can differentiate into conidiospore forming conidiophores (Figure 3). The conidiophore stalk of *A. nidulans* extends

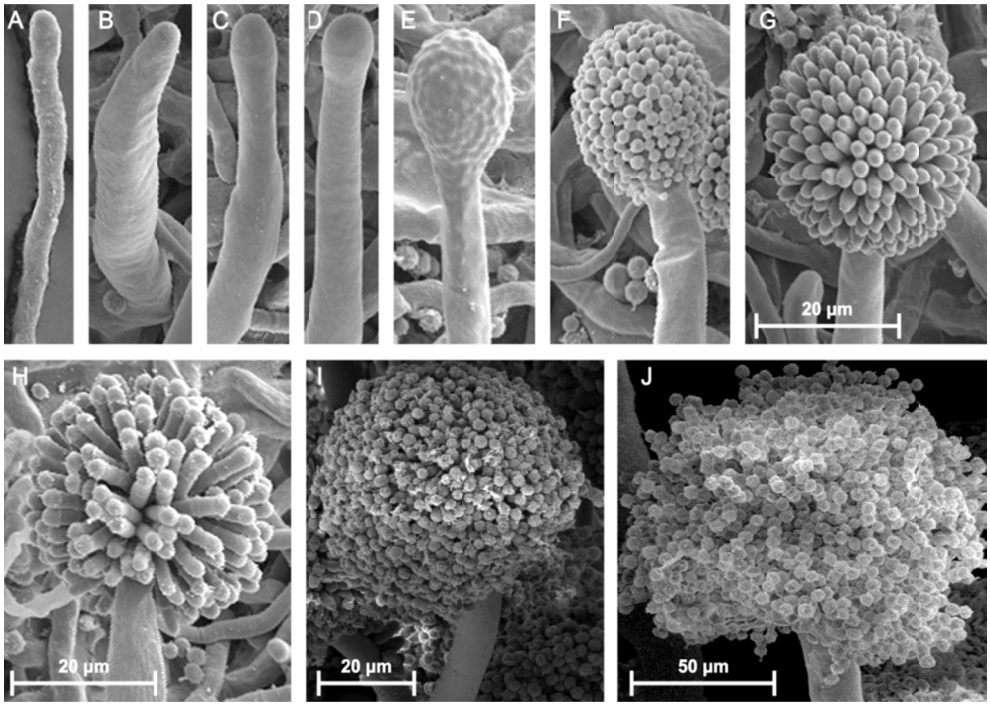


Figure 3: Development of *A. niger* monitored by scanning electron microscopy. The vegetative mycelium forms two types of aerial hyphae. One type is similar to vegetative hyphae (A), while the other type is 2-3 times thicker (B). The tips of the latter aerial hyphae may swell to form a vesicle (C, D). Buds are formed on the vesicle (E) that develop into metulae (F, G). Phialides are formed on top of the metulae, which give rise to chains of conidiospores (H, I, J). The bar in G also holds for A-F.

about 100 µm into the air and is formed from a specialized foot-cell within the substrate mycelium (Adams et al., 1998). When the stalk has reached its maximum height, the tip swells and forms a vesicle with a diameter of 10 µm. In biserate species like *A. nidulans* and *A. niger*, the vesicle surface buds resulting in a layer of primary sterigmata termed metulae. The metulae in turn bud twice. This results in a second layer of sterigmata called phialides. The phialides give rise to chains of uninucleate conidiospores. As a result, more than 10.000 conidiospores can be produced per conidiophore. *A. oryzae* can be both uniserate and biserate. This means that spore producing phialides can also be positioned directly at the surface of the vesicle of *A. oryzae* conidiophores.

The 2-3 µm thick aerial hyphae of *A. nidulans* and *A. niger* are formed about 8 h after inoculation of spores on complete medium. Although timing of this type of aerial hyphae seems to be medium-independent, the density of aerial hyphae is lower in the case of minimal medium. The first stalks of *A. nidulans* and *A. niger* are formed 10 h after spore inoculation on complete medium and growth at 37 °C and 30 °C, respectively. In both cases, conidiophores are formed 20 h post-inoculation. Formation of aerial hyphae in both aspergilli starts in the center of the colony and moves outwards ending a few millimeters from the edge of the mycelium. This observation implies that the competence of hyphae to form aerial hyphae is acquired faster when a colony gets older (Adams et al., 1998). The process of aerial growth has been proposed to involve signaling of the cell density of the vegetative mycelium (Lee and Adams 1994b; Wösten et al., 1999a; Wösten and Willey, 2000). The signaling molecule would induce hydrophobin genes. These genes encode proteins that lower the water surface tension to enable hyphae to breach the interface to grow into the air (Wösten et al., 1999b; Wösten 2001). Which hydrophobin is secreted into the aqueous environment to lower the water surface tension is not yet known.

A. nidulans can also form conidia in submerged cultures (Adams et al., 1998). In this case, conidiation is induced when the culture gets stressed or when nutrients are limited (*e.g.* limitation of the carbon and the nitrogen source). On the other hand, formation of conidiophores in air-exposed colonies is assumed to be induced by an internal signal that activates a genetic program of sporulation (see below) (Adams et al., 1998). In both cases, competence to sporulate is acquired in a time-dependent way (Skromne et al., 1995). Like *A. nidulans, A. niger* can also form conidiophores in submerged conditions. However, these conidiophores hardly, if at all, form spore chains (Figure 1F).

Regulation of a-sexual development

Formation of conidiophores has been well studied in *A. nidulans.* Experimental evidence has shown that mechanisms underlying a-sexual development in *A. fumigatus* and *A. oryzae* are similar but not identical in *A. nidulans* (see below). So far, formation of conidiophores and conidia has not been studied in *A. niger.* However, its genomic sequence predicts that mechanisms of a-sexual development are also similar, if not identical, to that in *A. nidulans* (Pel et al., 2007). About 1300 genes have been found to be up-regulated in whole colonies of *A. nidulans* during a-sexual reproduction (Timberlake, 1980). Similar results have been obtained in *A. niger*

(Chapter 2; Bleichrodt et al., 2012). In the latter study, RNA was isolated from the vegetative mycelium and from aerial structures (aerial hyphae, conidiophores, and spores) of 7-day-old colonies. Micro-array analysis showed that 2056 genes had a fold change ≥ 2. Of these genes, 1144 genes were up-regulated in the aerial structures. Taken together, these studies indicate that several hundred genes are involved in a-sexual development of aspergilli.

Regulation by fluG, brlA, abaA, wetA, medA, stuA, and vosA

FluG is believed to be at the start of the developmental program leading to a-sexual sporulation in *A. nidulans*. *A. nidulans* strains in which the *fluG* (fluffy) gene is inactivated (*i.e.* a Δ*fluG* strain) do form aerial hyphae but conidiophores are not being formed in excess of nutrients (Lee and Adams, 1994b). During nutrient deprivation, however, some conidiophores are being formed on a solid medium. Similarly, submerged cultures of the Δ*fluG* strain start to sporulate in the absence of a carbon source (Lee and Adams, 1996). These data indicate that FluG is involved in a developmental program of sporulation but not in the stress-related sporulation pathway. Formation of conidiophores in the Δ*fluG* strain can be rescued by growing the mutant next to a wild-type strain. Complementation is also observed when the strains are physically separated by a dialysis membrane with a size exclusion of 6-8 kDa. This indicates that FluG is involved in the production of a low-molecular weight extracellular signaling molecule that is involved in the formation of conidiophores. The homology of *fluG* with bacterial glutamine synthetases suggests that the signaling molecule is related to this amino acid.

Overexpression of *fluG* in vegetative hyphae of *A. nidulans* is sufficient to cause sporulation under conditions that normally suppress conidia formation (Lee and Adams, 1996). In wild-type strains, *fluG* transcripts are present in relatively constant levels during late vegetative growth and conidiation. Notably, a 4-fold higher *fluG* expression level is found in germinating spores during their isotropic growth (3 h after inoculation) when compared to polar growing germlings (5 h after inoculation) (Breakspear and Momany, 2007). This suggests that *fluG* is not only involved in conidiophore formation but also in germination.

FluG activates the *brlA* (*bristle*) gene. A Δ*brlA* strain of *A. nidulans* forms stalks that do not stop their growth after they have reached a length of 100 µm. These stalks can reach a length 20-30 times longer than those of the wild-type, which results in the characteristic bristle phenotype (Adams et al., 1988). Moreover, isotropic growth is not initiated at the apex of the stalks of the Δ*brlA* strain. As a result, conidiophore vesicles are not being formed. Conidiophore development becomes independent from *fluG* by placing *brlA* under control of an inducible promoter (Adams et al., 1988). Similar results have been obtained in *A. oryzae* (Ogawa et al., 2010; Yamada et al., 1999). Inactivation of *brlA* in *A. oryzae* results in the inability to form conidiophores. In contrast, fully developed conidiophores are formed in submerged culture when the *brlA* gene is expressed under the control of the *amyB* promoter. BrlA is also essential for conidiophore formation in *A. fumigatus* (Mah and Yu, 2006). However, in contrast to *A. nidulans* (Adams et al., 1988) and *A. oryzae* (Ogawa et al., 2010), the *A. fumigatus* gene seems to function earlier in conidiophore

development. This is based on the fact that conidiophore development is completely abolished in a Δ*brlA* strain of *A. fumigatus*. In addition, the *A. fumigatus* gene seems to function independent from *fluG.* At least, a Δ*fluG* strain of *A. fumigatus* still sporulates in air-exposed cultures. Possibly, *A. fumigatus* has more than one *brlA* activating mechanism (Mah and Yu, 2006). The *brlA* gene of *A. fumigatus* has also been shown to be involved in suppressing ribosomal protein genes during nitrogen stress (Twumasi-Boateng et al., 2009). This finding conforms to the general starvation response in fungi, which involves both down-regulation of ribosomal protein biogenesis and induction of sporulation (Bahn et al., 2007; de la Serna et al., 1999; Gasch et al., 2000; Li et al., 1999; Mogensen et al., 2006; Warner, 1999). However, down-regulation of ribosomal protein encoding genes is not impaired during carbon stress in *A. fumigatus* (Twumasi-Boateng et al., 2009). Nevertheless, these findings suggest that *brlA* of *A. fumigatus* is not only a regulator of formation of conidiophores but also influences the vegetative mycelium by affecting its protein synthesizing capacity.

Transcription of *brlA* in *A. nidulans* results in two transcripts that are called *brlAα* and *brlAβ*. Both transcripts are essential for proper conidiophore development (Prade and Timberlake, 1993) and are controlled at the transcriptional (*brlAα* and *brlAβ*) and translational level (*brlAβ*) (Han and Adams, 2001). Transcript *brlAβ* contains a short upstream ORF (μORF) and a downstream ORF that encodes the same polypeptide as BrlAα but with an N-terminal extension of 23 aa (Prade and Timberlake, 1993). Both polypeptides contain two C_2H_2 zinc finger DNA binding motifs. The *brlAα* and *brlAβ* transcripts have different functions during a-sexual development. As mentioned above, inactivation of *brlA* results in indefinitely elongating stalks. In contrast, aberrant primary conidiophores develop in the Δ*brlAβ* strain that can form secondary conidiophores. A-sexual development proceeds further in the Δ*blrAα* strain but conidia are not produced (Fischer and Kües, 2006). So far, it is not known whether transcription of *brlA* of *A. oryzae* and *A. fumigatus* also results in two transcripts.

BrlA activates a central regulatory pathway controlling temporal and spatial expression of conidiation specific genes (Boylan et al., 1987, Mirabito et al., 1989). This cascade is complex and involves, amongst others, the regulatory genes *abaA*, *wetA, stuA, medA,* and *vosA* (Figure 4). Gene *abaA* (*abacus*) is a regulatory gene that is activated in *A. nidulans* by BrlA during sterigmata differentiation (Boylan et al., 1987, Breakspear and Momany, 2007). A Δ*abaA* strain forms metulae that bud apically resulting in chains of cells with metula-like, rather than phialide-like, properties. In other words, phialides are not produced and therefore conidia are not formed (Boylan et al., 1987; Clutterbuck, 1969; Sewall et al., 1990). The interaction of AbaA with *brlA* is complex (Figure 4A). Gene *abaA* is activated by BrlA and, in turn, AbaA stimulates formation of *brlAα* transcripts but represses *brlAβ* accumulation (Adams et al., 1998; Andrianopoulos and Timberlake, 1994; Han and Adams, 2001; Sewall et al., 1990). This is caused by AbaA binding to a responsive element in the *brlAβ* locus (Han and Adams, 2001). The net result of *abaA* inactivation is that *brlA* is over-activated (Aguirre, 1993). The positive feedback loop of *brlA* on itself is likely to be independent of AbaA because the over-expression of *brlAβ* activates expression of

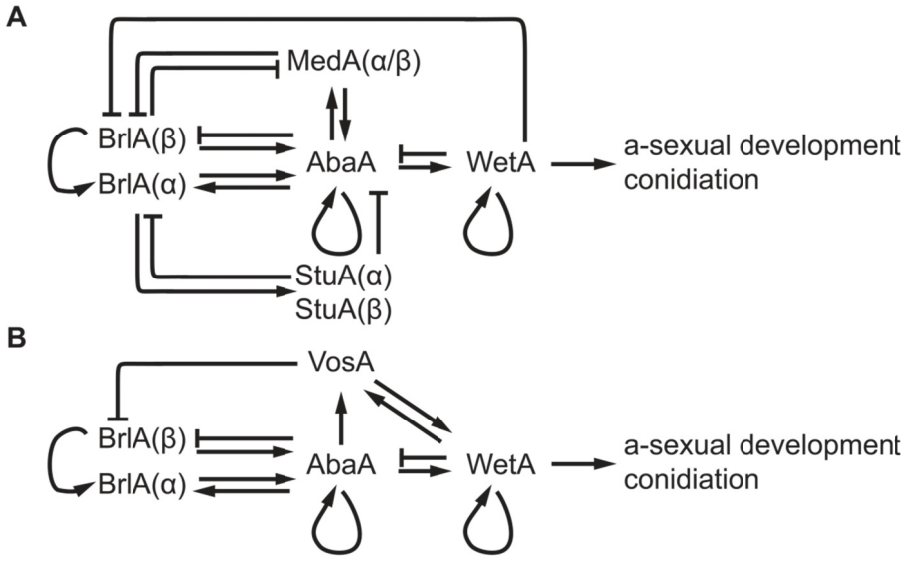


Figure 4: The central regulatory network consisting of BrlA, AbaA and WetA initiates a-sexual development in *A. nidulans*. StuA and MedA (A) and VosA (B) are regulators of *brlA, abaA,* and *wetA*.

brlAα in an *abaA* mutant (Han and Adams, 2001) (Figure 4A). Taken together, both BrlA and AbaA control transcript levels of *brlAα* and *brlAβ*. AbaA regulates several other genes including *abaA* itself, *medA, wetA* (Figure 4A), *vosA* (Figure 4B), and the structural genes *yA* and *rodA* (Andrianopoulos and Timberlake, 1994). Recently *abaA* was identified in *A. oryzae* (Ogawa et al., 2010) and *A. fumigates* (Tao and Yu, 2011). The role of *abaA* in *A. oryzae* is similar to that in *A. nidulans.* In the case of *A. fumigatus abaA* also delays autolysis and cell death.

During the late phase of conidiation, *wetA (wet white)* is activated by *abaA* (Figure 4A). Normal conidiophores are formed by *wetA* mutants. However, the conidia do not form pigments, are not water repellent, and go in autolysis (Marshall and Timberlake, 1991; Sewall et al., 1990). Gene *wetA* activates a set of genes in phialides and spores (*e.g. wA*), which are involved in making the conidial wall impermeable and mature (Marshall and Timberlake, 1991). In addition, WetA seems to activate itself (Adams et al., 1998; Boylan et al., 1987; Marshall and Timberlake, 1991; Ni and Yu, 2007) and represses *abaA* and *brlA* (Tao and Yu, 2011) (Figure 4A). Gene *wetA* of *A. oryzae* (Ogawa et al., 2010) has a role similar to that in *A. nidulans.* In the case of *A. fumigatus wetA* seems to have an additional role (Tao and Yu, 2011). It would also function in germ tube formation and reduced hyphal branching.

VosA (*viability of spore*) is a putative transcription factor of the velvet family (Tao and Yu, 2011). This family, which is conserved in filamentous fungi, also includes VeA and VelB of *A. nidulans*. Inactivation of *vosA* results in uncontrolled activation of a-sexual development, whereas its over-expression blocks sporulation. This may be the result of the observed inhibition of *brlA* by VosA (Tao and Yu, 2011) (Figure 4B). It should be noted that *vosA* is lowly expressed in the vegetative mycelium. Yet, these

expression levels may be sufficient to control *brlA.* Gene *vosA* is particularly expressed during the formation of conidia and sexual ascospores, where it plays a role in resistance to stress conditions (see below).

The *stuA* and *medA* genes are classified as developmental modifiers. Their encoded proteins affect *brlA* and *abaA* expression (Figure 4A). Mutations in *stuA* (*stunted*) of *A. nidulans* results in shortened aerial hyphae, shortened conidiophores and the absence of metulae and phialides. Conidiophores that are formed have reduced vesicles with abnormal numbers of nuclei. Only a few conidia can directly bud from the conidiophore vesicle. Thus, the morphology of the conidiophores is aberrant in *stuA* mutants, but neither temporal development nor conidiophore density is affected (Wu and Miller, 1997). Gene *stuA* has a similar role in a-sexual development in *A. fumigatus* (Sheppard et al., 2005). StuA is a helix-loop-helix transcription factor with two transcription start sites. This leads to *stuAα* and *stuAβ* transcripts, of which the former is most important for correct development (Aguirre, 1993; Miller et al., 1991; Miller et al., 1992). Expression of *stuA* depends on *brlA.* As a result, transcript levels of s*tuA* are increased 20-fold in conidiating cultures (Breakspear and Momany, 2007; Busby et al., 1996; Miller et al., 1992). In turn, StuA directly or indirectly represses and spatially restricts *brlA* and *abaA* expression (Figure 4A). With this ability *stuA* is involved in proper spatial distribution of AbaA and BrlA (Miller et al., 1992; Wu and Miller, 1997). The StuA protein also stimulates *stuA* expression. This seems to be an indirect effect because its responsive elements are absent in the promoter (Wu and Miller, 1997).

The *medA* (*medusa*) gene is conserved in filamentous fungi. Like other regulators, *medA* is transcribed at two initiation sites. While *stuA* of *A. nidulans* is required for proper spatial expression of *abaA* and *brlA, medA* is involved in proper temporal expression of these genes (Adams et al., 1998; Busby et al., 1996). Accumulation of both *brlA* transcripts is observed earlier in development in a Δ*medA* strain. Moreover, the mutant strain shows higher levels of *brlAβ,* but not *brlAα,* transcripts. As a result, the ratio of *brlA*α and *brlAβ* transcripts is lowered. Gene *medA* thus acts as a repressor of *brlA* expression. In contrast, it is an activator of *abaA* expression. This is concluded from the observation that *abaA* transcription levels are reduced or even absent in the *medA* mutant (Busby et al., 1996; Miller et al., 1992). The molecular basis of MedA function is still unclear. A Δ*medA* strain forms repeated layers of sterigmata and frequent reinitiated secondary conidiophores (Clutterbuck, 1969; Sewall et al., 1990). This phenotype resembles that of a strain of *A. nidulans* in which the chitin synthase genes *chsA* and *chsC* have been inactivated (Ichinomiya et al., 2005). In the latter strain, *abaA* expression is reduced. This indicates that *chsA* and *chsC* regulate expression of *abaA*, most probably in an indirect way. The Δ*chsA*Δ*chsC* mutant shows a defect in septum formation (Ichinomiya et al., 2005). Therefore, it was proposed that MedA is involved in septum formation on conidiophore structures. Taken together, conidiophore morphogenesis requires a finely tuned balance of at least BrlA, AbaA, MedA, and StuA (Busby et al., 1996), and possibly VosA and other velvet complex genes (Boylan et al., 1987; Ni and Yu, 2007).

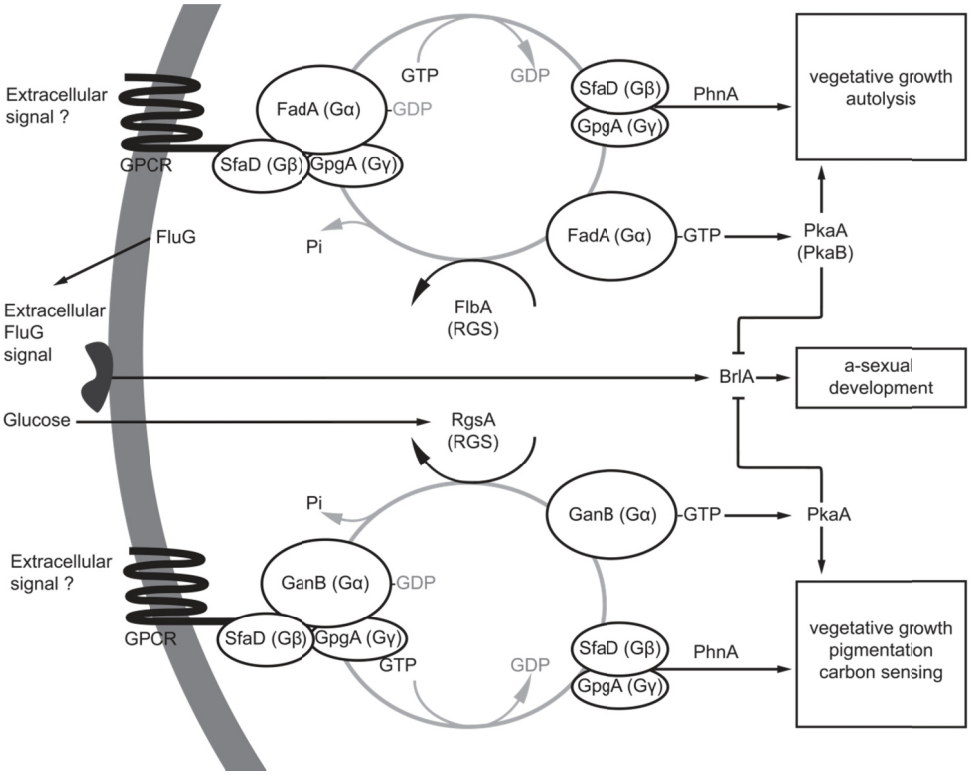


Figure 5: Signaling cascades resulting in vegetative growth or a-sexual reproduction in *A. nidulans*. Signalling involves FluG and independently, two heterotrimeric G-protein complexes, both consisting of SfaD and GpgA (the Gβγ subunits) and the Gα subunits FadA and GanB, respectively. GTP-bound FadA and GanB stimulate vegetative growth via the cAMP PkaA pathway and repress a-sexual reproduction via *brlA*. The RGS proteins FlbA and RgsA hydrolyze the GTP bound to FadA and GanB, respectively, thereby repressing vegetative growth and promoting a-sexual development. The SfaD-GpgA dimer also stimulates vegetative growth. This is regulated by PhnA (Adapted from Yu, 2006).

Trimeric G-protein signaling

Trimeric G-protein signaling is involved in the decision to grow vegetatively or to start a-sexual development. Gene *flbA* (*fluffy low brlA expression*) encodes a RGS domain protein, which negatively regulates vegetative growth signaling (Figure 5). It does so by stimulating the intrinsic GTPase activity of the Gα subunit FadA (*fluffy autolytic dominant*) of a heterotrimeric G-protein. As a result, the Gα subunit is converted into the inactive GDP bound state (D'Souza et al., 2001; Yu et al., 1996; Yu et al., 1999) (Figure 5). Overexpression of *flbA* in vegetative cells inhibits hyphal growth and stimulates conidiophore development even under conditions that normally prevent sporulation (Lee and Adams, 1994a; Lee and Adams, 1996). On the other hand, a mutation in *flbA* results in reduced *brlA* expression and a fluffy phenotype (hence the name *fluffy low brlA expression*). The Δ*flbA* strain does not form conidiophores. Instead, the mycelium proliferates uncontrolled and masses of undifferentiated aerial hyphae are formed. Both the submerged and aerial hyphae autolyse when colonies

mature (Lee and Adams, 1994a; Wieser et al., 1994). The autolytic phenotype of the *flbA* mutant can be partially overcome by deleting a class V endochitinase B (*chiB*). However, reduced cell viability cannot be restored in this way (Shin et al., 2009). Inactivation of *fadA* (Δ*fadA* or dominant-interfering *fadA* mutant) can also counteract the autolytic phenotype of the *flbA* mutant. This is in agreement with the function of FlbA to convert FadA into the inactive GDP bound state (Figure 5). A constitutively active *fadA* mutant, $fadA^{G42R}$, results in autolytic mutants similar to the *flbA* mutant (Hicks et al., 1997; Yu et al., 1996). The constitutively active $fadA^{G42R}$ mutant phenotype cannot be suppressed by overexpression of *flbA* (Yu et al., 1999).

In its inactive GDP-bound form, FadA of *A. nidulans* forms a heterotrimeric G-protein with the β- and γ-subunits encoded by *sfaD* and *gpgA*, respectively (Rosén et al., 1999; Seo et al., 2005; Yu et al., 1996; Yu et al., 1999). When FadA becomes GTP bound, this α-subunit dissociates from SfaD and GpgA (Figure 5). Inactivation of *sfaD* (Rosén et al., 1999) or *gpgA* (Seo et al., 2005) suppress the phenotype of the *flbA* mutant. Moreover, in a wild-type background reduced vegetative growth is observed in these deletion strains. Inactivation of *sfaD* (Rosén et al., 1999) but not *gpgA* (Seo et al., 2005) also causes hyper-sporulation. The Δ*sfaD*Δ*gpgA* double mutant showes a phenotype identical to those of the Δ*sfaD* mutant (Seo et al., 2005). This shows that Δ*sfaD* is epistatic to Δ*gpgA* and that SfaD can induce inappropriate conidiation even in the absence of GpgA. Notably, constitutive activation of *fadA* in the absence of *sfaD* is sufficient to cause proliferation of undifferentiated hyphae (Seo et al., 2005; Wieser et al., 1997). Taken together, FadA and SfaD-GpgA have overlapping functions in stimulating vegetative growth (Rosén et al., 1999; Seo et al., 2005). The phosducin like protein A (PhnA) seems to be involved in positively regulating Gβγ signaling in *A. nidulans*. Deletion of *phnA* results in a phenotype similar to that of a Δ*sfaD* strain (Seo and Yu, 2006). This would agree with the role of phosducin like proteins to act as chaperones for Gβγ assembly (Yu, 2006). Finally, deletions in *sfaD*, *fadA* or *gpgA* do not suppress conidiation defects in a *fluG* mutant. Therefore, the FadA/SfaD/GpgA vegetative growth-signaling cascade seems to be distinct from that of the FluG pathway (Seo et al., 2005).

GTP-bound-FadA promotes vegetative growth and inhibits a-sexual development by activating a cAMP-PKA signaling cascade (Shimizu and Keller, 2001) (Figure 5). The cAMP dependent protein kinase A catalytic subunit (PKA) encoded by *pkaA* has a major role in the stimulation of vegetative growth and the repression of conidiation (Lafon et al., 2005; Lafon et al., 2006; Seo et al., 2005; Yu and Keller, 2005). Inactivation of *pkaA* results in hyper-sporulation and reduced radial growth (Shimizu and Keller, 2001). On the other hand, over-expression of *pkaA* leads to decreased sporulation accompanied by a fluffy-like appearance. Deletion of the other *pka* gene in the genome of *A. nidulans, pkaB*, causes no apparent phenotype (Ni et al., 2005). However, over-expression of *pkaB* reduces conidiation and increases vegetative growth on solid medium. Moreover, it complements the reduced radial growth of the Δ*pkaA* strain. Apparently, PkaB functions as a backup for PkaA (Ni et al., 2005; Seo et al., 2003).

The FlbA/FadA/SfaD/GpgA pathway seems to be conserved in *A. nidulans, A. oryzae* and *A. fumigatus* (Mah and Yu, 2006; Ogawa et al., 2010; Yu, 2006). Like in *A.*

nidulans, the SfaD-GpgA complex is involved in stimulating vegetative growth in *A. fumigatus* (Shin et al., 2009). However, there are some differences in the case of the other components. Deletion of *flbA* in *A. nidulans* (Wieser et al., 1994), *A. oryzae* (Ogawa et al., 2010) or *A. fumigatus* (Mah and Yu, 2006) results in low *brlA* expression, and reduced conidiation. In contrast to *A. nidulans* and *A. oryzae,* the autolysis phenotype is missing in *A. fumigatus*. Moreover, in both *A. oryzae* and *A. fumigatus* hyphal proliferation is reduced in the Δ*flbA* strain, blocking formation of aerial hyphae in the case of *A. oryzae* (Ogawa et al., 2010). Like in *A. nidulans,* FadA of *A. oryzae* (Ogawa et al., 2010) and *A. fumigatus* (Mah and Yu, 2006) represses conidiation. Remarkably, in *A. oryzae* it also represses vegetative growth (Ogawa et al., 2010), while in *A. fumigatus* vegetative growth is stimulated (Liebmann et al., 2004; Shimizu and Keller, 2001).

Apart from FadA*,* two other Gα subunits are present in *A. nidulans* (GanA and GanB) and *A. fumigatus* (GpaA and GpaB), and three in *A. oryzae* (GanA, GanB, GaoC) (Chang et al., 2004; Lafon et al., 2006; Liebmann et al., 2003; Rosén et al., 1999; Seo et al., 2005; Yu et al., 1996). In contrast to *ganB,* the functions of *ganA* and *gaoC* have not been established yet (Chang et al., 2004; Han et al., 2004; Lafon et al., 2006). Like FadA, GanB in its inactive form interacts with SfaD and GpgA (Seo et al., 2005) (Figure 5), which in fact are the only β and γ subunits of trimeric G-proteins in *A. nidulans, A. fumigates*, and *A. oryzae* (Lafon et al., 2006). The Δ*ganB* strain shows hyper-sporulation in submerged cultures and earlier expression of the *brlA* transcripts. Constitutive activation of GanB results in reduced hyphal growth and a severe defect in a-sexual sporulation (Chang et al., 2004). Like FadA, GanB therefore seems to be involved in repression of *brlA* and inhibition of a-sexual sporulation (Chang et al., 2004).

RgsA (*regulator of G protein signaling*) is a repressor of GanB signaling. Colonies of the Δ*rgsA* strain are reduced in size, form more aerial hyphae, and accumulate dark brown pigments (Han et al., 2004). Expression of a constitutively active RgsA results in hyper-sporulation in submerged cultures and earlier expression of the *brlA* transcripts (Han et al., 2004). The presence of glucose is claimed to result in the increase of *rgsA* mRNA levels, and this would result in down-regulation of GanB mediated signaling. In cases of stress or unfavorable carbon sources, *rgsA* levels decrease and as a consequence GanB-GTP signaling is activated (Han et al., 2004). In *A. fumigatus* the outcome of the signaling pathway involving the GanB orthologue GpaB is different. Growth and conidiospore formation of a Δ*gpaB* strain is slightly decreased (Liebmann et al., 2004). This and other data show that GpaB signaling in *A. fumigatus* promotes a-sexual sporulation via PKA. The PkaC1 cascade in *A. fumigatus* is, however, complex, since it also promotes vegetative growth, when activated by GpaA (Liebmann et al., 2003).

Upstream activators of brlA

FluG activates sporulation by a derepression pathway that involves the SfgA protein (Figure 6). Gene *sfgA* (*suppressor of fluG*) is predicted to encode a transcription factor with a Gal4-type $Zn(II)_2Cys_6$ binuclear cluster DNA binding motif (Seo et al., 2006). Mutations in *sfgA* bypass the need for FluG during a-sexual development. The Δ*sfgA*

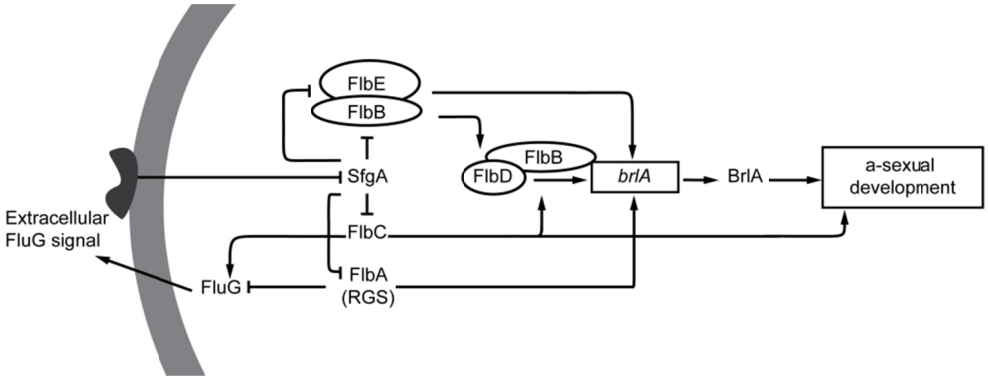


Figure 6: Model of upstream regulation of *brlA*. FluG is involved in the formation of an extracellular factor that activates an unknown receptor. At a certain concentration of FluG, the general suppressor SfgA is inhibited removing the repression of the *flb* genes. FlbB and FlbE form a complex that activates *brlA* leading to a-sexual development. FlbC activates *brlA* together with the FlbB/FlbD transcription complex. FlbC also activates *fluG* and regulatory genes that act downstream of BrlA. FlbA activates *brlA* by inactivating FadA and probably plays a role in repressing *fluG* (Adapted from Etxebeste *et al.* 2010).

strain shows hyperactive sporulation in liquid submerged cultures. Overexpression of *sfgA* results in delayed and reduced levels of *brlA* mRNA, and in colonies with reduced conidiation. Apparantly, the primary role of FluG is to remove the repressive effects of SfgA (Seo et al., 2006). The low FluG levels in young colonies would result in *sfgA*-mediated repression of conidiation. Once the FluG factor has accumulated above a certain threshold, it inhibits the repression of conidiation by SfgA (Seo et al., 2006). SfgA has also been proposed to negatively regulate FlbA (Figure 6). By this, the repression of SfgA by FluG will result in both activation of conidiation and inhibition of FadA-mediated stimulation of growth.

Apart from *flbA*, four other regulatory genes, *flbB*, *flbC, flbD,* and *flbE,* have been identified that act upstream of *brlA* (Wieser et al., 1994). All *flb* mutant strains show low *brlA* expression and a fluffy phenotype (Wieser et al., 1994). The mutants grow indeterminately and produce masses of aerial hyphae. The *flb* mutants can restore conidiation in *fluG* loss-of-function mutants (Wieser et al., 1994). These results indicate that the *flb* genes are involved in responding to the diffusible signaling molecule, produced by FluG, which is necessary for conidiation (Wieser et al., 1994). SfgA acts downstream of *fluG* but upstream of *flbA-D*. The position of *flbE* in the developmental pathway is not clear yet (Seo et al., 2006). As mentioned above, FluG is assumed to repress *sfgA* thus releasing the repression of the *flb* genes. Notably, *flb* genes are involved in regulation of *fluG* expression (Ruger-Herreros et al., 2011). A repressing function of FlbA on *fluG* expression is indicated by a 7-fold higher *fluG* expression in a Δ*flbA* strain. In contrast, *fluG* expression is stimulated by FlbB and FlbC. For instance, a Δ*flbC* shows no *fluG* mRNA accumulation (Ruger-Herreros et al., 2011).

The *flbC* gene encodes a transcriptional regulator containing two C_2H_2 zinc finger DNA binding domains (Kwon et al., 2010). The Δ*flbC* strain shows delayed and reduced conidiation, while overexpression causes restricted hyphal growth and

delayed conidiation. In wild type colonies, FlbC is localized in nuclei of vegetative hyphae and in conidiophores (*i.e.* not in conidiospores). Here, FlbC activates *brlA, abaA,* and *vosA* but not *wetA.* Overexpression of *flbC* not only inhibits vegetative growth in a wild-type strain but also in a Δ*abaA* or Δ*brlA* background. Thus, FlbC plays a direct role in repressing vegetative growth, independent of *brlA* or *abaA* (Figure 6). FlbC acts in a pathway parallel to that of *flbB, flbD*, and *flbE* (Garzia et al., 2010; Wieser and Adams, 1995). Absence of FlbC does not affect expression of *flbB* or *flbE* and vice versa. Moreover, double mutants cause additive effects, resulting in a prolonged delay in conidiation (Kwon et al., 2010). It has been proposed that FlbC coordinates activation of *brlA* together with a FlbB/FlbD transcriptional complex (Etxebeste et al., 2010a; Garzia et al., 2010) (Figure 6). Promoter binding regions of FlbC and FlbB/FlbD may overlap (Garzia et al., 2010; Han and Adams, 2001).

The *flbB* gene encodes a fungal specific bZIP-type transcription factor (Etxebeste et al., 2008). A Δ*flbB* strain shows defective branching patterns, delayed conidiation with a fluffy appearance and a high autolysis rate. Overexpression of *flbB* results in reduced conidiophore vesicle formation, and a reduced number of metulae (Etxebeste et al., 2008; Etxebeste et al., 2009). FlbB is located within the cytoplasm at the hyphal apex during early vegetative growth. In contrast, it resides in nuclei at hyphal tips during later stages of growth (Etxebeste et al., 2008). The repressor SfgA might be a key intermediate in the process of nuclear localization of FlbB. This is indicated by the fact that FlbB is found in all nuclei of Δ*sfgA* hyphae, rather than only at the hyphal tip as observed in the wild-type strain. Gene *flbE* encodes a protein without any known conserved domain (Garzia et al., 2009). Expression of *brlA* and *vosA* is delayed in the Δ*flbE* strain (Kwon et al., 2010). This is accompanied by absence of conidiophore formation, a fluffy appearance of the colonies, accelerated vegetative growth, and accelerated autolysis and cell death. FlbE is localized at hyphal tips. In fact, it co-localizes with FlbB. Localization of these proteins at the hyphal tip depends on the presence of F-actin. This is concluded from the fact that disintegration of the actin filaments causes mis-localization of FlbB and FlbE. Localization of FlbB and FlbE was also lost in a Δ*flbE* and a Δ*flbB* strain, respectively. These results indicate that these proteins depend on each other for proper localization at the hyphal apex. It has also been shown that FlbB stability is affected by the absence of a functional form of FlbE (Garzia et al., 2009). FlbE may thus protect FlbB from proteolytic degradation. It may do so by physical interaction with FlbB. At least, such an interaction was shown *in vivo* (Garzia et al., 2009). Taken together, FlbE and FlbB function in close association with each other and are functionally interdependent (Garzia et al., 2009).

The FlbB/FlbE complex is a requisite for *flbD* expression in the wild-type (Garzia et al., 2010) (Figure 6). FlbD is a c-Myb transcription factor. Deletion of its encoding gene results in delayed conidiation and a fluffy phenotype (Wieser et al., 1994; Wieser and Adams, 1995). Overexpression causes sporulation in liquid submerged cultures. This is due to inappropriate activation of *brlA* (Wieser and Adams, 1995). As mentioned above, the FlbB/FlbE complex is found at hyphal apices (Garzia et al., 2009). In contrast, FlbD is found in all nuclei of vegetative hyphae of *A. nidulans*. Thus, other transcription factors seem also to be involved in the regulation

of *flbD* expression (Etxebeste et al., 2010a; Garzia et al., 2009; Garzia et al., 2010). Not only *flbD* expression depends on the presence of FlbB, FlbD also interacts with this protein (Etxebeste et al., 2010a; Garzia et al., 2009; Garzia et al., 2010). The underlying mechanism is so far unknown but might involve a translationally modified form of FlbB (Garzia et al., 2010). Both FlbD and the FlbB/FlbE complex seem to activate *brlA* expression (Garzia et al., 2010) (Figure 6). Overexpression of *flbD* restores the conidiation defect in the Δ*flbE* strain (Kwon et al., 2010), suggesting an additive effect of both pathways. Interestingly *flbB* and *flbD* transcripts disappear shortly after *brlA* activation (Etxebeste et al., 2008; Wieser and Adams, 1995). However, the mechanism underlying this effect is independent of *brlA* levels (Garzia et al., 2010).

The *flb* genes are conserved in *A. fumigatus, A. oryzae* and *A. nidulans* (Kwon et al., 2010; Ogawa et al., 2010). The phenotypes of the Δ*flb* strains of *A. oryzae* are similar to those of *A. nidulans*. These results indicate that the functions of these regulatory genes are conserved between *A. oryzae* and *A. nidulans* (Ogawa et al., 2010). The *A. fumigatus flbE* could complement the Δ*flbE* phenotype in *A. nidulans* (Kwon et al., 2010), suggesting a similar role for these orthologues. Indeed, FlbE in *A. fumigatus* is necessary for proper control of conidiation and *brlA* and *vosA* expression. However, deletion of *flbE* does not cause an elevated vegetative proliferation or accelerated autolysis or cell death in *A. fumigatus* (Kwon et al., 2010). Inactivation of *flbB* in *A. fumigatus* results in delayed and reduced sporulation, and precocious cell death. Moreover, expression of *brlA* and *abaA* is affected. In contrast to *A. nidulans,* the FlbB protein is in *A. fumigatus* encoded by two transcripts, *flbBα* and *flbBβ*. The longest transcript, *flbBβ,* is constitutively expressed, while the *flbBα* transcript is found during progression of conidiation (Xiao et al., 2010). Both transcripts are needed for proper a-sexual development. The *flbB* gene of *A. nidulans,* encoding one transcript, only partially complements the Δ*flbB* strain of *A. fumigatus.* FlbC and FlbD functions in *A. fumigatus* are probably similar to *A. nidulans,* but characterization is ongoing at the moment (Xiao et al., 2010).

The role of hydrophobins in a-sexual development

Regulators activate target genes that fulfill a structural or enzymatic role in the formation of a-sexual structures. Genes have been identified that are specifically expressed in conidiophores and/or conidiospores (Chapter 2; Bleichrodt et al., 2012; van Leeuwen et al., 2012a; van Leeuwen et al., 2012b). Hydrophobin genes are examples of such target genes. Hydrophobins mediate the escape of hyphae into the air and make aerial structures such as conidiophores and conidiospores hydrophobic (Wösten, 2001). This hydrophobicity ensures that reproductive structures do not fall back in the substrate under humid conditions and serves dispersal of conidiospores by wind or vectors. Hydrophobins may also affect the cell wall architecture (van Wetter et al., 2000) and mediate attachment to hydrophobic substrates (Wösten, 2001). In the case of *A. fumigatus* it has also been shown that hydrophobins prevent immune recognition by the host (Aimanianda et al., 2009; Aimanianda and Latgé, 2010; Bruns et al., 2010; Dagenais et al., 2010; Paris et al., 2003). Moreover, the hydrophobin

RolA of *A. oryzae* recruits cutinase by adsorbing to the substrate of the enzyme. As a result, the substrate is efficiently degraded (Takahashi et al., 2005).

The *A. nidulans, A. fumigatus, A. oryzae* and *A. niger* genomes contain 6, 4-5, 2, and 8 hydrophobin genes, respectively (Jensen et al., 2010). One or more of the hydrophobins in each species enable hyphae to grow into the air by lowering the surface tension of the aqueous environment (see above). The aerial structures are then coated with hydrophobins to make them hydrophobic. So far, it has not been established which hydrophobins line aerial hyphae and the conidiophore stalk and vesicle of aspergilli. However, hydrophobins have been identified that coat sterigmata and conidiospores. The hydrophobin gene *rodA* (*rodlet)* of *A. nidulans* is expressed during the final stages of conidiophore formation. It is not expressed by vegetative hyphae and conidiospores (Boylan et al., 1987; Stringer et al., 1991). Expression of *rodA* is mediated by BrlA but not by AbaA or WetA. This is based on the finding that a Δ*brlA* strain does not express *rodA* but expression of the hydrophobin gene is not affected in the Δ*abaA* and Δ*wetA* strains. A Δ*rodA* strain forms wettable conidiophores and conidiospores. This is accompanied by the absence of the rodlet layer (Stringer et al., 1991). As a consequence, Δ*rodA* conidiospores adhere to each other in water. This affects their dispersal by air flow. The rodlet layer is also absent at the surface of metulae and phialides of the Δ*rodA* strain (Stringer et al., 1991). Experimental data imply that the RodA protein is produced by the sterigmata and diffuses to the outer surface of these structures as well as to that of the conidiospores to form the rodlet layer. *A. fumigatus* contains an ortholog of *rodA*. Inactivation of this gene results in a phenotype similar to that in *A. nidulans* (Paris et al., 2003; Thau et al., 1994). Moreover, *rodA* of *A. fumigatus* is involved in attachment of spores to particular substrates. The adhesion of the Δ*rodA* conidiospores is reduced in the case of collagen and bovine serum albumin but is not affected in the case of pneumocytes, fibrinogen, and laminin.

The *dewA* (*detergent wettable*) hydrophobin gene is expressed in sporulating cultures but not in cultures that grow vegetatively (Boylan et al., 1987; Stringer and Timberlake, 1995). Unlike RNA of *rodA*, transcripts of *dewA* are present in conidiospores (Breakspear and Momany, 2007). In agreement, immuno-detection showed that DewA hydrophobin is specifically present in cell walls of conidiospores, especially in mature spores. Expression of *dewA* is not only abolished in the Δ*brlA* strain but also in the Δ*abaA* and Δ*wetA* strains. Forced expression of *brlA* or *abaA* has only resulted in *dewA* expression in strains with an intact copy of *wetA.* Thus, *dewA* is regulated by *brlA* and *abaA* via *wetA* (Stringer and Timberlake, 1995). Conidiospores of Δ*dewA* are still covered with the rodlet layer. Thus, DewA is not essential for the rodlet layer on spores. Yet, when present, it may be part of it. The conidiospores of the Δ*dewA* strain do not wet in water. However, they wet more easily compared to wild-type spores when detergent is added to the water. A role of DewA in surface hydrophobicity is also indicated from the fact that wettability of the Δ*dewA*Δ*rodA* strain is much more pronounced when compared to the Δ*dewA* and the Δ*rodA* strains. *A. fumigatus* also contains a hydrophobin that is present at the surface of conidiospores but whose presence is not essential for the rodlet layer of conidiospores (Paris et al., 2003). This hydrophobin, RodB, is different in sequence when compared

to DewA. As mentioned above, the surface of Δ*rodA* conidia of *A. fumigatus* lacks the rodlet layer. Instead, the surface is granular. In contrast, the surface of Δ*rodA*Δ*rodB* conidia is amorphous. Taken together, RodB of *A. fumigatus* may be part of the rodlet layer of conidiospores when RodA is present. In the absence of RodA, RodB forms a granular structure and does not form rodlets.

OUTLINE OF THIS THESIS

The aim of this thesis was to study streaming of cytosol between compartments and between hyphae of *Aspergillus* and the effect of this on hyphal heterogeneity and a-sexual development.

Chapter 2 describes the RNA composition of vegetative hyphae and aerial structures (*i.e.* aerial hyphae, conidiophores and spores) of *A. niger* colonies. 2056 out of 14259 genes had a fold change in expression ≥ 2, of which 1144 genes were up-regulated and 912 were down-regulated in the aerial structures. Ratios of mRNA levels between the aerial structures and the vegetative mycelium as determined by micro-array analysis and a nuclear run-on transcription assay were different in 3 out of 7 analyzed genes. These data show that differences in mRNA composition between aerial structures and the vegetative mycelium do not only result from differential gene expression but also from differential RNA stability and/or RNA streaming/transport.

In **Chapter 3** it is described that 40% of the first, second and third septa of intact leading hyphae of *A. oryzae* are closed. Plugging did not depend on the environmental conditions but was shown to be reversible. Deletion of *Aohex1,* which encodes the main Woronin body protein, abolished the phenomenon of septal plugging. The inability to plug septa was irrespective of the environmental conditions. Taken together, it is concluded that Woronin bodies close septa in a reversible way in intact hyphae of *A. oryzae.*

Chapter 4 describes that part of the apical septa of intact growing hyphae is closed in a selected set of *Aspergillus* species. Changing the environmental conditions did not impact septal plugging in five out of six aspergilli. However, the incidence of septal plugging increased when *A. niger* was incubated below the optimal growth temperature and during hypertonic conditions. A wild-type strain of *Aspergillus oryzae* showed heterogeneous *glaA* expression at the periphery of the mycelium. Two populations of hyphae could be distinguished in this zone of the mycelium; one that highly and one that lowly expresses *glaA.* Heterogeneous *glaA* expression could not be shown in a Δ*Aohex1* strain. These data show that septal plugging maintains hyphal heterogeneity.

In **Chapter 5** inter- and intracellular streaming of glucose and GFP was studied in *Aspergillus*. Glucose and its inert analogue 3-OMG were shown to stream from the center to the periphery of colonies of wild-type and Δ*hex1* strains of *A. niger* and *A. oryzae*, but not vice versa. Short-distance inter-compartmental streaming of a glucose

analogue (2-NBDG) was not affected by conditions that normally plug septa in *A. niger* (*i.e.* incubation at 4 °C and 45 °C). This shows that plugged septa are not a barrier for sugar transport. In *A. niger* GFP was shown to stream from the vegetative mycelium to conidiophores but not vice versa. Streaming of PA-GFP in the vegetative mycelium and the conidiophores occurred at a rate of 10-15 μm s^{-1}. The streaming rate of PA-GFP decreased to about 4-6 μm s^{-1} when the reporter protein was fused to the glyceraldehyde-3-phosphate dehydrogenase (GPD) protein. Moreover, it was shown that PA-GFP streaming was affected by septal plugging resulting from incubation at 4 °C and 45 °C. In the former case, septa opened again after a 30 min incubation at 30 °C, thus restoring PA-GFP streaming.

Chapter 6 summarizes and discusses the results.

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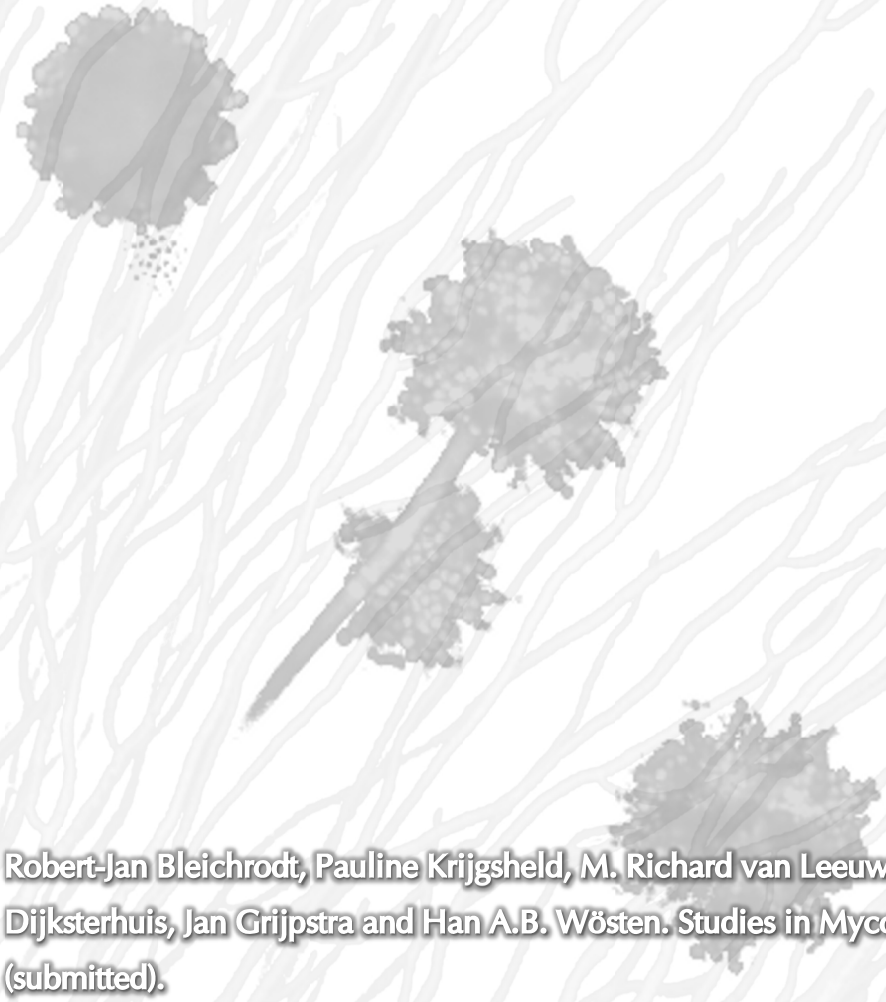
Chapter 2

Aerial structures and vegetative mycelium of the filamentous fungus *Aspergillus niger* have distinct RNA profiles



Robert-Jan Bleichrodt, Pauline Krijgsheld, M. Richard van Leeuwen, Jan Dijksterhuis, Jan Grijpstra and Han A.B. Wösten. Studies in Mycology (submitted).

Abstract

Aspergillus niger forms a vegetative mycelium consisting of hyphae that are separated by porous septa. After a period of vegetative growth, this fungus forms aerial hyphae and conidiospore forming conidiophores. Here, the RNA composition was determined of vegetative hyphae and aerial structures (*i.e.* aerial hyphae, conidiophores and spores) of 7-day-old colonies of *A. niger.* 2056 out of 14259 genes had a fold change in expression ≥ 2, of which 1144 genes were up-regulated in the aerial structures. Among these genes were the transcriptional regulators *brlA, abaA* and *wetA,* as well as 5 out of 7 hydrophobins. Genes encoding polysaccharide degrading enzymes (*e.g.* the glucoamylase gene *glaA*, and the acid α-amylase *aamA*) and monosaccharide permeases were among the genes that were down-regulated in the aerial structures. Ratios of mRNA levels between the aerial structures and the vegetative mycelium as determined by micro-array analysis and a nuclear run-on transcription assay were different in 3 out of 7 analyzed genes. These data indicate that differences in mRNA composition between aerial structures and the vegetative mycelium not only result from differential gene expression but also from differential RNA stability and/or RNA streaming/transport.

Introduction

Germination of a fungal spore leads to the formation of hyphae that grow by apical extension and that branch sub-apically. As a result, a vegetative mycelium is formed. This interconnected hyphal network forms aerial reproductive structures such as conidiophores or fruiting bodies. Growth of these structures depends on the translocation of nutrients and water from the vegetative mycelium (Jennings, 1984, 1987; Wösten and Wessels, 2006). Translocation in the higher fungi (*i.e.* the ascomycetes and the basidiomycetes) is possible because of the fact that the compartments within and between hyphae are separated by porous septa. In fact, the diameter of the pores often allows passage of organelles (Moore and McAlear, 1962; Lew, 2005). The cytoplasm within the vegetative mycelium of fungi is thus considered

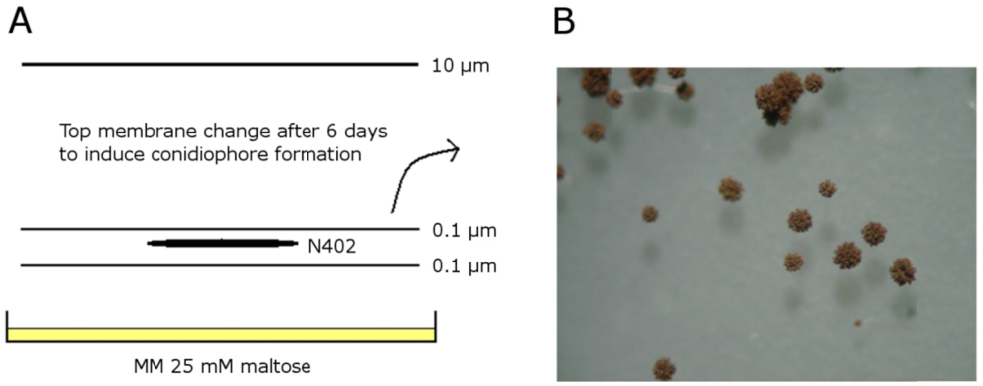


Figure 1: Growth method to obtain vegetative mycelium or aerial structures for RNA isolation.
A. niger N402 was grown between two porous polycarbonate membranes (0.1 µm pores) on top of solid minimal medium (MM) with 25 mM maltose as a carbon source (A). After 6 days the top membrane was replaced by a polycarbonate membrane with pores of 10 µm (A) allowing aerial growth (B). Growth was prolonged for 24 h.

to be continuous. Yet, it has been shown that the vegetative mycelium is highly heterogeneous with respect to growth, protein secretion and RNA composition (Wösten et al., 1991; Moukha et al., 1993; Vinck et al., 2005, 2011; Masai et al., 2006; Levin et al., 2007a, b; Kasuga and Glass, 2008; de Bekker et al., 2011a, b).

A-sexual sporulation is widespread in the fungal kingdom. Regulation of a-sexual sporulation has been extensively studied in *Aspergillus nidulans* (Timberlake, 1990; Adams et al., 1998; Krijgsheld et al., 2012; Chapter 1). The regulatory mechanisms governing a-sexual sporulation in this ascomycete probably also apply for other aspergilli including *Aspergillus niger* (Krijgsheld et al., 2012; Chapter 1). The formation of the conidiospore forming conidiophore of *A. nidulans* begins with the formation of a conidiophore stalk that elongates by apical extension. The stalk extends from a specialized thick-walled cell, termed a foot cell that anchors the developing conidiophore to the substrate mycelium. After apical extension of the conidiophore stalk has come to an end, the tip swells and forms the conidiophore vesicle. Depending on the *Aspergillus* species, phialides develop directly from the vesicle or from metulae (Mims, 1988). The phialides give rise to chains of uni- or di-nucleate conidiospores (Maruyama et al., 2001; van Leeuwen et al., 2012a, b) that are dispersed by wind and that can give rise to a new mycelium.

In this study, the RNA composition of vegetative hyphae and aerial structures (*i.e.* aerial hyphae, conidiophores and conidiospores) of *A. niger* was determined. 2056 genes had a fold change in expression ≥ 2, of which 1144 genes were up-regulated in the aerial structures. Nuclear run-on analysis indicates that differences in RNA composition of aerial structures and vegetative mycelium do not only result from differential gene expression, but also from differential RNA stability and/or from RNA streaming/transport.

Materials and Methods

Strains and growth conditions

A. niger strain N402 (Δ*cspA1*) (Bos et al., 1988) was grown at 30 °C in the light on solid minimal medium (de Vries and Visser, 1999) containing 1.5% agar and 25 mM maltose as carbon source. Cultures were inoculated with 1000 spores taken up in 2 µl 0.8% NaCl containing 0.005% v/v Tween-80. *A. niger* was grown as a sandwiched culture (Wösten et al., 1991) in a 0.25 mm layer of 0.6% agarose between two porous polycarbonate membranes (diameter 76 mm, pore size 0.1 µm; Profiltra; www.profiltra.nl). After six days of growth, the top membrane of the sandwich was replaced by a membrane with pores of 10 µm (Profiltra), allowing formation of aerial hyphae and conidiophores for 24 h (Figure 1).

Plasmids

Genes were amplified by PCR using *A. niger* N402 chromosomal DNA as template, Phusion® High-Fidelity DNA polymerase (Finnzymes; www.finnzymes.com), and oligonucleotides presented in Table 1. The blunt end PCR products of genes *gpdA*, *mpdA*, *ayg1* (also known as *olvA*), flavohemoprotein (flavo), *adhA*, FAD binding oxidoreductase (oxi), *glaA* and 18S rDNA were inserted in the *Sma*I site of pUC19.

This resulted in plasmids pRB001, pRB002, pRB003, pRB004, pRB005, pRB006, pRB007 and pRB008, respectively. In all cases, inserts were sequenced to confirm that the correct fragments had been cloned.

Table 1: Primers used in this study.

Gene ID	Gene	Primer	Primer Sequence
An02g05830	*gpdA*	RB1	GCGGCCGCTCCAGAAAGGAG
		RB2	CCATGGGGGCATCAACCTTGG
An16g01830	*mpdA*	RB3	GCGGCCGCTGCTCGTTCCCG
		RB4	CCATGGTCGTCCCCTGCTGCACCTTG
An14g05350	*ayg1*/ *olvA*	RB5	GCGGTTAATTAAGTCAGCTTACCGGAACAATG
		RB6	TATTGGCGCGCCGTTCTTGAGAGGCTCTTGG
An14g02460	flavo	RB7	GGGCGTTAATTAAGCCCACACATTGACCATCAACGAGAACCC
		RB8	TTAAGGCGCGCCAGCGGGCACACCACCAGTGCCAAAC
An17g01530	*adhA*	RB9	GCGGTTAATTAAGCGGCTGATGGTTACATAC
		RB10	TATTGGCGCGCCCTGAGGCACCTCAAGGACATACC
An18g00510	oxi	RB11	GCGATTAATTAACTGGAAGACCACCAGCAAGAAG
		RB12	TTAAGGCGCGCCAATCTGAACATCTTCCTCGGGAAG
An03g06560	*glaA*	RB13	CCAGCATCATTACACCTCAG
		RB14	TGCACACCCACTACATAC
An03e03260	18S rRNA	RB15	CCTGCGGCTTAATTTGACTC
		RB16	CCTCTAAATGACCGGGTTTG

RNA isolation

Vegetative mycelium and aerial structures of 7-day-old maltose grown cultures of *A. niger* were harvested from 3 and 5 sandwiched colonies, respectively. The aerial structures were scraped from the top membrane of the sandwiched culture with a razor blade. From other colonies, vegetative mycelium was harvested by flipping over the top membrane and scraping it off with a razor blade. In the case of the aerial structures, colonies were first submerged in RNAlater-ICE (Ambion; www.ambion.com). Vegetative mycelium and aerial structures were frozen in liquid nitrogen and homogenized in a TissueLyser II (Qiagen; www.qiagen.com; setting 2 min, 30 Hz) using stainless steel 10 ml buckets. RNA of the vegetative mycelium was isolated using TRIzol® reagent (Invitrogen; www.invitrogen.com) according to the instructions of the manufacturer followed by purification using RNeasy spin columns (Qiagen). RNA of the aerial structures was extracted using a modified protocol of the MasterPure Yeast RNA Purification Kit (Epicentre Biotechnologies; www.epibio.com). To this end, homogenized material was taken up in 1.8 ml T&C lysis buffer and vortexed vigorously. 525 µl MPC Protein Precipitation Reagent was added and the samples were incubated on ice for 5 min. After centrifugation at 4 °C for 10 min at 14.000 rpm in an Eppendorf microcentrifuge, the supernatant was transferred to a new tube and 1 ml isopropanol was added. After centrifugation (see above for the conditions), the RNA was re-suspended in DNAseI solution and incubated at 37 °C for 15 min. 400 µl T&C lysis solution was added. After vortexing, 400 µl of MPC Protein Precipitation Reagent was added and samples were placed on ice for 5 min. After centrifugation, 800 µl isopropanol was added to the supernatant, immediately

followed by centrifugation. The RNA pellet was washed twice with 70% ethanol and re-suspended in 100 µl TE buffer. After addition of 350 µl RLT buffer (RNeasy kit, Qiagen) and 250 µl ethanol, samples were purified using RNeasy spin columns according to the instructions of the manufacturer.

Micro-array analysis

Micro-array analysis was performed on biological triplicates at ServiceXS (Leiden, The Netherlands) according to Affymetrix protocols. In brief, RNA concentration was determined by absorbance at 260 nm using the Nanodrop ND-1000 (Thermo Scientific; www.thermo.com). Quality and integrity of the RNA was verified using the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies; www.agilent.com). Biotin-labeled antisense cRNA was produced from 2 µg of total RNA with the Eukaryotic One-Cycle Target Labeling kit (Affymetrix; www.affymetrix.com). The quality of the cRNA was checked using the Agilent 2100 bioanalyzer. 12.5-20 µg cRNA was used for fragmentation and 10 µg of this was hybridized to Affymetrix *A. niger* Genome Gene chips. After an automated process of washing and staining, absolute values of expression were calculated from the scanned array using the Affymetrix Command Console v1.1 software. The array data has been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and is accessible through GEO Series accession number GSE32123 (www.ncbi.nlm.nih.gov/geo/). RNA normalization was done using the MAS5.0 algorithm using a baseline correction of the median. CEL files were imported into the GeneSpring v.10 software package (Agilent Technologies). An unpaired T-test with Benjamini-Hochberg correction for multiple testing was performed to analyze differential expression between the vegetative mycelium and the aerial structures. A Fisher's exact test was used to identify overrepresented functional gene classes in the Functional Catalogue FunCat 2.0 (Ruepp et al., 2004; www.mips.helmholtz-muenchen.de/projects/funcat).

Northern analysis

Five µg of RNA was incubated for 1 h at 50 °C in a total volume of 20 µl containing 2.2 µl 8.8 M glyoxal, 10 µl DMSO and 2.0 µl 0.1 M sodium phosphate pH 7.0. Samples were run on an 1.5% agarose gel containing 10 mM phosphate buffer pH 7.0. RNA was blotted onto an Amersham Hybond™-N+ nitrocellulose membrane (GE Healthcare; www.gelifesciences.com) using 10x SSC (1.5 M NaCl and 150 mM sodium citrate pH 7.0). The blot was air dried and RNA was cross-linked to the membrane by a 30 sec exposure to 0.28 J UV-light. Blots were stained with 0.04% methylene blue in 0.5 M NaAc buffer pH 5.2 to check for equal loading. Probes for *gpdA* (1.1 kb), *mpdA* (1.0 kb), *glaA* (0.7 kb) and 18S *rDNA* (0.6 kb) were obtained by digesting pRB001 with *Hind*III and *Xho*I; pRB002 with *Eco*RI and *Sac*I; pRB007 with *Pvu*I and *Hin*cII; and pRB008 with *Eco*RI and *Bam*HI, respectively. DNA Probes were labeled with 25 µCi ^{32}P-dCTP for 1 h at room temperature using 5 units of Klenow fragment. Probes were separated from free nucleotides using a Sephadex G50 (medium) column. Blots were pre-hybridized for 2 h at 65 °C in 20 ml pre-hybridization buffer (6x SSC, 5x Denhardt's [0.1% Ficoll, 0.1% polyvinilpyrolidone, and 0.1% bovine serum albumin], 0.01 M EDTA, 0.5% SDS and 100 µl single

stranded herring sperm DNA). Hybridization was performed for 16 h at 42 °C in 20 ml hybridization buffer (50% formamide, 6x SSC, 2x Denhardt's, 0.1% SDS, 10% dextrane sulfate). The ^{32}P-labeled DNA probes had been denatured at 95 °C. Blots were washed twice at 65 °C for 20 min in 50 ml 2x SSC, 0.5% SDS, followed by two washes for 20 min with 0.2x SSC, 0.5% SDS. Blots were exposed to X-OMAT (Kodak; www.kodak.com) at -80 °C using intensifying screens.

Dot-blotting of plasmid DNA

Plasmid DNA was isolated from *E. coli* cultures using a NucleoBond® PC 100 kit (Macherey-Nagel; www.mn-net.com). For each plasmid, 5 µg of DNA was taken up in 180 µl of water. 80 µl 4 M NaOH was added, after which the mixture was incubated for 15 min at rt. This was followed by adding 800 µl of ice cold 2 M NH_4Ac. Dot-blot equipment was incubated for 1 h in 3.5% H_2O_2 and rinsed with RNase free water. The dot-blot apparatus was loaded with two Whatmann papers and an Amersham Hybond™-N+ nitrocellulose membrane that had been washed with RNAse free water and 2x SSC. The dot-blot apparatus was put under vacuum, using a standard vacuum pump and wells were washed with 200 µl 2x SSC. This was followed by washing with 200 µl 1 M NH_4Ac. 800 µl of each DNA sample (*i.e.* 3.8 µg) was spotted. Wells were washed with 200 µl 1 M NH_4Ac, after which the nitrocellulose membrane was air dried. DNA was cross-linked to the membrane by a 30 sec exposure to 0.28 J UV-light. DNA was stained with 0.04% methylene blue in 0.5 M NaAc buffer pH 5.2 to confirm equal loading, after which the membrane was de-stained with RNAse free water.

Isolation of nuclei

Vegetative mycelium and aerial structures were isolated from 7-day-old colonies as described above and frozen in liquid nitrogen. The material was homogenized in a TissueLyser II (setting 2 min, 30 Hz) using stainless steel 10 ml buckets and re-suspended in ice cold HB 0.5 buffer (10 mM PIPES pH 6.9, 5 mM $CaCl_2$, 5 mM $MgSO_4$, 0.5 M sucrose, complete protease inhibitor (Roche; www.roche.com), 0.1% 2-mercaptho-ethanol). From now on all steps were performed at 4 °C. Mycelial fragments were removed from the homogenate by centrifugation for 10 min at 160 g in a swing-out rotor (Harrier; www.mseuk.co.uk) followed by filtering the supernatant over glass wool twice. The filtrate was centrifuged 20 min at 5900 g. The pellet was re-suspended in 2 ml HB 2.1 buffer (10 mM PIPES pH 6.9, 5 mM $CaCl_2$, 5 mM $MgSO_4$, 2.1 M sucrose, complete protease inhibitor (Roche), 0.1% 2-mercaptho-ethanol) and centrifuged 20 min at 5900 g to pellet mycelial fragments. The supernatant was transferred to a new tube and brought to a volume of 2 ml with HB 2.1 buffer. Samples were centrifuged for 1 h at 128000 g in 1 ml tubes in a TLA100.1 rotor (Beckman Coulter; www.beckmancoulter.com) to pellet the nuclei. The pellet was taken up in 200 µl nuclei re-suspension buffer (50 mM Tris-HCl pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA), divided in 100 µl portions, and stored at -80 °C. Nuclei were stained with DAPI for quantification using a haemocytometer. About 2.5 x 10^7 and 7 x 10^6 nuclei were isolated from the vegetative mycelium and the aerial structures, respectively, from one 7-day-old colony.

Nuclear run-on transcription assay

Nuclei (4.5 x 10^7 in 100 µl nuclei re-suspension buffer) were thawed on ice and mixed with 67 µl 3x reaction buffer (15 mM Tris HCl pH 8.0, 0.45 M KCl, 7.5 mM $MgCl_2$, and 0.75 mM of each of the nucleotides, except dUTP). 27 µl DEPC treated demi water and 6.25 µl α-^{32}P-UTP (100 µCi, 6000 Ci/mmol, PerkinElmer; www.perkinelmer.com) were added. After mixing carefully by pipetting up and down, the mixture was incubated at 30 °C for 30 min. The nuclear DNA was degraded by incubating with 5 µl RNAse free DNAseI (1U/µl) for 10 min at rt. Nuclei were lysed by adding 1/9th volume of 10% SDS and 4 M NaCl, after which 1 ml of TRIzol® was added. After incubation for 5 min at rt, 210 µl chloroform was added. Samples were centrifuged at 10000 g for 10 min after 3 min incubation at rt. The aqueous phase was transferred to a new tube and centrifuged again. 250 µl of 2-propanol was added to the aqueous phase. After mixing well, the RNA was precipitated at 10000 g for 10 min. The RNA was washed with 70% ethanol and centrifuged for 5 min. Pellets were taken up in 150 µl RNAse free water and the RNA was dissolved by incubation for 15 min at 65 °C.

The nitrocellulose membrane containing plasmid DNA was pre-hybridized in 20 ml hybridization buffer (see Northern analysis) for 2 h at 42 °C. Radioactively labeled RNA, resulting from the run-on transcription, was added to the hybridization buffer after incubating the RNA for 2 min at 100 °C and 5 min on ice. After hybridization for 16 h at 42 °C, the membrane was washed once with 6x SSC and 0.2% SDS (5 min at rt), twice with 2x SSC and 0.2% SDS (20 min at 65 °C), and twice with 0.2x SSC and 0.2% SDS (20 min at 65 °C). The blots were exposed to X-OMAT film at -80 °C using intensifying screens.

Results

Total RNA of vegetative mycelium of 7-day-old maltose-grown sandwiched colonies of *A. niger* was isolated using TRIzol® (Figure 2). However, extraction of total RNA from aerial structures was not successful with this commonly used method. Therefore, a novel RNA extraction method was developed, which was based on the MasterPure Yeast RNA Purification Kit (Epicentre Biotechnologies; see Material and Methods). This extraction method yielded high quality total RNA from aerial structures (Figure 2), but not from vegetative mycelium. Therefore, RNA extraction was performed with TRIzol® and the MasterPure Yeast RNA Purification Kit to isolate RNA from vegetative mycelium and aerial structures, respectively.

Total RNA of biological triplicates was hybridized to Affimetrix whole genome micro-arrays representing 14259 unique *A. niger* ORFs (Pel et al., 2007; Jacobs et al., 2009). 5095 and 5939 of the probe sets yielded a present call after hybridization with RNA from the vegetative mycelium and the aerial structures, respectively. These probe sets represented a total of 6476 genes. Of these genes, 2056 genes had a fold change ≥ 2, 644 genes a fold change ≥ 4, 200 genes a fold change ≥ 10, and 3 genes a fold change ≥ 100 (Table 2, Supplementary Table 1; see http://hdl.handle.net/10411/10107). The genes in the latter group represented the

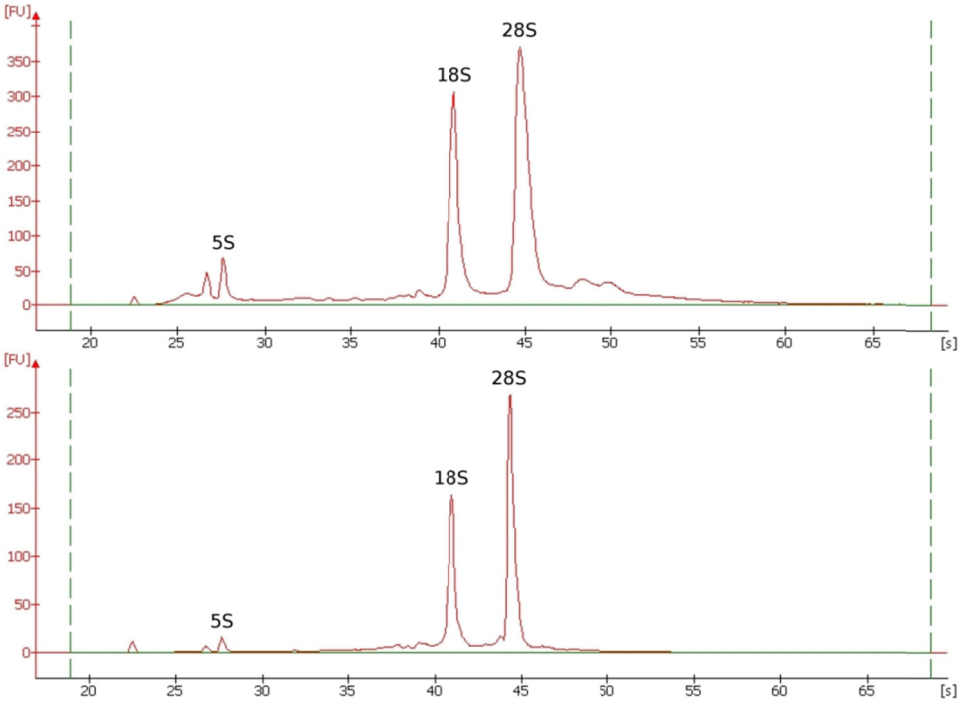


Figure 2: Bioanalyzer graphs of RNA isolated from vegetative mycelium (upper panel) and aerial structures (lower panel).

cellobiohydrolase gene *cbhB* that was down-regulated in the aerial structures, and a strong homologue of an obtusifoliol 1,4-α-demethylase and a hypothetical protein (recently described as a hydrophobin; Jensen et al., 2010) that were up-regulated. The top 100 of most up- and down-regulated genes are shown in Table 3 and Table 4.

Northern analysis confirmed the micro-array data. Accumulation of mRNA of the glucoamylase gene *glaA* and the mannitol-1-phosphate dehydrogenase gene *mpdA* was higher in the vegetative mycelium compared to the aerial structures. The RNA level of the glyceraldehyde-3-phosphate dehydrogenase gene *gpdA* was similar in the vegetative mycelium and in the aerial structures (data not shown).

Table 2: Number of genes that are up- or down-regulated in the aerial structures when compared to the vegetative mycelium.

Fold change ≥	Down-regulated	Up-regulated	Total
2	912	1144	2056
4	254	390	644
10	48	152	200
50	5	28	33
100	1	2	3

Table 3: The top 100 of genes up-regulated in the aerial structures when compared to the vegetative mycelium. The fold change was also calculated when a gene had an absent call. Also known as: [a]*brnA*, [b]*olvA*, [c]*ctcB*, [d]*fwnA*, [e]*rotA*, [f]*A. fumigatus* laccase homologue *abr2*, [g]*kslA* and [h]*knlA*.

Gene ID	Fold change	Annotation
An01g10940	111.20	hypothetical protein
An18g00500	107.34	strong similarity to obtusifoliol 1,4-alpha demethylase CYP51 – *Sorghum bicolor*
An04g07530	93.20	hypothetical protein
An15g07370	87.26	similarity to hypothetical protein encoded by CG4090 – *Drosophila melanogaster*
An09g05330	80.95	similarity to hypothetical protein 4MeS – *Metarhizium anisopliae*
An15g07370	80.59	similarity to hypothetical protein encoded by CG4090 – *Drosophila melanogaster*
An07g00510	76.38	similarity to hypothetical lipoprotein SC4A2.13c - *Streptomyces coelicolor*
An18g00510	76.17	similarity to 6-hydroxy-d-nicotine oxidase 6-HDNO - *Arthrobacter oxidans*
An13g02530	74.55	similarity to carbonic anhydrase CAH - *Neisseria gonorrhoeae*
An08g02170	74.26	hypothetical protein
An02g03690	72.79	hypothetical protein
An13g02470	71.81	hypothetical protein
An07g08670	69.22	weak similarity to hypothetical protein RtoA - *Dictyostelium discoideum*
An12g04700	68.65	strong similarity to allergen Tri r 4 - *Trichophyton rubrum*
An12g02680	67.75	weak similarity to hypothetical protein encoded by An02g12900 - *Aspergillus niger*
An11g02720	66.68	similarity to hypothetical protein C50F7.2 - *Caenorhabditis elegans*
An12g02740	60.43	weak similarity to ATP-dependent proteinase Clp from patent WO9743303-A1 - *Streptococcus pneumoniae*
An07g03340	58.27	strong similarity to hydrophobin hYP1 - *Aspergillus fumigatus*
An04g00710	57.45	weak similarity to hypothetical protein CAC28773.2 - *Neurospora crassa*
An16g07330	57.00	weak similarity to hypothetical extracellular matrix protein AAL47843.1 - *Fusarium oxysporum*
An06g02650	56.86	strong similarity to hypothetical protein CAD70532.1 - *Neurospora crassa*
An09g02420	55.59	hypothetical protein
An18g00490	55.36	similarity to salicylate hydroxylase nahW - *Pseudomonas stutzeri*
An09g06360	54.04	hypothetical protein
An08g06730	53.19	weak similarity to hypothetical protein CAD29600.1 - *Aspergillus fumigatus*
An18g00480	52.12	strong similarity to cycloheximide resistance protein CYHR - *Candida maltosa*
An14g05370[a]	51.38	strong similarity to cell surface ferroxidase precursor Fet3 - *Saccharomyces cerevisiae*
An01g15020	50.94	similarity to O-methyltransferase omtB - *Aspergillus flavus*
An12g07050	49.57	weak similarity to dihydrofolate reductase dfr1p - *Schizosaccharomyces pombe*
An06g02700	46.86	strong similarity to cytochrome P450 eln2 - *Coprinus cinereus*
An16g00940	45.29	strong similarity to fluconazole resistance protein FLU1 - *Candida albicans*
An15g02350	43.79	strong similarity to hypothetical precursor of spore coat protein sp96 - *Neurospora crassa*
An14g05340	43.34	strong similarity to hypothetical protein BH0485 - *Bacillus halodurans*
An01g10540	41.13	strong similarity to developmental regulatory protein brlA - *Aspergillus nidulans*
An14g05350[b]	39.45	strong similarity to hypothetical yellowish-green 1 ayg1 - *Aspergillus fumigatus*
An02g11240	39.15	hypothetical protein
An18g06360	38.95	similarity to mycelial surface antigen Csa1 - *Candida albicans*
An19g00230	37.81	similarity to monophenol monooxygenase melC2 - *Streptomyces antibioticus*

An02g00330	36.91	hypothetical protein
An06g02660	36.78	strong similarity to cytochrome P450 eln2 - *Coprinus cinereus*
An02g05790	36.54	strong similarity to hypothetical protein encoded by An09g03310 - *Aspergillus niger*
An01g03750	36.51	strong similarity to protein abaA - *Aspergillus nidulans*
An12g02750	36.50	similarity to FK520 biosynthetic gene cluster polyketide synthase fkbB - *Streptomyces hygroscopicus*
An09g05920[c]	35.71	strong similarity to chitinase precursor chit33 - *Trichoderma harzianum*
An09g05730[d]	34.57	strong similarity to polyketide synthase alb1 - *Aspergillus fumigatus*
An02g14040	34.50	hypothetical protein
An04g09520	34.32	strong similarity to O-methyltransferase omtB - *Aspergillus flavus*
An16g00600	33.89	similarity to saframycin Mx1 synthase safA - *Myxococcus xanthus*
An06g02680	33.85	similarity to hypothetical protein CAD70532.1 - *Neurospora crassa*
An14g03290	32.70	strong similarity to bile acid transporter Ybt1 - *Saccharomyces cerevisiae*
An12g02700	32.51	strong similarity to gluconate 5-dehydrogenase GNO - *Gluconobacter oxydans*
An12g02720	32.15	similarity to hypothetical protein C25G4.2 - *Caenorhabditis elegans*
An07g00020	31.76	strong similarity to hypothetical protein Z - *Streptomyces hygroscopicus*
An07g01540[e]	31.25	strong similarity to hypothetical protein YMR200w - *Saccharomyces cerevisiae*
An09g05530	29.94	hypothetical protein
An11g09750	29.79	weak similarity to neurofilament protein NF-220 - *Loligo pealei*
An07g03590	29.69	hypothetical protein
An08g03150	29.08	strong similarity to hypothetical purine-cytosine permease Fcy2 - *Saccharomyces cerevisiae*
An03g03750[f]	28.51	strong similarity to brown 2 protein abr2 - *Aspergillus fumigatus*
An02g03980[g]	28.10	strong similarity to beta-glucan synthesis associated protein Kre6 - *Saccharomyces cerevisiae*
An05g00130[h]	28.10	similarity to cell wall synthesis protein KRE9 - *Candida albicans*
An08g06740	27.76	weak similarity to hypothetical 1,4-beta-cellobiosidase XF1267 - *Xylella fastidiosa*
An02g08880	27.31	similarity to hypothetical protein PFB0705w - *Plasmodium falciparum*
An09g05040	27.15	similarity to hypothetical protein B1D4.110 - *Neurospora crassa*
An02g08690	26.46	strong similarity to galactose permease Gal2 - *Saccharomyces cerevisiae*
An15g05360	26.41	similarity to actVA-ORF4-like protein from patent WO9911793-A1 - *Homo sapiens*
An12g07070	26.38	strong similarity to polyketide synthase PKS1 - *Cochliobolus heterostrophus*
An01g08900	25.87	strong similarity to regulator protein wetA - *Aspergillus nidulans*
An03g02870	25.34	strong similarity to hypothetical transmembrane protein L2185.03 - *Leishmania major*
An15g03980	24.49	strong similarity to oxalate decarboxylase - *Collybia velutipes*
An04g08500	23.91	strong similarity to rodletless protein rodA - *Aspergillus nidulans*
An16g06170	23.65	hypothetical protein
An04g04700	23.33	strong similarity to catechol-O-methyltransferase COMT from patent WO9111513-A - *Homo sapiens*
An04g09540	22.81	similarity to fatty acid omega-hydroxylase cytochrome P-450 CYP4A4 - *Oryctolagus cuniculus*
An01g15140	22.78	strong similarity to agmatinase speB - *Escherichia coli*
An05g01410	21.59	similarity to acyl-CoA oxidase ACX2 - *Arabidopsis thaliana*
An07g01390	21.56	similarity to hypothetical conserved protein PA2839 - *Pseudomonas aeruginosa*
An08g03890	21.40	strong similarity to hypothetical sdowneroxid Cu/Zn dismutase B24P7.320 - *Neurospora crassa*

An06g02710	21.38	strong similarity to cyclohexanol dehydrogenase chnA - *Acinetobacter* sp. putative frameshift
An15g03500	19.68	weak similarity to hypothetical protein AAP68395.1 - *Oryza sativa*
An16g01540	19.52	strong similarity to hypothetical membrane protein YMR266w - *Saccharomyces cerevisiae*
An03g06020	19.03	strong similarity to 4-carboxymuconolactone decarboxylase pcaC - *Acinetobacter calcoaceticus*
An01g15120	18.74	hypothetical protein
An12g07060	18.71	similarity to hypothetical protein YMR222c - *Saccharomyces cerevisiae*
An09g05340	18.68	strong similarity to polyketide synthase FUM5 - *Gibberella moniliformis*
An04g06880	18.62	similarity to hypothetical myoinositol-dehydrogenase spcB - *Streptomyces spectabilis*
An13g03640	18.46	strong similarity to polyamine transport protein Tpo1 - *Saccharomyces cerevisiae*
An08g11100	18.36	hypothetical protein
An16g06390	18.11	strong similarity to hypothetical protein SPAC2C4.17c - *Schizosaccharomyces pombe*
An04g09530	17.90	strong similarity to melanin polyketide synthase PKS - *Nodulisporium* sp.
An07g10410	17.88	strong similarity to metalloprotease Mep1 - *Metarhizium anisopliae*
An18g00470	17.47	strong similarity to sequence 11 from patent WO0073470 - *Zea mays*
An10g00800	17.42	strong similarity to purine nucleoside permease Ndown - *Candida albicans*
An12g02670	16.76	strong similarity to polyketide synthase FUM5 - *Gibberella fujikuroi*
An01g06650	16.68	strong similarity to hypothetical protein PA4204 - *Pseudomonas aeruginosa*
An09g06390	16.31	strong similarity to lipase LipP - *Pseudomonas* sp.
An18g00520	16.30	strong similarity to polyketide synthase PKS1 - *Cochliobolus heterostrophus*
An01g14990	16.25	strong similarity to cytochrome P450 monooxygenase TRI11 - *Fusarium sporotrichioides*
An07g01430	15.88	strong similarity to hypothetical protein Rv1215c - *Mycobacterium tuberculosis*
An09g02510	15.75	strong similarity to hypothetical membrane protein YLR251w - *Saccharomyces cerevisiae*

Table 4: The top 100 of genes down-regulated in the aerial structures when compared to the vegetative mycelium. The fold change was also calculated when a gene had an absent call. Also known as: [a]*aamA*, [b]*mstF*.

Gene ID	Fold change	Annotation
An01g11660	106.79	1,4-beta-D-glucan cellobiohydrolase B precursor cbhB - *Aspergillus niger*
An07g08640	77.63	strong similarity to mutanase mutA - *Penicillium purporogenum*
An11g04810	67.31	alternative oxidase aox1 - *Aspergillus niger*
An16g07080	58.02	hypothetical protein
An09g00840	55.46	similarity to plastic-degradation enzyme within SEQ ID NO:6 from patent WO2004038016-A1 – *Aspergillus oryzae*
An18g04090	49.65	strong similarity to hypothetical protein encoded by An01g14640 - *Aspergillus niger*
An16g01880	37.01	strong similarity to lysophospholipase - *Aspergillus foetidus*
An15g04990	34.70	similarity to EST an_2920 - *Aspergillus niger*
An03g06560	34.60	strong similarity to triacylglycerol lipase Lip2 - *Candida rugosa*
An11g03340[a]	28.31	acid alpha-amylase - *Aspergillus niger*
An01g00530	25.47	proteinase aspergillopepsin II - *Aspergillus niger*
An04g09490	24.69	hypothetical protein
An12g09270	24.67	strong similarity to lactose permease Lac12 - *Kluyveromyces lactis*
An07g05460	24.34	strong similarity to hypothetical protein CAE47856.1 - *Aspergillus fumigatus*
An03g06410	23.74	strong similarity to methyl sterol oxidase Erg25 - *Saccharomyces cerevisiae*
An14g02610	23.03	strong similarity to ATP-binding cassette multidrug transport protein atrB - *Aspergillus nidulans*
An01g00850	22.30	similarity to xylose permease xylT - *Bacillus megaterium*
An12g00660	22.02	similarity to indoleamine 2,3-dioxygenase IDO - *Mus musculus*
An08g10780	21.59	strong similarity to hypothetical protein T16K5.230 - *Arabidopsis thaliana*
An12g01870	17.71	similarity to positive regulator of the lactose-galactose regulon LAC9 - *Kluyveromyces lactis*
An03g04190	16.88	similarity to cellulase #2 from patent US2003036176-A1 - *Xanthomonas campestris*
An16g07040	16.47	similarity to beta-1,3-glucanosyltransferase bgt1 - *Aspergillus fumigatus* truncated ORF
An08g04600	16.02	strong similarity to peptide transporter Ptr2 - *Saccharomyces cerevisiae*
An04g06070	15.72	strong similarity to EST an_1548 - *Aspergillus niger*
An14g02540	15.46	weak similarity to sterol regulatory element-binding protein-1 SREBP-1 - *Mus musculus*
An12g06130	13.93	similarity to glutamyl endoprotease polypeptide from patent WO200268623-A2 - *Aspergillus niger*
An01g07980	13.56	strong similarity to EST SEQ ID NO:4248 from patent WO200056762-A2 - *Aspergillus niger*
An15g05000	13.55	hypothetical protein
An01g12380	13.54	strong similarity to ATP-binding cassette transporter PMR1 - *Penicillium digitatum*
An14g02460	13.47	strong similarity to flavohemoglobin Fhp - *Alcaligenes eutrophus*
An01g00650	13.28	hypothetical protein
An11g03680	13.24	strong similarity to cinnamyl alcohol dehydrogenase CAD - *Eucalyptus gunnii*
An01g07980	12.80	strong similarity to EST SEQ ID NO:4248 from patent WO200056762-A2 - *Aspergillus niger*
An18g03430	12.22	similarity to EST n1a10a1.r1 - *Aspergillus nidulans*
An15g07700	12.12	strong similarity to aspergillopepsin II precursor (acid proteinase A) - *Aspergillus niger*

An15g02040	11.99	strong similarity to EST an_3318 - *Aspergillus niger*
An16g02930	11.93	strong similarity to C-5 sterol desaturase Erg3 - *Saccharomyces cerevisiae*
An03g01050	11.81	similarity to endo-beta-1,4-glucanase - *Bacillus polymyxa*
An12g01210	11.72	strong similarity to peptide transport protein Ptr2 - *Saccharomyces cerevisiae*
An12g10630	11.66	similarity to acid phosphatase aphA - *Aspergillus ficuum*
An03g01010	11.20	strong similarity to lysosomal pepstatin insensitive protease CLN2 - *Homo sapiens*
An03g00640	11.13	similarity to neutral amino acid permease mtr - *Neurospora crassa* truncated ORF
An12g05010	10.74	acetyl xylan esterase axeA - *Aspergillus niger*
An12g08930	10.74	strong similarity to epoxide hydrolase hyl1 - *Aspergillus niger*
An15g01580	10.72	weak similarity to cell envelope protein 05ae20220orf32 from patent WO9640893-A1 - *Helicobacter pylori*
An14g02660	10.49	strong similarity to hypothetical necrosis and ethylene inducing protein BH0395 - *Bacillus halodurans*
An16g05970	10.42	similarity to UDP-glucuronosyltransferase UGT2B11 - *Homo sapiens*
An01g11670	10.30	strong similarity to endo-beta-1,4-glucanase A eglA - *Aspergillus nidulans*
An03g04400	9.85	weak similarity to hypothetical protein encoded by An16g02860 - *Aspergillus niger*
An14g01370	9.77	hypothetical protein
An02g00590[b]	9.61	strong similarity to high-affinity glucose transporter HGT1 - *Kluyveromyces lactis*
An11g02190	9.37	strong similarity to hypothetical protein CAD70480.1 - *Neurospora crassa*
An14g04040	9.28	strong similarity to hypothetical protein SPAC1093.01 with conserved domain PF01535 duf17p - *Schizosaccharomyces*
An12g08630	9.18	strong similarity to cytochrome P450 monooxygenase TRI11 - *Fusarium sporotrichioides*
An15g06150	9.11	strong similarity to hypothetical protein EAA64210.1 - *Aspergillus nidulans*
An07g08950	9.01	endoglucanase B eglB - *Aspergillus niger*
An12g07450	9.00	strong similarity to glucose permease Rgt2 - *Saccharomyces cerevisiae*
An09g00670	8.82	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanosyltransferase gel3 - *Aspergillus fumigatus*
An06g00990	8.72	strong similarity to soluble cytoplasmic fumarate reductase YEL047c - *Saccharomyces cerevisiae*
An14g04910	8.60	weak similarity to GABA-A receptor epsilon-like subunit epsilon - *Rattus norvegicus*
An11g02230	8.60	strong similarity to lanosterol 1,4 alpha-demethylase (P450(14DM)) CYP51 - *Penicillium italicum*
An11g02160	8.57	strong similarity to maleylacetoacetate isomerase maiA - *Aspergillus nidulans*
An03g06550	8.42	glucan 1,4-alpha-glucosidase glaA - *Aspergillus niger*
An14g04040	8.37	strong similarity to hypothetical protein SPAC1093.01 with conserved domain PF01535 duf17p - *Schizosaccharomyces*
An13g00840	8.31	strong similarity to general amino acid permease Gap1 - *Saccharomyces cerevisiae*
An03g00690	8.28	hypothetical protein
An11g02200	8.27	strong similarity to 4-hydroxyphenylpyruvate dioxygenase tcrP - *Coccidioides immitis*
An06g00190	8.24	strong similarity to lysosomal pepstatin insensitive protease CLN2 - *Homo sapiens*
An14g02580	8.22	hypothetical protein
An09g02160	8.14	rhamnogalacturonan acetyl esterase rgaeA - *Aspergillus niger*
An02g02760	8.12	weak similarity to skeleton binding protein 1 Pfsbp1 - *Plasmodium falciparum*

An02g13400	7.94	weak similarity to antigen abe from patent WO9850531-A1 - *Salmonella enterica*
An16g01320	7.81	similarity to integral membrane protein PTH11 - *Magnaporthe grisea*
An04g09810	7.80	strong similarity to choline monooxygenase CMO from patent WO9830702-A2 - *Beta vulgaris*
An18g06650	7.66	strong similarity to heat shock protein hsp30 - *Aspergillus nidulans*
An12g07920	7.57	hypothetical protein
An06g02270	7.55	similarity to arabinose transport protein araE - *Escherichia coli*
An12g08280	7.54	exo-inulinase inu1 - *Aspergillus niger*
An04g04540	7.52	similarity to hypothetical protein YLR352w - *Saccharomyces cerevisiae*
An03g06550	7.31	glucan 1,4-alpha-glucosidase glaA - *Aspergillus niger*
An02g07610	7.27	strong similarity to mannitol transporter mat1 - *Apium graveolens*
An14g01820	7.20	strong similarity to hypothetical cell wall protein binB - *Aspergillus nidulans*
An01g05810	7.09	strong similarity to hypothetical protein CAE85516.1 - *Neurospora crassa*
An01g11320	7.09	similarity to dopa decarboxylase 3 DDC3 from patent WO9960136-A1 - *Aspergillus oryzae*
An12g10830	6.97	similarity to hypothetical protein EAA74834.1 - *Gibberella zeae*
An18g05700	6.94	strong similarity to hypothetical protein CAD70818.1 - *Neurospora crassa*
An01g00640	6.93	strong similarity to hypothetical protein CAD21096.1 - *Neurospora crassa*
An03g00130	6.91	strong similarity to FAD-dependent L-sorbose dehydrogenase SDH - *Gluconobacter oxydans*
An02g08520	6.90	strong similarity to allantoicase alc - *Neurospora crassa*
An11g09000	6.78	strong similarity to glutathione transferase GST - *Escherichia coli*
An01g14000	6.58	strong similarity to cyclohexanol dehydrogenase chnA - *Acinetobacter sp.*
An05g01730	6.56	hypothetical protein
An04g07200	6.44	strong similarity to kynureninase - *Rattus norvegicus*
An01g02280	6.43	hypothetical protein
An07g09330	6.41	1,4-beta-D-glucan cellobiohydrolase A precursor cbhA - *Aspergillus niger*
An08g04490	6.40	endoprotease Endo-Pro - *Aspergillus niger*
An08g10000	6.39	strong similarity to cytochrome b245 beta chain CYBB - *Homo sapiens* putative frameshift
An08g09610	6.37	similarity to mutanase mutA - *Penicillium purpurogenum*
An08g08840	6.25	strong similarity to glutamate decarboxylase GAD1 - *Arabidopsis thaliana*
An12g08940	6.22	strong similarity to biotin biosynthesis gene bioS1 from patent DE19806872-A1 - *Escherichia coli*

Expression analysis of functional gene classes

The Functional Catalogue (FunCat) consists of 28 main functional categories of genes (Ruepp et al., 2004). These classes are subdivided by a tree like structure with up to six levels of increasing specificity. The Fisher's exact test showed that 8 main functional FunCat categories are over-represented in the gene set that showed a fold change ≥ 2 ($p \leq 0.01$; Table 5). The categories energy, cell cycle and DNA processing, protein synthesis, protein fate, cellular transport and transport mechanisms, and subcellular localization were over-represented in the genes that were up-regulated in the aerial structures (Table 5). The categories metabolism, protein fate, and cell fate were over-represented in the genes that were down-regulated in the aerial structures (Table 5). Within the functional category energy, the sub-category aerobic respiration was overrepresented in the up-regulated genes. Within the FunCat category metabolism the sub-categories amino acid biosynthesis, transport and degradation,

purine nucleotide metabolism, C-compound and carbohydrate utilization, and breakdown of lipids, fatty acids and isoprenoids were overrepresented in the down-regulated genes. In contrast, lipid, fatty acid and isoprenoid biosynthesis, and biosynthesis of acetic acid derivatives were overrepresented in the up-regulated gene pool (Table 5).

The top 100 of up-regulated genes (Table 3) contains 34 and 51 genes belonging to the functional gene categories metabolism and unclassified proteins, respectively (Supplementary Table 2). In the case of the top 100 of down-regulated genes (Table 4) these numbers were 45 and 38, respectively (Supplementary Table 3).

Table 5: Over-representation of functional FunCat classes (Ruepp et al., 2004) in the set of genes that have a fold change ≥ 2 when expression of aerial structures and the vegetative mycelium is compared ($p \leq 0.01$). The analysis was performed using the FunCat main-categories (bold) and the FunCat 3 sub-categories.

Functional classes over-represented in up-regulated genes in aerial structures.
01.06.01 lipid, fatty-acid and isoprenoid biosynthesis
01.20.05 biosynthesis of acetic acid derivatives
02 ENERGY
02.13.03 aerobic respiration
03 CELL CYCLE AND DNA PROCESSING
03.01.03 DNA synthesis and replication
03.01.05 DNA recombination and DNA repair
03.01.09 DNA restriction or modification
03.03.01 mitotic cell cycle and cell cycle control
05 PROTEIN SYNTHESIS
06 PROTEIN FATE (folding, modification, destination)
06.07 protein modification
06.10 assembly of protein complexes
06.13.01 cytoplasmic and nuclear degradation
08 CELLULAR TRANSPORT AND TRANSPORT MECHANISMS
14.04 cell differentiation
30.10.03 organization of chromosome structure
40 SUBCELLULAR LOCALISATION

Functional classes over-represented in down-regulated genes in aerial structures.
01 METABOLISM
01.01.01 amino acid biosynthesis
01.01.07 amino acid transport
01.01.10 amino acid degradation (catabolism)
01.03.01 purine nucleotide metabolism
01.05.01 C-compound and carbohydrate utilization
01.06.04 breakdown of lipids, fatty acids and isoprenoids
06 PROTEIN FATE (folding, modification, destination)
06.07.05 modification by ubiquitination, deubiquitination
06.13.99 other proteolytic degradation
11.07.99 other detoxification
14 CELL FATE

Expression analysis of selected gene groups

Genes related to regulation and formation of conidiophores

The *A. niger* genome contains 39 genes that have been classified as involved in a-sexual reproduction when hydrophobin and pigmentation genes are excluded (Table 6; Pel et al., 2007). 33 of these genes had a present call in the vegetative mycelium and/or in the aerial structures. Genes *gprD*, *rgsA*, *rasB*, *vosA*, *devR* and *apsB* had an absent call in both the vegetative mycelium and the aerial structures. On the other hand, *flbD*, *flbE*, *ppoD*, *brlA* and *abaA* were exclusively expressed in the aerial structures. In contrast, *phiA* and *csnD* were only expressed in the vegetative mycelium (Table 6, Supplementary Table 4). The homologs of the central transcriptional activators of conidiophore development in *A. nidulans brlA, abaA* and *wetA* (Adams et al., 1998) are among the top 100 of up-regulated genes in the aerial structures (Table 3). Other genes involved in conidiophore development were not changed in expression except for the cell wall protein encoding gene *phiA*, and the Psi factor gene *ppoC*, that showed a down-regulation ≥ 4 and an up-regulation ≥ 8-fold, respectively (Table 6, Supplementary Table 4). Of the genes predicted to be involved in a-sexual signal transduction (Pel et al., 2007) the expression of the red light phytochrome *fphA* and the developmental regulator *flbE* was more than 2-fold up-regulated in the aerial structures.

Hydrophobins

Hydrophobins fulfil a wide spectrum of functions in the life cycle of filamentous fungi. For instance, they enable hyphae to escape the aqueous environment to grow into the air by reducing the water surface tension (Wösten et al., 1999) and they coat aerial structures with a hydrophobic lining (Wösten, 2001). Moreover, hydrophobins can affect the architecture of the cell wall (van Wetter et al., 2000). The genome of *A. niger* strain CBS513.88 contains 8 putative hydrophobin genes (Pel et al., 2007; Jensen et al., 2010). Seven hydrophobin genes had a present call in both the vegetative mycelium and the aerial structures, whereas the hydrophobin gene An09g05530 was only expressed in the aerial structures (Table 7, Supplementary Table 4). Gene An15g03800 was more than 2-fold up-regulated in the aerial structures (Table 7, Supplementary Table 4), whereas four other genes were even ≥ 8-fold up-regulated. One of them is the homolog of *rodA* of *A. nidulans* (Stringer et al., 1991). Another is homologous to *hyp1* of *A. fumigatus* (Parta et al., 1994). RodA and Hyp1 form rodlets at the surface of conidiospores. RodA has also been shown to coat metulae and phialides. The *A. niger* homologues of *rodA* and *hyp1* are among the top 100 of up-regulated genes (Table 3). One of the hydrophobin genes (An01g10940) was 111.2 fold up-regulated in the aerial structures (Table 3). This makes this gene the highest differentially expressed gene in this study.

Table 6: Expression of genes involved in a-sexual reproduction (Pel et al., 2007; excluding hydrophobin and pigmentation genes) in the aerial structures of 7-day-old maltose-grown colonies of *A. niger* when compared to the vegetative mycelium. The fold change was also calculated when a gene had an absent call. Dark shading indicates genes that had an absent call in both the vegetative mycelium and the aerial structures. Light shading indicates genes that had a present call in either the vegetative mycelium (m) or the aerial structures (a). *Gz = Gibberella zea*.

Gene ID	Gene name	Annotation	Fold change
Signal Transduction			
An14g03390	*fluG*	Similarity to glutamine synthetase; signalling molecule	<2
An02g09780	*sfgA*	C6 transcription factor	<2
An02g01560	*gprD*	Receptor prevents improper sexual development	<2
An08g06130	*fadA (GPA1)*	Alpha-subunit G protein	<2
An02g08000	*ganA*	guanine nucleotide-binding protein subunit alpha	<2
An08g05820	*ganB*	Alpha-subunit G protein	<2
An18g02090	*sfaD (STE 4)*	Beta-subunit G protein	<2
An07g06040	*phnA*	phosducin-like protein	<2
An18g06110	*rgsA*	Regulator of G-protein signaling	<2
An02g03160	*flbA*	Regulator of G-protein signaling	<2
An15g03710	*flbB*	bZIP transcription factor FlbB	<2
An02g05420	*flbC*	Putative regulator containing two zinc-fingers	<2
An01g04830	*flbD*	strong similarity to myb-like DNA binding protein	<2 a
An08g05850	*sakA*	MAP kinase represses sexual development	<2
An17g01280	*steC (STE11)*	Serine/threonine protein kinase MKKK	<2
An02g04270	*pkaA*	cAMP-dependent protein kinase type 2	<2
An11g01520	*cyaA*	adenylate cyclase	<2
An16g03740	*pkaR*	protein kinase A regulatory subunit	<2
An01g02320	*rasA*	RAS protein *A. fumigatus*	<2
An05g00370	*rasB*	RAS protein *A. fumigatus*	<2
An08g07210	*flbE*	developmental regulator FlbE	>2 up a
An14g02970	*fphA*	Red light phytochrome	>2 up
Conidiophore Development			
An05g00480	*stuA /phd1*	APSES-transcription factor (spatial expression of abaA)	<2
An02g02150	*medA*	Transcription factor	<2
An04g05790	*vosA*	hypothetical protein	<2
An11g04750	*dopA*	Leucine zipper-like domain regulator (initiation conidiogenesis)	<2
An15g03490	*devR*	bHLH transcription factor (phialide development)	<2
An02g08420	*hymA /hym1*	Cell cycle regulation and polarity	<2
An15g02740	*apsA*	Microtuble-cortex interaction (nuclear migration into metulae)	<2
An06g01620	*apsB*	Spindle pole associated protein (nuclear migration into metulae)	<2
An16g07210	*csnD*	Signalosome subunit 4 (regulation of conidiophore number)	<2 m
An15g06660	*csnE*	Signalosome subunit 5 (regulation of conidiophore number)	<2
An04g05880	*ppoA*	Fatty acid oxygenase for Psi factor production	<2
An12g01320	*Gz ppoD*	Fatty acid oxygenase	<2 a
An01g10540	*brlA*	Zn (II) finger transcription factor (transition stalk to vesicle)	>8 up a
An01g03750	*abaA /tec1*	ATTS/TEA DNA binding domain transcription regulator	>8 up a
An01g08900	*wetA*	Probable regulator of spore maturation	>8 up
An02g07930	*ppoC*	Fatty acid oxygenase for Psi factor production	>8 up
An14g01820	*phiA*	Strong similarity to hypothetical cell wall protein binB	>4 down m

Table 7: Expression of predicted hydrophobin genes (Pel et al., 2007; Jensen et al., 2010) in the aerial structures of 7-day-old maltose-grown colonies of *A. niger* when compared to the vegetative mycelium. The fold change was also calculated when a gene had an absent call. Light shading indicates a gene that had a present call only in the aerial structures.

Gene ID	Gene name	Annotation	Fold change
An03g02400	*dewA*	strong similarity to spore-wall fungal hydrophobin dewA - *Aspergillus nidulans*	<2
An03g02360		weak similarity to spore-wall fungal hydrophobin dewA - *Aspergillus nidulans*	<2
An08g09880		weak similarity to hydrophobin CoH1 - *Coprinus cinereus*	<2
An15g03800		strong similarity to EST an_3228 - *Aspergillus niger*	>2 up
An04g08500	*rodA*	strong similarity to rodletless protein rodA - *Aspergillus nidulans*	>8 up
An09g05530		hypothetical protein	>8 up
An07g03340	*hyp1*	strong similarity to hydrophobin hYP1 - *Aspergillus fumigatus*	>8 up
An01g10940		hypothetical protein	>8 up

Genes involved in pigmentation

Four genes have been described that are involved in the black pigmentation of conidiospores of *A. niger* (Jørgensen et al., 2011). Inactivation of *fwnA* resulted in fawn-coloured spores, while inactivation of *olvA, brnA* and *pptA* resulted in olive, brown-coloured, and white spores, respectively. Pel et al. (2007) classified 13 other genes as genes involved in pigmentation. These genes are homologous to pigmentation genes of *A. fumigatus.* This *Aspergillus* species is known to have 6 genes involved in formation of its bluish-green pigment (Tsai et al., 1999). From the 17 pigmentation genes (Table 8), 12 had an absent call in both the vegetative mycelium and the aerial structures. Gene *pptA* was among these genes (Table 8, Supplementary Table 4). Only *fwnA, olvA, brnA,* an *abr2* homolog and an *arp1* homolog had a present call in the vegetative mycelium and/or the aerial structures. The former four genes were more than 8-fold up-regulated in the aerial structures and were all shown to be among the top 100 of up-regulated genes (Table 3 and Table 8). The scytalone dehydratase homologue *arp1* of *A. fumigatus* was ≥ 2-fold down-regulated in aerial structures (Table 8, Supplementary Table 4).

Genes encoding cell wall modifying proteins

The *A. niger* genome contains 9 chitin synthases (*csmA-B*, c*hsA-G*), 13 chitinases (*ctcA-B*, *cfcA-K*), 5 genes involved in α-1,3-glucan synthesis (*agsA-E*), 1 gene involved in β-1,3-glucan synthesis (*knrA*) and three genes involved in β-1,6-glucan synthesis and processing (*kslA*, *knlA*, *rotA*) (Pel et al., 2007). Twelve of these 31 genes had an absent call in both the vegetative mycelium and the aerial structures (Table 9, Supplementary Table 4). Among them were 8 out of the 13 chitinases. Nineteen out of the 31 genes had a present call in the vegetative mycelium and/or the aerial structures (Table 9, Supplementary Table 4). Genes *kslA*, *knlA*, *rotA*, and *agsD* were exclusively expressed in aerial structures, while *ctcA*, *agsA* and *agsE* were only expressed in the vegetative mycelium (Table 9, Supplementary Table 4). Eleven of the 19 expressed genes had a fold change ≥ 2 when expression in the aerial structures was compared to the vegetative mycelium. Gene *chsA* (≥ 2 fold), *knrA* (≥ 4 fold) and *chsC*, *ctcB*, *knlA*,

Table 8: Expression of predicted pigmentation genes of *A. niger* (Pel et al., 2007; Jørgensen et al., 2011) in the aerial structures of 7-day-old maltose-grown colonies of *A. niger* when compared to the vegetative mycelium. The fold change was also calculated when a gene had an absent call. Dark shading indicates genes that had an absent call in both the vegetative mycelium and the aerial structures. *Afum* = *Aspergillus fumigatus*.

Gene ID	Gene or homolog	Annotation	Fold change
An12g03950	*pptA*	4'phosphopantetheinyl transferase	<2
An12g02050	*wA*	Polyketide synthase	<2
An07g01830	*Afum arp2*	HNreductase	<2
An16g01650	*Afum arp2*	HNreductase	<2
An02g00220	*Afum arp2*	HNreductase	<2
An06g01980	*Afum arp2*	HNreductase	<2
An16g06450	*Afum arp2*	HNreductase	<2
An01g14010	*Afum abr2/yA*	Laccase	<2
An05g02540	*Afum abr2*	Laccase	<2
An04g10400	*Afum abr2*	Laccase	<2
An01g13660	*Afum abr2*	Laccase	<2
An14g05270	*brnA-like*	Multicopper oxidase	<2
An09g05730	*fwnA*	Polyketide synthase	>8 up
An14g05350	*olvA/ayg1*	YWA1 hydrolase	>8 up
An03g03750	*Afum abr2*	Laccase	>8 up
An14g05370	*brnA*	Sc cell surface ferroxidase precursor *Fet3*	>8 up
An08g09920	*Afum arp1*	Scytalone dehydratase	>2 down

kslA and *rotA* (≥ 8-fold) were up-regulated in the aerial structures (Table 9, Supplementary Table 4). Of these genes *ctcB*, *knlA*, *kslA* and *rotA* were among the top 100 of genes that are up-regulated in aerial structures (Table 3). *chsD*, *csmB* and *cfcA* were ≥ 2 fold and *ctcA* was ≥ 4-fold down-regulated in the aerial structures.

Genes related to the secretion of proteins

Levin et al. (2007a) analysed the expression of 12 genes that are involved in the secretion pathway in concentric zones of the *A. niger* colony. In our analysis, all genes had a present call in the vegetative mycelium and/or the aerial structures, except *ssp120* (Table 10, Supplementary Table 4). *Rab6* is only expressed in the aerial structures, while all other genes are expressed in both sample types. Four genes, including *bipA* and *rab6*, are not changed in expression when comparing the vegetative mycelium with the aerial structures. On the other hand, seven genes are up-regulated in the aerial structures. These genes include the disulfide isomerase genes *tigA* (≥ 2-fold) and *pdiA* (≥ 4-fold); the ER membrane translocator genes *sec61a* and *sec61b* (≥ 2-fold); and *hacA* which is involved in the unfolded protein response (≥ 2-fold) (Table 10, Supplementary Table 4).

Table 9: Expression of selected genes involved in cell wall modification (Pel et al., 2007) in the aerial structures of 7-day-old maltose-grown colonies of *A. niger* when compared to the vegetative mycelium. The fold change was also calculated when a gene had an absent call. Dark shading indicates genes that had an absent call in both the vegetative mycelium and the aerial structures. Light shading indicates genes that had a present call in either the vegetative mycelium (m) or the aerial structures (a).

Gene ID	Gene name	Annotation	Fold change
Chitin synthases			
An09g04010	*chsB*	Putative chitin synthase ClassIII; EnChsB-like	<2
An12g10380	*chsE*	Putative chitin synthase ClassIII; EnChsB-like	<2
An03g06360	*chsF*	Putative chitin synthase ClassIII; EnChsB-like	<2
An08g05290	*chsG*	Putative chitin synthase ClassVI;	<2
An02g02360	*csmA*	Putative chitin synthase with a myosin motor domain ClassV; EnCsmA-like	<2
An07g05570	*chsA*	Putative chitin synthase ClassII; EnChsA-like	>2 up
An14g00660	*chsC*	Putative chitin synthase ClassI; EnChsC-like	>8 up
An09g02290	*chsD*	Putative chitin synthase ClassIV; EnChsD-like	>2 down
An02g02340	*csmB*	Putative chitin synthase with a myosin motor domain ClassV; EnCsmA-like	>2 down
Chitinases			
An08g09030	*cfcB*	Putative ClassV Chitinase (GH18); ScCts2-like	<2
An04g04670	*cfcC*	Putative ClassV Chitinase (GH18); ScCts2-like	<2
An01g05360	*cfcD*	Putative ClassV Chitinase (GH18); ScCts2-like	<2
An15g00840	*cfcE*	Putative ClassV Chitinase (GH18); ScCts2-like	<2
An11g01160	*cfcF*	Putative ClassV Chitinase (GH18); ScCts2-like	<2
An19g00100	*cfcG*	Putative ClassV Chitinase (GH18); ScCts2-like	<2
An14g07420	*cfcH*	Putative ClassV Chitinase (GH18); ScCts2-like	<2
An02g13580	*cfcI*	Putative ClassV Chitinase (GH18); ScCts2-like	<2
An11g05860	*cfcJ*	Putative ClassV Chitinase (GH18); ScCts2-like	<2
An12g05330	*cfcK*	Putative ClassV Chitinase (GH18); ScCts2-like	<2
An09g05920	*ctcB*	Putative ClassIII Chitinase (GH18); ScCts1-like	>8 up
An09g06400	*ctcA*	Predicted GPI-anchored protein. Putative ClassIII Chitinase (GH18); ScCts1-like	>4 down m
An02g07020	*cfcA*	Putative ClassV Chitinase (GH18); ScCts2-like	>2 down
Ags-family			
An04g09890	*agsA*	Putative catalytic subunit alpha1,3-glucan synthase complex; SpAgs1-like	<2 m
An15g07810	*agsB*	Putative catalytic subunit alpha1,3-glucan synthase complex; SpAgs1-like	<2
An12g02450	*agsC*	Putative catalytic subunit alpha1,3-glucan synthase complex; SpAgs1-like	<2
An02g03260	*agsD*	Putative catalytic subunit alpha1,3-glucan synthase complex; SpAgs1-like	<2 a
An09g03070	*agsE*	Putative catalytic subunit alpha1,3-glucan synthase complex; SpAgs1-like	<2 m
Beta-1,3-glucan synthesis and processing			
An17g02120	*knrA*	strong similarity to 1,3-beta-glucan synthase gs-1 - *Neurospora crassa*	>4 up
Beta-1,6-glucan synthesis and processing			
An05g00130	*knlA*	Pytative glycoprotein required for beta-1,6 glucan biosynthesis; ScKre9-like	>8 up a
An02g03980	*kslA*	Putative transglycosidase required for beta-1,6 glucan biosynthesis; ScKre6-like	>8 up a
An07g01540	*rotA*	Putative protein involved in beta-1,6 glucan biosynthesis; ScRot1-like	>8 up a

Table 10: Expression of selected genes involved in secretion (Pel et al., 2007; Levin et al., 2007a) in the aerial structures of 7-day-old maltose-grown colonies of *A. niger* when compared to the vegetative mycelium. The fold change was also calculated when a gene had an absent call. Dark shading indicates a gene that had an absent call in both the vegetative mycelium and the aerial structures. Light shading indicates a gene that had a present call only in the aerial structures.

Gene ID	Gene name	Annotation	Fold change
An11g04180	*bipA*	dnaK-type molecular chaperone bipA - *Aspergillus niger*	<2
An15g05740	*rab6*	strong similarity to GTP-binding protein Rab6 - *Homo sapiens*	<2
An16g04330	*dpm1*	strong similarity to mannose phospho-dolichol synthase dpm1 - *Hypocrea jecorina*	<2
An11g09620	*rho3*	strong similarity to GTPase Rho3 - *Saccharomyces cerevisiae*	<2
An18g03100	*ssp120*	strong similarity to hypothetical precursor of secretory protein Ssp120 - *Saccharomyces cerevisiae*	<2
An18g02020	*tigA*	disulfide isomerase tigA - *Aspergillus niger*	>2 up
An01g08420	*cnx1*	strong similarity to calcium-binding protein precursor cnx1p - *Schizosaccharomyces pombe*	>2 up
An04g02020	*cypB*	strong similarity to cyclophilin cypB - *Aspergillus nidulans*	>2 up
An01g11630	*sec61a*	strong similarity to translocation complex component Sss1 - *Saccharomyces cerevisiae*	>2 up
An03g04340	*sec61b*	strong similarity to ER membrane translocation facilitator Sec61 - *Yarrowia lipolytica*	>2 up
An01g00160	*hacA*	similarity to regulator of unfolded protein response (UPR) Hac1 - *Saccharomyces cerevisiae*	>2 up
An02g14800	*pdiA*	protein disulfide isomerase A pdiA - *Aspergillus niger*	>4 up

Amylolytic genes and their regulator AmyR

The transcriptional regulator AmyR induces genes involved in starch and maltose degradation (Fowler et al., 1990; Tani et al., 2001) as well as genes encoding D-glucose and D-galactose releasing enzymes (van Kuyk et al., 2011). The genome of *A. niger* contains 16 genes that are regulated by AmyR (Table 11; van Kuyk et al., 2011), of which one (*i.e.* a β-glucosidase gene) is repressed by this transcriptional regulator. In this study, 10 genes, including *amyR*, had a present call in both the vegetative mycelium and the aerial structures. Six genes had an absent call in both the vegetative mycelium and the aerial structures, including all β-glucosidases, an amylase, a predicted galactosidase and a glucoamylase (Table 11, Supplementary Table 4). Gene *aglA* was only expressed in the vegetative mycelium. From the six genes that were differentially expressed, all were down-regulated in the aerial structures. Gene *aglA* was ≥ 2, *aglU* and *amyR* ≥ 4, and *aamA*, *glaA* and *mstF* ≥ 8-fold down-regulated (Table 11, Supplementary Table 4). Genes *glaA*, *aamA* and sugar transporter *mstF* were among the top 100 of most down-regulated genes (Table 4).

Table 11: Expression of AmyR regulated genes (van Kuyk et al., 2011) in the aerial structures of 7-day-old maltose-grown colonies of *A. niger* when compared to the vegetative mycelium. The fold change was also calculated when a gene had an absent call. Dark shading indicates genes that had an absent call in both the vegetative mycelium and the aerial structures. Light shading indicates a gene that had a present call only in the vegetative mycelium. BGL = β-glucosidase, AMY = α-amylase, AGD = α-glucosidase, GLA = glucoamylase, AGL = α-galactosidase, LAC = β-galactosidase. GH = glycoside hydrolase (www.cazy.org). *repressed by AmyR.

Gene ID	Gene name	Annotation	Family	Function	Fold change
An01g10930		strong similarity to enzyme with sugar transferase activity from patent JP11009276-A - *Acremonium* sp.	GH31	AGD	<2
An04g06920	*agdA/ aglU*	extracellular alpha-glucosidase aglU - *Aspergillus niger*	GH31	AGD	>4 down
An04g02700		similarity to wheat raffinose synthase from patent WO200024915-A2 - *Triticum aestivum*	GH36	AGL	<2
An06g00170	*aglA*	alpha-galactosidase aglA - *Aspergillus niger*	GH27	AGL	>2 down
An09g00260	*aglC*	alpha-galactosidase aglC - *Aspergillus niger* [truncated ORF]	GH35	AGL	<2
An09g00270	*aglC*	alpha-galactosidase aglC - *Aspergillus niger* [truncated ORF]	GH36	AGL	<2
An04g06930	*amyA*	strong similarity to extracellular alpha-amylase amyA/amyB - *Aspergillus niger*	GH13	AMY	<2
An05g02100		extracellular alpha-amylase amyA/amyB - *Aspergillus niger*	GH13	AMY	<2
An11g03340	*aamA*	acid alpha-amylase - *Aspergillus niger*	GH13	AMY	>8 down
An06g02040		strong similarity to beta-glucosidase cbg1 - *Agrobacterium tumefaciens*	GH3	BGL	<2
An11g06090*		strong similarity to beta-glucosidase2 BGL2 - *Saccharomycopsis fibuligera*	GH3	BGL	<2
An14g01770		strong similarity to beta-glucosidase bgln - *Candida molischiana*	GH3	BGL	<2
An03g06550	*glaA*	glucan 1,4-alpha-glucosidase glaA - *Aspergillus niger*	GH15	GLA	>8 down
An12g03070		strong similarity to hypothetical protein SPAC4H3.03c - *Schizosaccharomyces pombe*	GH15	GLA	<2
An06g00290		strong similarity to beta-galactosidase lacA - *Aspergillus niger*	GH35	LAC	<2
An02g00590	*mstF*	strong similarity to high-affinity glucose transporter HGT1 – *K. lactis*	MFS	sugar trans-port	>8 down
An04g06910	*amyR*	transcription regulator of maltose utilization amyR - *Aspergillus niger*	Zn2Cys regulator		>4 down

Secreted proteases

The genome of *A. niger* contains 198 genes involved in proteolytic degradation of which 31 encode a secreted protease (Pel et al., 2007). The secreted proteases belong to the aminopeptidase, aspartyl protease, serine carboxypeptidase and the di- and tri-peptidylaminopeptidase classes. The expression of several of these secreted proteases, including the major acid protease gene *pepA*, is regulated by the transcriptional regulator PrtT (Punt et al., 2008). Gene *prtT* is ≥ 5-fold higher expressed in the vegetative mycelium when compared to the aerial structures (Supplementary Table 4). From the 31 secreted proteases, 20 had a present call in the vegetative mycelium

Table 12: Expression of secreted proteases (Pel et al., 2007) in the aerial structures of 7-day-old maltose-grown colonies of *A. niger* when compared to the vegetative mycelium. The fold change was also calculated when a gene had an absent call. Dark shading indicates genes that had an absent call in both the vegetative mycelium and the aerial structures. Light shading indicates genes that had a present call in either the vegetative mycelium (m) or the aerial structures (a).

Gene ID	Annotation	Activity	Fold change
An02g07210	aspartic protease pepE - *Aspergillus niger*	secreted aspartyl protease	<2
An04g01440	strong similarity to precursor of pepsin A3 - *Homo sapiens*	secreted aspartyl protease	<2
An13g02130	strong similarity to aspartic proteinase Yps3 - *Saccharomyces cerevisiae*	secreted aspartyl protease	<2
An11g00310	similarity to aspartyl proteinase candidapepsin - *Candida albicans*	secreted aspartyl protease	<2
An07g00950	similarity to aspartyl proteinase candidapepsin - *Candida albicans*	secreted aspartyl protease	<2
An05g01870	strong similarity to carboxypeptidase y CPY - *Candida albicans*	secreted serine carboxypeptidase	<2
An05g02170	strong similarity to serine-type carboxypeptidase F CPD-II - *Aspergillus niger*	secreted serine carboxypeptidase	<2
An06g00310	similarity to carboxypeptidase D - *Penicillium janthinellum*	secreted serine carboxypeptidase	<2
An07g08030	serine carboxypeptidase pepF - *Aspergillus niger*	secreted serine carboxypeptidase	<2
An11g06350	strong similarity to carboxypeptidase C cpy1p - *Schizosaccharomyces pombe*	secreted serine carboxypeptidase	<2
An14g02150	strong similarity to serine-type carboxypeptidase precursor cpdS - *Aspergillus phoenicis*	secreted serine carboxypeptidase	<2
An16g09010	strong similarity to carboxypeptidase I protein from patent WO9814599-A1 - *Aspergillus oryzae* [putative frameshift]	secreted serine carboxypeptidase	<2
An09g02370	similarity to proteinase SlpE - *Streptomyces lividans*	secreted tripeptidylaminopeptidase	<2
An12g08560	strong similarity to proteinase SlpE - *Streptomyces lividans*	secreted tripeptidylaminopeptidase	<2
An13g02620	strong similarity to proteinase P5-6 from patent WO9517512-A - *Streptomyces lividans*	secreted tripeptidylaminopeptidase	<2
An08g04640	strong similarity to hypothetical lysosomal pepstatin insensitive protease CLN2 - *Canis lupus*	secreted tripeptidylaminopeptidase	<2
An11g01110	strong similarity to lysosomal pepstatin insensitive protease CLN2 - *Homo sapiens*	secreted tripeptidylaminopeptidase	<2
An17g00390	strong similarity to aminopeptidase from patent WO9628542-A1 - *Aspergillus oryzae*	secreted aminopeptidase	<2
An06g00780	weak similarity to aminopeptidase N - *Felis catus*	secreted aminopeptidase	<2
An08g08750	carboxypeptidase Y cpy from patent WO9609397-A1 - *Aspergillus niger*	secreted serine carboxypeptidase	>2 up
An14g00620	strong similarity to aminopeptidase from patent WO9628542-A1 - *Aspergillus oryzae*	secreted aminopeptidase	>2 up
An16g02250	strong similarity to lysosomal pepstatin insensitive protease CLN2 - *Homo sapiens*	secreted tripeptidylaminopeptidase	>8 up a
An12g05960	strong similarity to dipeptidyl peptidase II DPPII - *Rattus norvegicus*	secreted dipeptidylaminopeptidase	>2 down
An03g05200	strong similarity to carboxypeptidase S1 - *Penicillium janthinellum*	secreted serine carboxypeptidase	>2 down

An15g06280	strong similarity to aspartic proteinase aspergillopepsin I pepA - *Aspergillus niger* [truncated ORF]	secreted aspartyl protease	>2 down m
An14g04710	aspartic proteinase aspergillopepsin I pepA - *Aspergillus niger*	secreted aspartyl protease	>2 down
An12g03300	strong similarity to aspartic protease pr1 - *Phaffia rhodozyma*	secreted aspartyl protease	>2 down m
An02g04690	strong similarity to serine-type carboxypeptidase I cdpS - *Aspergillus saitoi*	secreted serine carboxypeptidase	>4 down
An01g01750	similarity to lysosomal protease CLN2 - *Rattus norvegicus*	secreted tripeptidylaminopeptidase	>4 down m
An06g00190	strong similarity to lysosomal pepstatin insensitive protease CLN2 - *Homo sapiens*	secreted tripeptidylaminopeptidase	>8 down
An03g01010	strong similarity to lysosomal pepstatin insensitive protease CLN2 - *Homo sapiens*	secreted tripeptidylaminopeptidase	>8 down

and/or the aerial structures (Table 12, Supplementary Table 4). Of these 20 genes, *pepA*, *pr1* and the *cln2* homolog from *Rattus norvegicus* had a present call in the vegetative mycelium only, and transcripts of one *cln2* homolog from *Homo sapiens* were exclusively found in aerial structures (Table 12, Supplementary Table 4). Twelve genes encoding a secreted protease showed differential expression (Table 12). Three genes were up-regulated in the aerial structures. Gene *cpy* and an aminopeptidase had a fold change ≥ 2, and a homolog of human *cln2* was ≥ 8-fold up-regulated. RNA levels of 9 out of the 12 differentially expressed genes were higher in the vegetative mycelium (Table 12). The aspartic proteinase aspergillopepsin I *pepA* was one of them. Two human *cln2* homologs were among the top 100 of down-regulated genes (Table 4).

Nuclear run-on transcription assay

Differences in RNA levels determined by micro-array analysis may not only result from transcription, but also from differential RNA stability and streaming/transport of RNA from the vegetative mycelium to the aerial structures and vice versa. A nuclear run-on transcription assay was performed to monitor whether differential RNA turnover and/or RNA streaming/transport contributes to the differences in RNA composition of the vegetative mycelium and the aerial structures. To this end, active transcription of 7 genes (Table 13) was determined in the nuclei of the vegetative mycelium and the aerial structures of 7-day-old maltose-grown colonies. According to the micro-array analysis, these genes were either up-regulated (*ayg1/olvA*, FAD binding oxidoreductase gene (oxi)), or down-regulated (*mpdA*, *adhA*, *glaA*, flavohemoprotein gene (flavo)) in the aerial structures or their expression was unaffected (*gpdA*) (Table 13, Supplementary Table 4). Nuclei were isolated from vegetative mycelium and aerial structures and were incubated with nucleotides including ^{32}P-UTP. These nucleotides were incorporated in the RNA of actively transcribed genes by native DNA polymerase. The radioactively labeled RNA was isolated from the nuclei and hybridized with the coding sequences of the selected genes that had been spotted on a Nylon membrane (Figure 3). Autoradiography showed that the hybridization signals of *ayg1/olvA,* the FAD binding oxidoreductase gene, *adhA,* and *glaA* were similar to that obtained with the micro-array analysis

Table 13: Genes selected for nuclear run-on transcription analysis. Genes are divided in groups based on no change in expression, ≥ 2-fold up-regulation or ≥ 2 down-regulation. The fold change was also calculated when a gene had an absent call. Light shading indicates a gene that only had a present call in the aerial structures. Expression values are given as the mas5-Signal.

Gene ID	Gene/ Alias	Annotation	Fold change	Expression mycelium	Expression aerial
An16g01830	*gpdA*	glyceraldehyde-3-phosphate dehydrogenase *gpdA* - *Aspergillus niger*	<2	2561.5	2000.9
An14g05350	*ayg1/ olvA*	pigment biosynthesis protein *ayg1* – *Aspergillus niger*	39.4 up	101.4	2578.3
An18g00510	oxi	FAD binding oxidoreductase – *Aspergillus niger*	76.2 up	12.3	1178.5
An02g05830	*mpdA*	mannitol-1-phosphate 5-dehydrogenase *mpdA* – *Aspergillus niger*	2.8 down	1058.5	526.8
An17g01530	*adhA*	alcohol dehydrogenase 2 - *Aspergillus niger*	4.2 down	1091.9	395.8
An03g06550	*glaA*	glucoamylase *glaA* - *Aspergillus niger*	7.3 down	2234.8	477.6
An14g02460	flavo	flavohemoprotein – *Aspergillus niger*	13.5 down	756.1	89.4

(Figure 3). In contrast, signals were different in the case of *gpdA, mpdA,* and the flavohemoprotein gene. Run-on transcription showed that glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) was higher expressed in the vegetative mycelium (Figure 3), whereas micro-array analysis showed constitutive expression (Table 13). Mannitol-1-phosphate dehydrogenase *mpdA*, which is down-regulated in aerial structures according to micro-array analysis (Table 13), was higher expressed in these structures in the run-on analysis. The gene encoding a putative flavohemoprotein (flavo) was similarly expressed in the mycelium and the aerial structures according to the run-on analysis, but micro-array analysis showed higher mRNA levels in the vegetative mycelium. Taken together, these results show that not only transcription, but also differential RNA stability and/or RNA streaming/transport contribute to the differences in mRNA composition of the vegetative mycelium and the aerial structures.

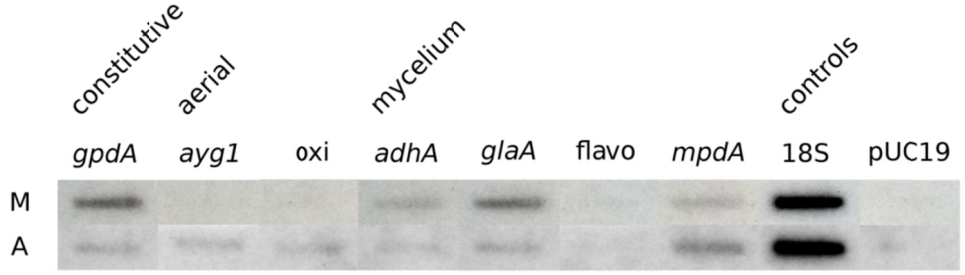


Figure 3: Nuclear run-on transcription assay. Transcription in nuclei isolated from vegetative mycelium (M) and aerial structures (A) was continued by labeling with ^{32}P-UTP. Radioactively labeled RNA was isolated and used as a probe for plasmids that either or not contained the coding sequence of 1 of the 7 genes used in the analysis (see also Table 13). 18S rDNA and the plasmid backbone of pUC19 served as a control (controls). Above the figure is indicated whether a gene is constitutively expressed (constitutive), up-regulated in the aerial structures (aerial) or up-regulated in the vegetative mycelium (mycelium) according to micro-array data (see Table 13, Supplementary Table 4). oxi = FAD binding oxidoreductase. flavo = flavohemoprotein.

Discussion

In this study, the RNA composition was analyzed of the vegetative mycelium and the aerial structures (*i.e.* aerial hyphae, conidiophores and conidiospores) of 7-day-old maltose-grown colonies of *A. niger*. From the 14259 genes, a total of 6476 were expressed in the colony. Of these genes, 5095 and 5939 were expressed in the vegetative mycelium and the aerial structures, respectively. 2056 genes had a fold change ≥ 2, of which 1144 were up-regulated in the aerial structures. This finding is in line with the estimation that approximately 1300 genes are up-regulated during a-sexual spore formation in *A. nidulans* (Timberlake, 1980). The high number of genes that are expressed in the aerial structures and the number of genes that are differentially expressed may be explained by the different cell types that make up the aerial structures. Recently, it was found that aerial hyphae of the basidiomycete *Ustilago maydis* have a RNA composition very similar to that of vegetative hyphae. Only 31 genes were differentially expressed (Teertstra et al., 2011). It would be of interest to perform a similar study in *A. niger.* Possibly, also *A. niger* aerial hyphae have a RNA profile similar to that of the vegetative mycelium. At least, the conidiophore and conidiospores are expected to have a RNA profile different from that of the vegetative mycelium.

The vegetative mycelium feeds the aerial structures. It is therefore not surprising that the functional gene category C-compound and carbohydrate utilization was over-represented in the vegetative mycelium. Part of these genes encodes secreted enzymes that are involved in acquisition of carbon source. AmyR regulates genes involved in starch and maltose degradation (Fowler et al., 1990; Tani et al., 2001) as well as genes encoding D-glucose and D-galactose releasing enzymes (van Kuyk et al., 2011). From the 16 genes that are regulated by AmyR (van Kuyk et al., 2011), 6 were not expressed, 4 did not show a fold change in expression ≥ 2 and 6 were up-regulated in the vegetative mycelium. The glucoamylase gene *glaA* and the acid amylase gene *aamA* were among the up-regulated genes. Gene *amyR* was also up-regulated in the vegetative mycelium. A similar differential expression was observed for *prtT*, which encodes the regulator of protease genes (Punt et al., 2008). Twelve out of the 31 genes encoding a secreted protease were differentially expressed. Nine of these genes were up-regulated in the vegetative mycelium, among which was *pepA.* Taken together, a set of genes involved in starch degradation and a set of genes encoding secreted proteases are up-regulated in the vegetative mycelium. Yet, many representatives of these classes of genes are not switched of or are only down-regulated in the aerial structures. Some are even up-regulated in the aerial structures. Future studies should reveal whether these mRNAs are translated into proteins. If so, these proteins fulfil other functions or represent a regulatory inefficiency of *A. niger.*

Several genes involved in secretion are up-regulated in the aerial structures including the disulfide isomerase encoding genes *pdiA* and *tigA*, the ER membrane translocator genes *sec61a* and *sec61b*, and the unfolded protein response gene *hacA*. Intuitively, one would expect to detect up-regulation of this gene class in the vegetative mycelium to support secretion of enzymes that are involved in acquisition of carbon source. Yet, a highly active secretory machinery may also be required in the aerial structures. These structures secrete hydrophobins that provide a hydrophobic

coating (Wösten, 2001). In the basidiomycete *Schizophyllum commune* these proteins comprise up to 10% of the total protein. This amount of protein is produced by the aerial hyphae that make up a minor part of the *S. commune* mycelium. In this study, 7 out of 8 hydrophobin genes were shown to be expressed. Five of them were up-regulated in the aerial structures, 4 of which even ≥ 8-fold. In fact, a hydrophobin gene that has not yet been characterized showed the highest up-regulation in the aerial structures. Genes involved in pigmentation of the conidiospores were also highly up-regulated in the aerial structures. Recently, four genes have been described that are involved in this process (Jørgensen et al., 2011). Inactivation of *fwnA* resulted in fawn-coloured spores, while inactivation of *olvA, brnA* and *pptA* resulted in olive, brown-coloured, and white spores, respectively. Of these genes *pptA* was not expressed. This gene encodes a polyketide synthase and it may be that low (*i.e.* undetectable) expression levels are sufficient to produce sufficient amount of enzyme. The other genes involved in pigmentation *fwnA, olvA* and *brnA* were more than 8-fold up-regulated in the aerial structures. In fact, they belonged to the top 100 of most up-regulated genes in the aerial structures.

A-sexual reproduction is regulated by a large set of genes (Adams et al., 1998; Krijgsheld et al., 2012). The transcriptional regulators encoded by *brlA, abaA,* and *wetA* play a central role in the formation of conidiophores. For instance, they regulate expression of hydrophobin genes in the aerial structures (Stringer et al., 1991; Stringer and Timberlake, 1995). In agreement, these genes were all shown to be ≥ 8-fold up-regulated in the aerial structures. The *brlA* gene is activated by a signaling pathway (Krijgsheld et al., 2012). The genes encoding these upstream regulatory proteins were shown to be expressed both in the vegetative mycelium and the aerial structures. This makes sense because the signaling pathway includes sensing of the nutrient status and signals secreted by vegetative hyphae.

Nuclear run-on transcription monitors genes that are actively transcribed in nuclei. Relative mRNA levels of *glaA*, *adhA, olvA* and the FAD binding oxidoreductase gene (oxi) in the aerial structures and the vegetative mycelium were similar in the nuclear run-on transcription and the micro-array analysis. In contrast, ratios found for *gpdA, mpdA* and the gene encoding the flavohemoprotein (flavo) were different between the run-on analysis and the micro-array analysis. Micro-array analysis showed that *gpdA* was constitutively expressed, but the run-on analysis showed higher expression in the vegetative mycelium. Possibly, *gpdA* transcripts are less stable in the vegetative mycelium. Alternatively, *gpdA* transcripts stream from or are transported from the vegetative mycelium to the aerial structures, thereby leveling out the differences of *gpdA* transcription. According to micro-array analysis, transcript levels of *mpdA* were higher in the vegetative mycelium than in the aerial structures. In contrast, they were found to be more abundant in the aerial structures in the run-on analysis. These results can be explained by lower transcript stability in the aerial structures or by streaming/transport of the transcripts from these structures towards the vegetative mycelium.This may also explain the discrepancy in the RNA ratios of the flavohemoprotein (flavo) according to the run-on analysis and the micro-array analysis. Stability of transcripts is primarily determined by co-transcriptional 5´ and/or 3' capping. Decapping of transcripts is triggered by shortening of the polyA tail, which

results in 5' to 3' (Tucker and Parker, 2000) or 3' to 5' (Mitchell and Tollervey, 2000; Butler, 2002) degradation. Deadenylation thus represents an important point of regulation in mRNA turnover. In *A. nidulans* deadenylase Caf1 is required for the regulated turnover of specific transcripts, while deadenylase Ccr4 mediates basal degradation (Morozov et al., 2010). Furthermore, CutA modifies adenylated mRNA at the 3' terminus by adding a CUCU sequence. Morozov et al. (2010) proposed that this 3' modification primes transcripts for decapping and efficient degradation. There is no evidence for differential expression of the nearest *A. niger* homologs of *caf1* (An04g09062; 4.4e-02), *ccr4* (An11g01520; 2.0e-08) and *cutA* (An03g03780; 0.0e+00) in the vegetative mycelium and the aerial structures (Supplementary Table 4; note that An04g09062 is not on the micro-array). PUB1 and NAB2 of *Saccharomyces cerevisiae* are known to stabilize specific transcripts (Apponi et al., 2007). The *NAB2* homolog in *A. niger* (An11g01570; 1.3e-16) is 1.7 fold up-regulated in the aerial structures, while the homolog of *PUB1* (An08g04280; 2.1e-54) was not differentially expressed (Supplementary Table 4). Taken together, there is no evidence yet for differential RNA stability within the colony of *A. niger*. The same holds for unidirectional streaming/transport of mRNA. Yet, this process has been described in other fungi. For example *ABP140* mRNA is transported from the daughter to the mother cell in *S. cerevisiae* (Kilchert and Spang, 2011). In contrast, *ASH1* mRNA is transported from the mother to the daughter cell in this yeast (Beach and Bloom, 2001). Moreover, in *U. maydis* the RNA-binding protein Rrm4 shuttles along microtubules and is involved in long-distance transport of *ubi1* and *rho3* mRNAs (König et al., 2009). Interestingly, *A. niger* contains a gene with five functional domains of Rrm4 (An01g03050). However, transcripts of this homolog are absent in the vegetative mycelium and in the aerial structures.

Taken together, our results show that the vegetative mycelium and the aerial structures of *A. niger* are heterogenic with respect to RNA composition. This heterogeneity is the result of differential gene expression and differential mRNA stability and/or mRNA streaming/transport.

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Chapter 3

Woronin bodies function in dynamic septal closure in intact vegetative hyphae of *Aspergillus oryzae*

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Abstract

Hyphae in the mycelium of ascomycetes are compartmentalized by septa. These septa contain a central pore, which allows for cytoplasmic streaming. Previously, it was shown that the central pore is closed by Woronin bodies upon damage. In this study we demonstrate by laser dissection that septa of *Aspergillus oryzae* can already be closed in intact growing hyphae. It was found that approximately 60% of the first, second and third septa of leading hyphae of *A. oryzae* are open. The number of open septa did not change upon exposure to a number of stresses. Deletion of *Aoso* also did not affect septal plugging under most growth conditions despite the fact that this protein accumulated at the septa during stress conditions. Inactivation of *Aohex1* did affect septal plugging. 100% of the septa were open in a Δ*Aohex1* strain both under standard growth conditions and during exposure to stress. Plugging did not increase when *Aoso* was deleted in the Δ*Aohex1* strain. Septal closure by Woronin bodies and AoSO localization at the septum were shown to be reversible. Taken together, Woronin bodies close septa during vegetative growth in a dynamic way but the AoSO protein does not have a role in this process.

Introduction

Filamentous fungi form a mycelium by means of hyphae that extend at their tips and that branch sub-apically. Hyphae of the Ascomycota are compartmentalized by septa. The diameter of the septal pore varies between 50 to 500 nm allowing passage of cytosol and even organelles (Shatkin and Tatum, 1959; Moore and McAlear, 1962; Gull, 1978; Lew, 2005). Upon mechanical injury, septa of ascomycetes close to prevent excessive cytoplasmic bleeding. The closure of septa is mediated by Woronin bodies (Trinci and Collinge, 1974; Collinge and Markham, 1985; Jedd and Chua, 2000; Maruyama et al., 2005) and by the protein SO (Fleissner and Glass, 2007).

Woronin bodies originate from peroxisomes (Jedd and Chua, 2000). Their budding from peroxisomes is mediated by Pex11 (Escaño et al., 2009) and WSC (Liu et al., 2008). Gene *pex14* also plays a role in Woronin body biogenesis. Deletion of this gene in *Neurospora crassa* resulted in the absence of Woronin bodies (Managadze et al., 2007). It was shown that Pex14 is involved in import of HEX1 in the peroxisomes (Managadze et al., 2007). In wild-type strains of *N. crassa*, the lumen of Woronin bodies is filled with hexagonal rods of HEX1 (Hoch and Maxwell, 1974; Jedd and Chua, 2000; Managadze et al., 2010). Deletion of *HEX1* results in the absence of Woronin bodies (Jedd and Chua, 2000; Tenney et al., 2000; Soundararajan et al., 2004; Maruyama et al., 2005). Polarized *HEX1* expression in *N. crassa* restricts Woronin body formation at the edge of the colony (Tey et al., 2005). These organelles are also predominantly found at the apex of germ tubes of *Aspergillus nidulans*. It is thought that Woronin bodies are transported from the apex to the more basal regions of the cell immediately before or during septation. Woronin bodies are usually located at the septal pore in this fungus (Momany et al., 2002). Hyphal compartments of *N. crassa* contain usually a few Woronin bodies that are tethered via WSC and Leashin to the cell cortex (Ng et al., 2009; Jedd, 2011). Upon hyphal rupture, Woronin bodies move from this rim to plug the pore in *N. crassa* (Jedd and Chua, 2000). Woronin bodies also plug septal pores of *A. oryzae*, *Magnaporthe grisea* and

Penicillium chrysogenum (Collinge and Markham, 1985; Soundararajan et al., 2004; Maruyama et al., 2005). After Woronin body plugging, cell wall deposition permanently seals the pore in *P. chrysogenum* and *N. crassa* (Collinge and Markham, 1987; Fleissner and Glass, 2007) and this is usually followed by re-initiation of growth (Buller, 1933; Markham, 1994; Fleisser and Glass, 2007).

The homologous proteins SO (SOFT) and Pro40 of *N. crassa* and *Sordaria macrospora,* respectively, have been localized at septal pores of injured compartments (Fleissner and Glass, 2007; Engh et al., 2007). PRO40 is thought to play a role in fruiting body formation and has been found associated with Woronin bodies. However, deletion of *pro40* did not result in increased cytoplasmic bleeding (Engh et al., 2007). Deletion of *so* in *N. crassa* resulted in impaired vegetative hyphal fusion. In this case, an effect on septal plugging was observed. SO helps sealing the plugged pore (Fleissner and Glass, 2007). In the wild-type, 50% of the septal pores seal immediately upon hyphal damage. This number is only 30% in the Δ*so* strain. In addition, more than 20% of the Δ*so* hyphae show cytoplasmic leakage through the septal pore after more than 30 sec, while only 6% of the wild-type septa were still bleeding at this time point (Fleissner and Glass, 2007). Deletion of the *so* homologue of *A. oryzae, Aoso,* also leads to a higher occurrence of cytoplasmic bleeding after hyphal injury (Maruyama et al., 2010). The AoSO protein is localized at the septum after exposure to stress conditions such as temperature shock, pH shock, starvation and hyphal rupture.

Here, it is shown that Woronin bodies of *A. oryzae* not only plug septa during mechanical damage but also in intact growing hyphae. Inactivation of *HEX1* abolishes the capacity of septal plugging, while inactivation of *Aoso* does not affect the plugging incidence. Woronin body and AoSO localization at the septal pore is reversible, which makes septal plugging in intact growing hyphae of *A. oryzae* a dynamic process.

Material and methods

Strains and growth conditions

Strains used in this study are listed in Table 1. RIB40 strain was used as wild-type (Machida et al., 2005). Strain NSRKu70-1-1A (Escaño et al., 2009) is a derivative of NSRKu70-1-1 (Δ*ku70*; Takahashi et al., 2006) and expresses the *adeA* seletion marker gene in this latter strain. NS4 (*niaD*$^{-}$ *sC*$^{-}$) (Yamada et al., 1997) is the parental strain of NSR13 (*niaD*$^{-}$ *sC*$^{-}$ *adeA*$^{+}$) (Jin et al., 2004). NSRK-ΔSO11 (Maruyama et al., 2010) has the *Aoso* deletion in the NSRKu70-1-1 strain. Inactivation of *Aohex1* in strain NSRKu70-1-1A, in NSRK-ΔSO11 or in NSR13, resulted in strains NSRK-ΔHx5, NSRK-ΔSOΔHx7 and NSR-ΔHx11, respectively. Strain NSRK-ΔHx5 has the *Aohex1* deletion. Strain NSRK-ΔSOΔHx7 is the double mutant of *Aohex1* and *Aoso*. Strain 11NSR-NAGHs expresses EGFP-AoHex1 under control of the *amyB* promoter in strain NSR-ΔHx11. Strain NSK-ASG1 (Maruyama et al., 2010) is a derivative of NSK-ΔSO11 (Maruyama et al., 2010) and expresses the AoSO-EGFP fusion protein under the control of the *amyB* promoter. Expression of AoSO-EGFP under the control of the *amyB* promoter in the double deletion strain NSRK-ΔSOΔHx7, resulted in NSRK-

Table 1: Strains used in this study.

Strain	Parental Strain	Genotype	Auxotrophy	Reporter plasmid	References
RIB40		Wild-type			Machida et al., 2005
NSR13	NS4		*niaD*⁻ *sC*⁻ *adeA*⁻		Jin et al., 2004
NSRKu70-1-1A	NSRKu70-1-1	Δ*ku70*	*niaD*⁻ *sC*⁻		Escaño et al., 2009
NSRK-ΔSO11	NSRKu70-1-1	Δ*ku70* Δ*Aoso*	*niaD*⁻ *sC*⁻		Maruyama et al., 2010
NSRKu70-1-1AS	NSRku70-1-1A	Δ*ku70*	*niaD*⁻		This study
NSRK-ΔHx5	NSRku70-1-1A	Δ*ku70* Δ*Aohex1*	*niaD*⁻		This study
NSRKS-ΔSO5	NSRK-ΔSO11	Δ*ku70* Δ*Aoso*	*niaD*⁻		This study
NSR-ΔHx11	NSR13	Δ*Aohex1*	*niaD*⁻ *sC*⁻		This study
NSRK-ΔSOΔHx7	NSRK-ΔSO11	Δ*ku70* Δ*Aoso* Δ*Aohex1*	*niaD*⁻		This study
NSK-ASG1	NSK-ΔSO11	Δ*ku70* Δ*Aoso*	*sC*⁻	P*amyB*::*Aoso-egfp*	Maruyama et al., 2010
11NSR-NAGHs	NSR-ΔHx11	Δ*Aohex1*	*sC*⁻	P*amyB*::*egfp-Aohex1*	This study
NSRK-ΔSOΔHx7-SO	NSRK-ΔSOΔHx7	Δ*ku70* Δ*Aoso* Δ*Aohex1*		P*amyB*::*Aoso-egfp*	This study

ΔSOΔHx7-SO. Expression of the *sC* selection marker gene in *A. oryzae* strains NSRKu70-1-1A and NSRK-ΔSO11, generated control strains NSRKu70-1-1AS and NSRKS-ΔSO5, respectively. The latter strain has the *Aoso* deletion.

To obtain spores for inoculation, *A. oryzae* strains were grown on 3.7% PDA (Potato Dextrose Agar, Sigma Aldrich, www.sigmaaldrich.com) plates. Spores were harvested in 0.9% NaCl (w/v) containing 0.05% (v/v) Tween-20 and diluted to a final concentration of $5*10^5$ spores ml^{-1}. For microscopy, *A. oryzae* was grown in glass bottom dishes (MatTek, www.glass-bottom-dishes.com, P35G-1.5-20-C) essentially as described by van Peer et al. (2009). Strains with nitrate prototrophy were grown on CD + Met medium (0.3% $NaNO_3$, 0.2% KCl, 0.1%, KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.002%, $FeSO_4.7H_2O$, 1% glucose, 0.0015% methionine, pH 5.5; Maruyama et al., 2010), while strains with nitrate auxotrophy (*niaD*) were grown on M medium (0.2% NH_4Cl, 0.1% $(NH_4)_2SO_4$, 0.05%, KCl, 0.05% NaCl, 0.1%, KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.002% $FeSO_4.7H_2O$, 1% glucose, pH 5.5; Ohneda et al., 2005). The glass bottom dishes were filled with 30 µl CD + Met medium or M medium containing 1% agarose. To this end, the glass bottom dishes and the agar medium were pre-warmed at 50 °C and 60 °C, respectively. Spores (0.5 µl, 500 spores/µl) were placed in the middle of an 18 mm cover glass and placed upside down on the non-solidified agarose medium. This resulted in a 118 µm thick agarose layer. After solidifying the agarose medium, 2 ml liquid medium was added on top of the culture. Cultures were grown for 2 days at 30 °C under water saturating conditions, after which they were either or not exposed to stress conditions. Temperature stress was imposed by incubation at 4 °C or 45 °C for 30 min. For pH stress, the liquid medium was replaced by CD + Met medium with a pH of 2.0 or 11.0 and subsequent incubation for 1.5 hours. To this end, the pH was adjusted with HCl or NaOH, respectively. Starvation stress was exposed from t = 0 onwards during 2 days of growth. For carbon-starvation, solid medium contained 0.2% glucose and liquid medium contained no glucose. For nitrogen-starvation solid medium contained nitrate/ammonium (see above), but liquid medium did not contain a nitrogen source.

Transformation

A. oryzae was transformed as described (Maruyama and Kitamoto, 2011). Transformants were selected on CD + Met medium for *niaD* prototrophy or on M medium for *adeA* prototrophy or *sC* prototrophy (containing 1.5% agar and 2% glucose; Juvvadi et al., 2007).

Construction of control and deletion strains

The 1.9 kb upstream flanking region of the *Aohex1* open reading frame was amplified with the primers aB4F_usAohex1 and aB1R_usAohex1 (Table 2) and RIB40 genomic DNA as template and inserted into pDONR™ P4-P1R (Invitrogen, www.invitrogen.com) by BP recombination reaction generating the 5' entry clone plasmid, pg5'Ahx1. The 1.6 kb downstream flanking region of the gene was amplified with the primers aB2F_dsAohex1 and aB3R_dsAohex1 (Table 2) and RIB40 genomic DNA as template and inserted into pDONR™ P2R-P3 (Invitrogen) by BP recombination reaction generating the 3' entry clone plasmid, pg3'Ahx1. The

Table 2: Primers used in this study.

Primer name	Sequence
aB4F_usAohex1	GGGGACAACTTTGTATAGAAAAGTTGGGACCAATGCGACCATGAAG
aB1R_usAohex1	GGGGACTGCTTTTTTGTACAAACTTGCAGTAGTAGTGCTAAGAACCTTGAC
aB2F_dsAohex1	GGGGACAGCTTTCTTGTACAAAGTGGATCATTTTCCCGCTTTGATCTGGTC
aB3R_dsAohex1	GGGGACAACTTTGTATAATAAAGTTG CAAGATTCTAGGTGCTTGGTTTGAG
5-hex1-F	GGGGACAACTTTGTATAGAAAAGTTGGGACCAATGCGACCATGAAG
5-hex1-R	GGGGACTGCTTTTTTGTACAAACTTGCAGTAGTAGTGCTAAGAACCTTGAC
3-hex1-F	GGGGACAGCTTTCTTGTACAAAGTGGATCATTTTCCCGCTTTGATTCGGTC
3-hex1-R	GGGGACAACTTTGTATAATAAAGTTGCAAGATTCTAGGTGCTTGGTTTGAG

underlined sequences show the Multisite Gateway *attB* recombination sites. The obtained 5' and 3' entry clones together with the center entry clone pgEsC (containing the *sC* marker gene) were subjected with LR clonase in the presence of pDEST R4-R3 (destination vector) to obtain the final plasmid pgΔdAoHex1. The *Aohex1* gene disruption fragment (~6.5 kb) was prepared by PCR using the plasmid pgΔdAoHex1 as template and primers aB4F_usAohex1 and aB3R_dsAohex1 and was introduced into *A. oryzae* strains NSRKu70-1-1A and NSRK-ΔSO11 (Escaño et al., 2009; Maruyama et al. 2010), generating NSRK-ΔHx5 and NSRK-ΔSOΔHx7, respectively. M-medium was used for selection of sC^+ transformants. Disruption of the *Aohex1* gene was confirmed by Southern blotting using restriction enzymes *Bam*HI and *Eco*T221.

For control stains the plasmid pgEsC with the *sC* selection marker gene was introduced into *A. oryzae* strains NSRKu70-1-1A and NSRK-ΔSO11, generating NSRKu70-1-1AS and NSRKS-ΔSO5, respectively.

Expression of EGFP-AoHex1 in the Δ*Aohex1* strain

The *Aohex1* deletion vector pgDAHx1 was constructed using the Multisite Gateway cloning system (Invitrogen) (Mabashi et al., 2006). The upstream (2.0 kb) and downstream (2.0 kb) regions of the *Aohex1* gene were amplified by PCR using the primer combinations 5-hex1-F and 5-hex1-R, and 3-hex1-F and 3-hex1-R, respectively (Table 2). The amplified upstream and downstream regions of *Aohex1* were introduced into pDNORP4-P1R and pDONRP2R-P3 (Invitrogen), respectively, with the Gateway BP clonase reaction. The resulting plasmids were subjected to Gateway LR clonase reaction together with the entry clone plasmid containing the *A. oryzae adeA* gene as a selection marker (Jin et al., 2007) and the destination vector pDESTR4-R3 (Invitrogen). The resulting plasmid pgDAHx1 was used as a template to amplify the deletion cassette by PCR with the primers 5-hex1-F and 3-hex1-R. The amplified deletion fragment was introduced into *A. oryzae* NSR13, and a representative transformant was selected in M medium for *adeA* prototrophy and named NSR-ΔHx11. Disruption of the *Aohex1* gene was confirmed by Southern analysis. For this, genomic DNA was digested with *Bam*HI and *Nco*I and the 2.0 kb fragment of the *Aohex1* upstream region was used as a probe, which was amplified by PCR with the primers 5-hex1-F and 5-hex1-R.

The expression vector pUNAGHs (Juvvadi et al., 2007) encompassing the

fusion of *eGFP* and *Aohex1* under the control of the *amyB* promoter was introduced into the Δ*Aohex1* strain NSR-ΔHx11 using *niaD* as a selection marker. Strain 11NSR-NAGHs (Table 1) showed fluorescence representative for the transformants obtained.

Expression of AoSO-EGFP in the Δ*Aoso* and the Δ*Aohex1*Δ*Aoso* strains

Strain NSK-ASG1 expressing the AoSO-EGFP fusion protein was previously described (Maruyama et al., 2010). *A. oryzae* strain NSRK-ΔSOΔHx7 was transformed with pgPaBSG (Maruyama et al., 2010), which harbors the *niaD* gene as selection marker and the *Aoso-eGFP* gene under control of the *amyB* promoter. Strain NSRK-ΔSOΔHx7-SO (Table 1) showed fluorescence representative for the transformants obtained.

Fluorescence microscopy

The reporter proteins EGFP-AoHex1 and AoSO-EGFP were monitored over periods of up to 16 h on a Zeiss confocal laser scanning microscope (CSLM; Zeiss LSM 5 PASCAL; Zeiss, www.zeiss.com) using a 488 nm laser and a LP505 filter. For Woronin body movement, images were taken in the Z-plane in three slices (slice thickness 0.4 µm) every 15 min using a Plan-Neofluor 25x/0.8 Imm corr objective. The pixel time was set at 2.51 µs, the laser power at 0.125 mW, and the pinhole at 136 µm. For localization of AoSO, images were taken in the Z-plane in four slices (slice thickness 0.6 µm) every 2 min using a Plan-Neofluor 100x/1.3 Oil objective. The pixel time was set at 1.60 µs, the laser power at 0.250 mW, and the pinhole at 164 µm. Images were taken with a resolution of 512 x 512 pixels and exported as tif files using the Zeiss LSM Image Browser v4.2 (www.zeiss.co.jp). Composition and layout of exported images was made with GIMP v2.6 (http://www.gimp.org/).

Analysis of plugging

Compartments were ruptured by laser dissection using the laser pressure catapulting function (LPC) of the P.A.L.M. laser dissecting microscope (Zeiss, www.zeiss.com). To this end, 60-70% of the power of the pulsed UV-laser was used. The septum was scored as 'open', 'quickly closed' or 'closed' directly after hyphal injury. The septum was scored as 'open' when cytoplasm was streaming out of the septum adjacent to the ruptured compartment (see Supplemental Movie 1; see http://hdl.handle.net/10411/10107). When cytoplasmic bleeding stopped within 3 seconds, the septum was scored as 'quickly closed' (Supplemental Movie 2). When no cytoplasmic movement was observed the septum was scored as 'closed' (Supplemental Movie 3). The percentage of open septa was defined as the number of 'open' and 'quickly closed' septa divided by the total number of septa x 100. Each experiment was done *in duplo* using 20 hyphae in each experiment. An ANOVA analysis with Bonferroni post-hoc correction was used when multiple comparisons were made. An ANOVA analysis with a Dunnett post-hoc correction was used to assess significant differences in septal plugging comparing the control situation with environmental stresses. Effects of the same treatments between two specific strains were assed using an independent-samples T-test. In all cases, a difference was assumed significant when $p < 0.05$.

Results

The wild-type *A. oryzae* RIB40 strain was grown for two days at 30 °C in a glass bottom microscopy dish in CD + Met medium. The apical compartments of leading hyphae of the RIB40 colonies were dissected using a UV laser. Streaming of cytoplasm from the sub-apical, adjacent compartment through the septum of the damaged compartment was monitored to assess whether it was open. The septum was scored as 'open' when cytoplasm was moving through the septum towards the ruptured compartment (Supplemental Movie 1; see http://hdl.handle.net/10411/10107). When cytoplasmic movement quickly ceased within seconds, the septum was scored 'quickly closed' (Supplemental Movie 2). In the case cytoplasmic streaming was not observed, the septum was scored as 'closed' (Supplemental Movie 3). Using these criteria it was found that 57.5% ± 10.6 of the apical septa of the wild-type strain were open (Figure 1). In more detail, 5% ± 7.1, 52.5% ± 17.7 and 42.5% ± 10.6 of the septa were scored as 'open', 'quickly closed' and 'closed', respectively. Similar percentages of open septa were found for the second and third septa (67.5% ± 3.5 and 50% ± 7.1, respectively; Figure 1). Taken together, these data show that there is no difference in septal plugging state between the first three septa of intact growing hyphae at the periphery of an *A. oryzae* RIB40 colony.

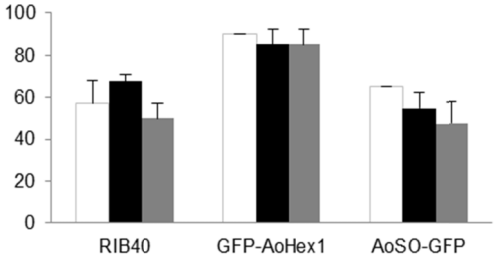

Figure 1: Percentage of open septa of leading hyphae of the wild-type strain RIB40 and the Δ*Aohex1* and Δ*Aoso* strains that had been complemented with EGFP-AoHex1 and AoSO-EGFP, respectively. White, black and grey bars show percentages of the first, second and third septa, respectively, that are open. Error bars indicate standard deviations.

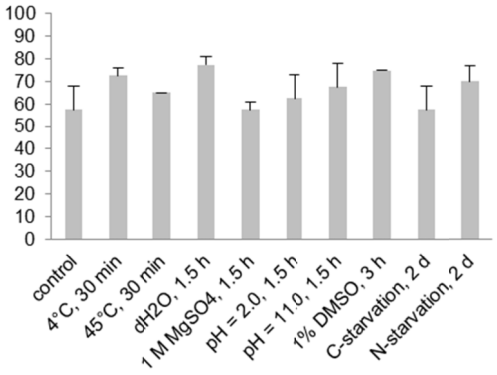

Figure 2: Percentage of open apical septa of leading hyphae of RIB40 under stress conditions. RIB40 was grown for two days on CD + Met medium, after which it was exposed to temperature stress, hypo- or hypertonic conditions, pH stress or 1% DMSO. Alternatively, RIB40 was grown for two days with C- or N-limitation. Error bars indicate standard deviations.

A. oryzae RIB40 was grown on CD + Met medium for two days at 30 °C, after which it was subjected to temperature stress, hypo- or hypertonic stress, pH stress or 1% DMSO. Alternatively, RIB40 was grown for two days with C- or N-limitation. Laser dissection showed that none of the stresses were significantly influencing the septal plugging state of the first (*i.e.* the apical) septum (Figure 2). After incubating the mycelium of RIB40 for 30 min at 4 °C or 45 °C, 72.5% ± 3.5 and 65% ± 0 of the septa were open,

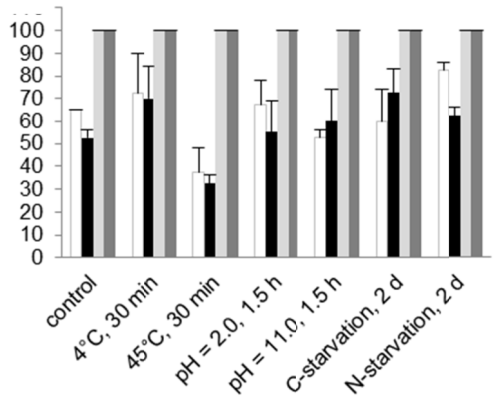

Figure 3: Percentage of open apical septa of leading hyphae that had either or not been subjected to stress. Strains were grown for two days on M medium, after which they were either or not exposed to temperature stress or pH stress. Alternatively, strains were grown for two days with C- or N-limitation. White, black, light grey and dark gray bars represent strains Δ*ku70*, Δ*Aoso*, Δ*Aohex1* and Δ*Aohex1*Δ*Aoso*, respectively. Error bars indicate standard deviations.

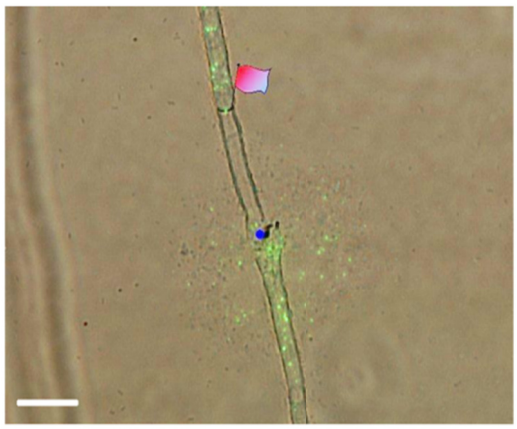

Figure 4: Woronin body closes a septum as monitored with laser dissection. Strain 11NSR-NAGHs was grown for two days on CD + Met medium. A septum with a Woronin body containing EGFP-AoHex1 (flag) was selected. No cytoplasmic bleeding was observed after dissection of the hypha at the spot indicated by the blue dot. Bar represents 25 µm.

respectively (Figure 2). When it was subjected to 1 M $MgSO_4$ or to H_2O for 1.5 hours, 57.5% ± 3.5 and 77.5% ± 3.5 of the septa were open, respectively. Similarly, 62.5% ± 10.6 and 67.5% ± 10.6 of the septa were open after incubation of the mycelium at pH 2.0 or pH 11.0 for 1.5 h, respectively. These numbers were 57.5% ± 10.6, 70% ± 7.1, and 75% ± 0 when the mycelium was exposed to carbon- and nitrogen starvation for 2 days, or to 1% DMSO for 3 hours (Figure 2). The relative number of 'open', 'quickly closed' and 'closed' septa also did not significantly change during the stress conditions when compared to the control (data not shown).

Woronin bodies are responsible for septal closure during vegetative growth

The Δ*Aohex1* deletion strain NSRK-ΔHx5 was grown for two days at 30 °C on M medium and was exposed to standard environmental conditions and to 4 °C, 45 °C, pH 2.0, pH 11.0, and to carbon- and nitrogen starvation (for culture conditions see above). In all cases 100% ± 0 of the apical septa were open (Figure 3). In contrast, 65% ± 0 of the septa of the control strain NSRKu70-1-1AS were open during these conditions. Notably, cytoplasmic movement was observed throughout minimally seven compartments after damaging the apical compartment of leading hyphae of NSRK-ΔHx5. In contrast, cytoplasmic movement was only observed in the second and sometimes the third compartment of strain NSRKu70-1-1AS (data not shown).

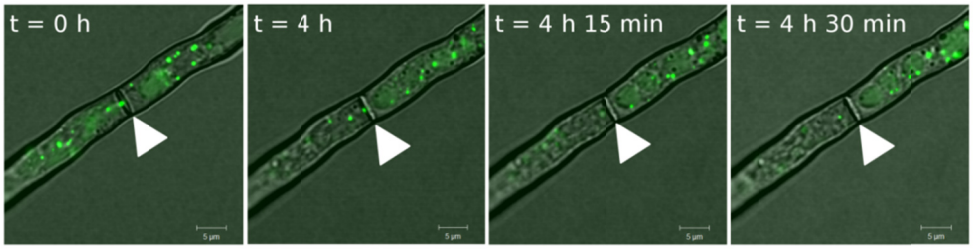

Figure 5: Position of Woronin body at the septum is dynamic. Strain 11NSR-NAGHs was grown for two days on CD + Met medium. A Woronin body was selected that was localized in the septal pore. Its localization was monitored in time using CLSM. After 4 hours the Woronin body was still localized at the septum but 15 minutes later the organelle had lost its position. Arrowhead indicates septum. Bar represents 5 µm.

Septal closure in strain NSRK-ΔHx5 was partially rescued after introduction of a vector encompassing a gene encoding EGFP-AoHex1 under control of the *amyB* promoter. In the resulting strain 11NSR-NAGHs 90% ± 0, 85% ± 7.1 and 85% ± 7.1 of the first, second and third septa of the leading hyphae, respectively, were open under standard growth conditions (Figure 1). Notably, laser dissection showed that septa of the 11NSR-NAGHs strain with a Woronin body localized at the septal pore (visualized by the reporter protein EGFP-AoHex1) were always closed (Figure 4, Supplemental Movie 4). In contrast, in the absence of a Woronin body, the septum was always open (data not shown). These results show that Woronin bodies of *A. oryzae* close septa during vegetative growth under standard conditions or when exposed to stress. CLSM life cell imaging showed that Woronin body localization at the septum was dynamic. In our experiments, Woronin bodies lost their localization at the septum after 15 minutes to several hours of growth (Figure 5).

AoSO localizes to septa upon stress

The Δ*Aoso* deletion strain NSRKS-ΔSO5 was grown for two days on M medium at 30 °C. Using laser dissection it was shown that 52.5% ± 3.5 of the apical septa were open (Figure 3). This was significantly different from the control strain NSRKu70-1-1AS of which 65% ± 0 of the septa were open. Exposure to stress did not significantly change the incidence of septal plugging in NSRKS-ΔSO5, except for nitrogen starvation where more septa were closed (Figure 3). Inactivation of *Aoso* in the *Aohex1* deletion strain did not affect septal closure. In all cases, septa of NSRK-ΔSOΔHx7 were open irrespective of the growth conditions (Figure 3). Introduction of *Aoso-eGFP* under the control of the *amyB* promoter in the NSRKS-ΔSO5 strain resulted in a septal plugging incidence similar to that of the RIB40 strain both under normal growth conditions (Figure 1) and during exposure to stress (data not shown).

AoSO-EGFP localized to 2.5% ± 3.5 of the apical septa under standard growth conditions (Figure 6). Localization of AoSO-EGFP at apical septa altered significantly when NSK-ASG1 was subjected to temperature stress or C-starvation. Localization of AoSO-EGFP was observed in the case of 67.5% ± 10.6 and 82.5% ± 10.6 of the apical septa after exposure to 4 °C and 45 °C for 30 min, respectively.

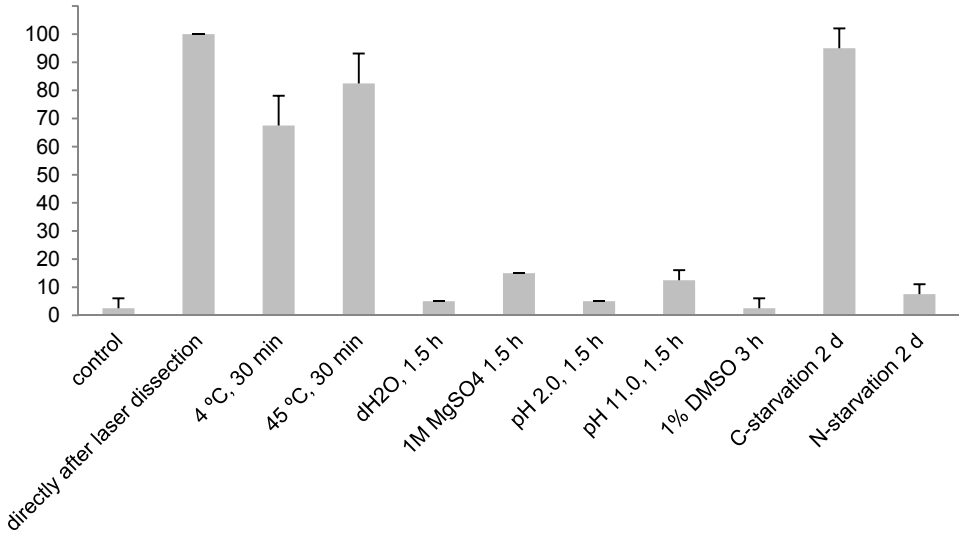

Figure 6: Percentage of apical septa of leading hyphae with AoSO-EGFP localization under normal and stress conditions. Strain NSK-ASG1 was grown for two days on CD + Met medium, after which it was exposed to temperature stress, hypo- or hypertonic conditions, pH stress or 1% DMSO. Alternatively, NSK-ASG1 was grown for two days with C- or N- limitation. Error bars indicate standard deviations.

Similarly, 95% ± 7.1 of the apical septa showed AoSO localization after C-starvation. This percentage increased to 100% ± 0 when fluorescence was monitored within 5 seconds after laser dissection. In this case AoSO-EGFP localized to the septum neighboring the ruptured compartment. Other stresses (hypo- and hypertonic stress, exposure to pH 2.0 or 11.0 or to DMSO or N-starvation) did not affect AoSO localization (Figure 6). Septa with punctuate AoSO-EGFP localization after cold-stress were selected and were followed in time with life cell imaging after bringing the culture back to room temperature. In all cases accumulation of AoSO-EGFP at the septum was lost within 1-6 hours (Figure 7). The time needed to loose AoSO accumulation correlated with the initial fluorescence intensity. In the next step, the culture was again subjected to cold shock. By monitoring the same septum it was shown that AoSO-EGFP re-localized at the septum after 20 minutes (Figure 7). These findings show that AoSO reversibly localizes to septa upon cold shock.

AoSO-EGFP was expressed in the Δ*Aoso*Δ*Aohex1* strain NSRK-ΔSOΔHx7 to assess whether AoSO-EGFP localization to septa depends on the presence of Woronin bodies. Strain NSRK-ΔSOΔHx7-SO was grown for two days on CD + Met medium and was subjected to 4 °C for 30 minutes. Using CSLM it was observed that AoSO-EGFP accumulated at septa as observed in the Δ*Aoso* strain NSK-ASG1 (data not shown). This shows that AoSO-EGFP localization at septa does not depend on the presence of Woronin bodies.

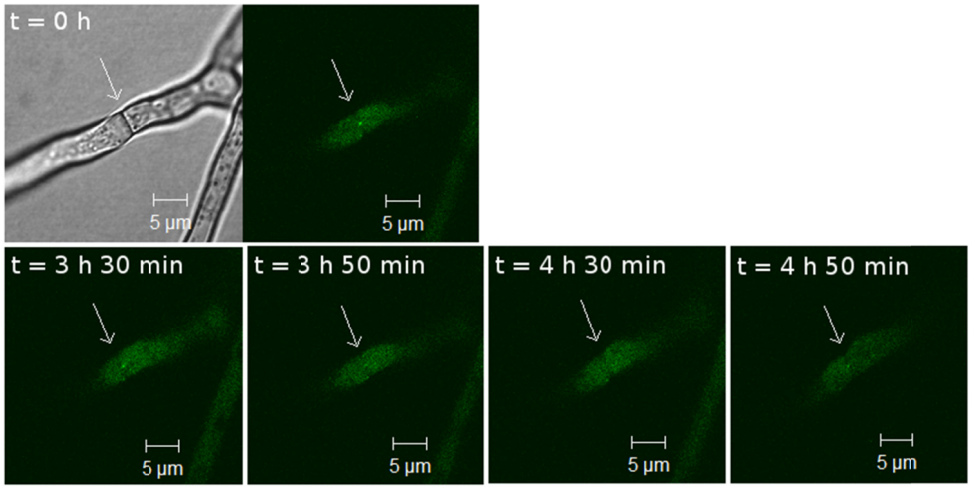

Figure 7: AoSO-EGFP reversibly accumulates at septum after cold shock. Strain NSK-ASG1 was grown for two days on CD + Met medium and was subjected for 30 minutes to cold shock. A septum was selected with AoSO-EGFP localization. In time, the septal punctuate localization of AoSO-EGFP gradually disappeared. After 4 h 30 min, AoSO-EGFP localization was completely lost. At this time point the culture was again exposed to cold shock. This resulted in punctuate AoSO-EGFP localization at the septum after 20 minutes (at 4 h 50 min). Arrow indicates septum. Bar represents 5 µm.

Discussion

It is well established that septa of basidiomycetes and ascomycetes close to prevent cytoplasmic bleeding after hyphal damage. Recently, it was shown that septa of *Schizophyllum commune* can already be in a closed state in intact growing hyphae (van Peer et al., 2009). It was demonstrated that apical septa are open in intact vegetative hyphae of this basidiomycete. In contrast, only 50% and 10% of the second and third septa are open, respectively. This number increases when *S. commune* is grown under low glucose conditions. The apical septa of *S. commune* were also shown to be able to plug. These septa close upon exposure to hypertonic conditions, to elevated temperature, to an antibiotic or to ethanol (van Peer et al., 2009). Plugging induced by heat shock was shown to be reversible. The apical septa re-opened within a period of 15-30 minutes when the culture was transferred back to the normal growth temperature. In this study it was shown that septa in intact hyphae of an ascomycete can also be in a closed state and that this process is reversible. In contrast to *S. commune,* no differences were found in the plugging incidence of the first, second and third septa of hyphae at the periphery of an *A. oryzae* colony. In all cases, about 60% of the septa were open. This was irrespective of the environmental conditions. The fact that septa can be in a closed state impacts the concept of cytoplasmic continuity of the fungal mycelium.

The SO protein plays a role in sealing septal pores in *N. crassa* (Fleissner and Glass, 2007). A similar role has been shown for its ortholog AoSO in *A. oryzae* (Maruyama et al., 2010) but not for PRO40 in *S. macrospora* (Engh et al., 2007). In our study, there was no evidence for a role of AoSO in septal plugging during

standard growth conditions or during exposure to stress. In fact, there is some evidence that AoSO has a role in keeping septa open. The differences of septal closure observed for the Δ*Aoso* strain in our study and that of Maruyama et al. (2010) may be related to the assay that was used to monitor septal plugging. In the study of Maruyama et al. (2010) the phenotype of the Δ*Aoso* strain was assessed microscopically after growing for 24 h under hypotonic conditions, whereas in this study hyphae were injured by laser dissection. Taken together, there is conflicting evidence for a role of SO in plugging of septa in ascomycetes. It should be noted that AoSO accumulated at septal pores when hyphae were exposed to temperature stress and carbon starvation. This localization did not depend on the presence of Woronin bodies. In contrast, SO localization in *N. crassa* does depend on Woronin bodies in the septal pore (Fleissner and Glass, 2007). These data suggest that the mechanisms underlying septal plugging may be different in ascomycete species. This is supported by the finding that *N. crassa* showed a delocalized pattern of Woronin bodies at the cell cortex (Ng et al., 2009) compared to septal pore rim Woronin body localization in *A. oryzae*.

A role of HEX1 in septal plugging has been firmly established. Inactivation of *HEX1* eliminates Woronin bodies in ascomycetes (Jedd and Chua, 2000; Tenney et al., 2000; Maruyama et al., 2005; Soundararajan et al., 2004). As a result, prolonged cytoplasmic bleeding is observed after hyphal damage. In this study, it was shown that AoHex1 is essential for plugging septa in intact vegetative hyphae. All septa were open in a Δ*Aohex1* strain, irrespective of the growth conditions. Live cell imaging showed that AoSO and Woronin body localization near septa was dynamic in intact vegetative hyphae. This implies that closure of septa in *A. oryzae* is reversible. Plugging of the first septum is not related to closure of the second or third septum. In other words, there is less than 5% chance of cytoplasmic continuity between two hyphae that are separated by 6 septa. This would explain why the hyphae within a mycelium of *Aspergillus niger* are heterogenic with respect to mRNA composition and processes like growth and secretion (Wösten et al. 1991; Moukha et al. 1993; Vinck et al. 2005, 2011; Masai et al. 2006; Levin et al. 2007a, b; Kasuga and Glass, 2008; de Bekker et al. 2011a, b). In the case septa would be open, mRNA and proteins would freely move through the mycelium thereby abolishing heterogeneity. Indeed, in Chapter 4 it is shown that heterogenic *glaA* expression is abolished in a Δ*Aohex1* strain.

Acknowledgments

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Chapter 4

Septal plugging is a general phenomenon in intact vegetative hyphae of aspergilli and promotes hyphal heterogeneity

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Abstract

Septa that compartmentalize hyphae of ascomycetes contain a central pore that allows cytoplasmic streaming. In this study we show by laser dissection that septa that separate the first and second compartment of intact growing hyphae of six *Aspergillus* species are partly closed. For instance, about 50% of the septa of *Aspergillus niger* were open during standard growth conditions. Septal plugging increased when *A. niger* was incubated below the optimal growth temperature or when exposed to hypertonic conditions. During these conditions, 10% and 17.5% of the septa were open, respectively. Changing the environmental conditions did not affect the incidence of septal plugging in the other aspergilli. A wild-type strain of *Aspergillus oryzae* showed heterogeneous *glaA* expression at the outer part of the colony. Two populations of hyphae could be distinguished in this zone of the mycelium; one that highly and one that lowly expresses *glaA.* In contrast, *glaA* expression showed a normal distribution in the case of a Δ*HEX1* strain that does not form Woronin bodies and therefore cannot plug septa. These data show that septal plugging causes hyphal heterogeneity in the mycelium by impeding cytoplasmic continuity.

Introduction

Hyphae of filamentous fungi grow at their tips and branch sub-apically. This and the phenomenon of hyphal fusion gives rise to a network of interconnected hyphae called a mycelium. A vegetative fungal mycelium is heterogeneous with respect to gene expression, growth and secretion (Wösten et al. 1991; Moukha et al., 1993a, b; Teertstra et al., 2004; Vinck et al., 2005, 2011; Masai et al., 2006; Levin et al., 2007a, b; Kasuga and Glass, 2008; Extebeste et al., 2009; de Bekker et al., 2011a, b). For instance, only part of the hyphae at the periphery of a vegetative mycelium of *Aspergillus niger* secrete glucoamylase (Wösten et al. 1991). This is due to heterogeneous expression of *glaA* in this part of the colony. In fact, two populations of hyphae can be distinguished; those that highly and those that lowly express this gene (Vinck et al., 2005). Hyphae that show high *glaA* expression also highly express other genes encoding secreted proteins. Moreover, they have a high rRNA content and highly express the glyceraldehyde-3-phosphate dehydrogenase gene *gpdA* (Vinck et al., 2011). From these studies it was concluded that two populations of hyphae can be distinguished at the outer part of the vegetative mycelium; those with a "high" and those with a "low" transcriptional and translational activity. The low activity would be sufficient to support growth but a high activity would be needed to support secretion of high amounts of protein.

Fungi belonging to the Ascomycota and the Basidiomycota have hyphae that are compartmentalized by septa. The diameter of the septal pore varies between 50 and 500 nm allowing passage of cytosol and even organelles (Shatkin and Tatum, 1959; Moore and McAlear, 1962; Gull, 1978; Lew, 2005). Upon mechanical injury, septa of ascomycetes are plugged by Woronin bodies to prevent excessive cytoplasmic bleeding (Trinci and Collinge, 1974; Collinge and Markham, 1985; Jedd and Chua, 2000; Tenney et al., 2000; Soundarajan et al., 2004; Maruyama et al., 2005). The Woronin bodies of *Aspergillus nidulans* are usually located at the septal pore or in apical regions (Momany et al., 2002). In the case of *Neurospora crassa,*

they are tethered via WSC and Leashin to the cell cortex (Ng et al., 2009; Jedd, 2011). Woronin bodies originate from peroxisomes by Pex11 and WSC mediated budding (Jedd and Chua, 2000; Liu et al., 2008; Escaño et al., 2009). Pex14 plays an important role in biogenesis of Woronin bodies in *N. crassa* by mediating import of HEX1 in the organelle (Managadze et al., 2007). The lumen of Woronin bodies of this fungus is filled with hexagonal rods of the HEX1 protein (Hoch and Maxwell, 1974; Jedd and Chua, 2000; Managadze et al., 2010). Deletion of *HEX1* in *N. crassa*, *Magnaporthe grisea* and *A. oryzae* results in the absence of Woronin bodies (Jedd and Chua, 2000; Tenney et al., 2000; Soundararajan et al., 2004; Maruyama et al., 2005).

In Chapter 3 it was shown that part of the septa of intact vegetative hyphae of *Aspergillus oryzae* are in a closed state. It was shown that plugging is reversible, that it is not affected by environmental conditions and that it is mediated by Woronin bodies. In this study it is shown that part of the septa of five other *Aspergillus* species are also plugged during vegetative growth. Notably, septal plugging is influenced by temperature and hypertonic conditions in *A. niger*. It is also shown that septal plugging causes hyphal heterogeneity in *A. oryzae*.

Material and Methods

Strains and growth conditions

Strains used in this study are listed in Table 1. The strains of *Aspergillus clavatus*, *A. oryzae*, *Aspergillus terreus*, *Aspergillus fumigatus* and *Neosartorya fischeri* have been sequenced. *A. niger* N402 was derived as a short conidiophore mutant from *A. niger* strain NRRL 3 (Δ*cspA1*; Bos et al., 1988), which has been sequenced (www.integratedgenomics.com; Baker, 2006). Strains NSRKu70-1-1AS and NSRK-ΔHx5 have been described in Chapter 3 and were constructed from the parental strain NSRku70-1-1A (Escaño et al., 2009). Strains RB#140.1 and RB#140.2 are derivatives of *A. oryzae* NSRKu70-1-1AS (Δ*ku70*; Chapter 3) that express *sGFP* under

Table 1: Strains used in this study. Wild-type (WT) strains have been sequenced as well as the parental strain of *A. niger* N402.

Strain	Parental strain	Genotype	Reference
Aspergillus oryzae RIB40		WT	Machida et al., 2005
Aspergillus clavatus NRRL 1		WT	www.aspgd.org
Aspergillus terreus NIH 2624		WT	www.aspgd.org
Aspergillus fumigatus Af293		WT	Nierman et al., 2005,
Neosartorya fischeri NRRL 181		WT	www.aspgd.org
Aspergillus niger N402	NRRL 3	Δ*cspA1*	Bos et al., 1988
A. niger N402Δ*Anhex1*	N402	Δ*cspA1* Δ*Anhex1*	This study
NSRKu70-1-1AS	NSRku70-1-1A	Δ*ku70*	Chapter 3
NSRK-ΔHx5	NSRku70-1-1A	Δ*ku70* Δ*Aohex1*	Chapter 3
RB#140.1	NSRKu70-1-1AS	Δ*ku70 glaA::eGFP*	This study
RB#140.2	NSRKu70-1-1AS	Δ*ku70 glaA::eGFP*	This study
RB#141.3	NSRK-ΔHx5	Δ*ku70* Δ*Aohex1 glaA::eGFP*	This study
RB#141.4	NSRK-ΔHx5	Δ*ku70* Δ*Aohex1 glaA::eGFP*	This study

the control of the *A. niger glaA* promoter. Similarly, RB#141.3 and RB#141.4 are derivatives of *A. oryzae* NSRK-ΔHx5 (Δ*ku70* Δ*Aohex1*; Chapter 3) that express *sGFP* under the control of the *A. niger glaA* promoter.

To obtain spores for inoculation, fungi were grown on 3.7% PDA (Potato Dextrose Agar, Sigma Aldrich, www.sigmaaldrich.com). Spores were harvested in 0.9% NaCl (w/v) containing 0.05% (v/v) Tween-20 and diluted to a final concentration of $5*10^5$ spores ml^{-1}. Microscopy dishes were inoculated with 0.5 µl spore suspension, whereas 2 µl was used for plates and sandwiched cultures.

For microscopy, fungi were grown in glass bottom dishes (MatTek, www.glass-bottom-dishes.com, P35G-1.5-20-C) essentially as described by van Peer et al. (2009). Fungi were grown on CD + Met medium (0.3% $NaNO_3$, 0.2% KCl, 0.1%, KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.002%, $FeSO_4.7H_2O$, 1% glucose, 0.0015% methionine, pH 5.5; Maruyama et al., 2010). To this end, the glass bottom dishes were filled with 30 µl CD + Met medium containing 1% agarose. The glass bottom dishes and the agarose medium were pre-warmed at 50 °C and 60 °C, respectively. Spores (0.5 µl, 500 spores/µl) were placed in the middle of an 18 mm cover glass and placed upside down on the non-solidified agar medium. This resulted in a 118 µm thick agarose layer. After solidifying the agarose medium, 2 ml liquid medium was added on top of the culture. For the septal plugging experiments, *Aspergillus* species were grown at 25, 30 or 37 °C for three days under water saturating conditions. In all other cases cultures were grown for 2 days at 30 °C under water saturating conditions, after they were either or not exposed to stress conditions. Temperature stress was imposed by incubation at 4 °C for 30 min or 2 h or at 45 °C for 30 min. For pH stress, the liquid medium was replaced by standard medium with a pH of 2.0 or 11.0 and subsequent incubation for 1.5 hours. The pH of the medium was adjusted with HCl or NaOH, respectively. Starvation stress was exposed from t = 0 onwards during 2 days of growth. For carbon-starvation, solid medium contained 0.2% glucose and liquid medium contained no glucose. For nitrogen-starvation solid medium contained nitrogen, but liquid medium did not contain a nitrogen source.

Transformation

Protoplasting of *A. oryzae* and *A. niger* was performed according to de Bekker et al. (2009) and transformed using polyethylene glycol (Punt and van den Hondel, 1992). *A. oryzae* strain NSRKu70-1-1AS and NSRK-ΔHx5 were co-transformed with pAN52-10S65TGFPn/s (Siedenberg et al., 1999) and pNR10 (Yoon et al., 2010). These plasmids contain *sGFP*(S65T) under the regulation of the *glaA* promoter of *A. niger* and *niaD* under the control of the *amyB* promoter of *A. oryzae*, respectively. *A. oryzae* nitrate prototrophic (*niaD*$^+$) strains were selected on MMS medium (minimal medium pH 6.0, 0.95 M sucrose and 1.5% agar; de Bekker et al., 2009).

Construction of the *Anhex1* deletion strain of *A. niger*

The 0.9 kb upstream flanking region of the *Anhex1* (An07g04570) open reading frame was amplified with the primers BN090 and BN091 (Table 2) and N402 genomic DNA as template and inserted into pDONR™ P4-P1R (Invitrogen, www.invitrogen.com) by BP recombination reaction generating the 5' entry clone plasmid, pDONR_P-Hex1.

Table 2: Primers used in this study.

Primer name	Sequence
BN088	GGGGACAGCTTTCTTGTACAAAGTGGGCCCACACATCCACCTTCCC
BN089	GGGGACAACTTTGTATAATAAAGTTGACAAGGACACCTCCAGCTCTTC
BN090	GGGGACAACTTTGTATAGAAAAGTTGCAGCCCAAGTGGAGAGCGACAAG
BN091	GGGGACTGCTTTTTTGTACAAACTTGCTTGCGAGAAGGAGGGGAATCAA
hygroFW	GGGGACAAGTTTGTACAAAAAAGCAGGCTAGGATTTCGGCACGG
hygroREV	GGGGACCACTTTGTACAAGAAAGCTGGGTTGTGGAGTGGGCGCTTACAC

The 1.0 kb downstream flanking region of the gene was amplified with the primers BN088 and BN089 (Table 2) and N402 genomic DNA as template and inserted into pDONR™ P2R-P3 (Invitrogen) by BP recombination reaction generating the 3' entry clone plasmid, pDONR_T-Hex1. The hygromycin resistance cassette (*hygR*) was amplified with the primers hygroFW and hygroREV (Table 2) and pAN7.1 (Punt et al., 1987; Punt and van den Hondel, 1992) plasmid DNA as template and inserted into pDONR™ P1-P2R (Invitrogen) by BP recombination reaction generating the center entry clone plasmid, pDONR_hygR. The underlined sequences show the Multisite Gateway *attB* recombination sites (Table 2). The obtained 5' and 3' entry clones together with the center entry clone plasmid pDONR_hygR (containing the *hygR* marker gene) were subjected with LR clonase in the presence of pDEST R4-R3 (destination vector) to obtain the final plasmid pΔAnHex1. The *Anhex1* gene disruption fragment (5.0 kb) was prepared by PCR using the plasmid pΔAnHex1 as template and primers BN090 and BN089 and was introduced into *A. niger* strain N402, generating strain N402Δ*Anhex1*. MMS medium containing 100 µg/ml hygromycin was used for selection of *hygR*$^+$ transformants. Disruption of the *Anhex1* gene was confirmed by Southern blotting using restriction enzymes *Bam*HI and *Kpn*I.

Heterogenic expression of GFP under the control of the *glaA* promoter

A. oryzae was grown on minimal medium (0.6% $NaNO_3$, 0.15% KH_2PO_4, 0.05% KCl, 0.05% $MgSO_4.7H_2O$, 0.2 ml/l Vishniac (per liter: 10 g EDTA, 4.4 g $ZnSO_4$, 1.01 g $MnCl_2$, 0.32 g $CoCl_2$, 0.315 g $CuSO_4$, 0.22 g ammonium heptamolybdate, 1.47 g $CaCl_2$ and 1.0 g $FeSO_4$; Vishniac and Santer, 1957), pH 6.0; de Vries and Visser, 1999) containing 3% agar and 200 mM xylose as a sandwiched culture (de Bekker et al., 2011a). To this end, a polycarbonate membrane (76 mm; Profiltra, www.profiltra.nl) was placed on top of the solidified medium and was inoculated in the middle. After 24 h a Lumox membrane (20 x 20 mm, manually cut; Greiner Bio-One, www.greinerbioone.com) was placed on top of the culture with the hydrophobic side facing the colony. After 42 h of growth, the sandwiched colony was transferred for 6 h to minimal medium plates containing 25 mM maltose. The Lumox membrane was removed and a piece of the polycarbonate membrane (approximately 10 x 10 mm) carrying the colony was cut out and placed up-side-down in a glass bottom microscopy dish (MatTek, P35G-1.5-20-C) on a 20 µl drop of minimal medium. GFP fluorescence was monitored on an inverted Zeiss LSM 5 system (Carl Zeiss, www.zeiss.de) equipped with a Plan-Neofluar 16x/0.5 oil immersion objective. GFP was excited with a 488 nm laser and images were captured as a Z-stack of optical

slices (pinhole 1 airy unit; optimal interval 2.02 µm; 4x line average; 8 bit scan depth). Maximum intensity projections of the Z-sections (1024 x 1024 pixels) were used for further analysis. The fluorescence intensity was quantified by measuring the mean pixel values of hyphae using a macro in the KS400 software (Version 3.0; Carl Zeiss Vision, www.zeiss.de). Leading hyphae were selected by hand and fluorescence was automatically quantified as the sum grey value per hypha with the background value from an equivalent area subtracted (Vinck et al., 2005). Signals were normalized with a custom Python script by dividing single hyphal fluorescence by the total fluorescence of all selected hyphae per image. The normalized data were log transformed and subsequently modelled using 5 parameters (p, μ_1, σ_1, μ_2 en σ_2) as described (Vinck et al., 2005) to examine whether hyphal fluorescence followed a bimodal distribution. 95% Confidence Intervals (CI) of the parameters were estimated by bootstrapping (1000 replicates). Customs scripts in the Scilab programming language were used to fit the data (web.science.uu.nl/microbiology/links/).

Analysis of plugging

Compartments were ruptured by laser dissection using the laser pressure catapulting function (LPC) of the P.A.L.M. laser dissecting microscope (Zeiss, www.zeiss.com). To this end, 60-70% of the power of the pulsed UV-laser was used. Each experiment was done *in duplo* using 20 hyphae in each experiment. ANOVA analysis was used with Bonferroni post-hoc correction when multiple comparisons were made between treatments or strains. ANOVA analysis was used with Dunnet's post-hoc correction when differences in septal plugging in a strain were assessed between a control condition and stress conditions. In all cases, a difference was assumed significant when $p < 0.05$.

Growth of N402 and N402Δ*Anhex1* during stress and recovery

A. niger strains N402 and N402Δ*Anhex1* were grown for three days as sandwiched colonies (Wösten et al., 1991) on CD + Met medium with 1.5% agar. Colonies were transferred and subjected to different stresses for 3 days. For temperature stress, colonies were transferred to fresh CD + Met medium with 1.5% agar and exposed to 4 °C or 45 °C. For acid stress and hypertonic conditions, colonies were transferred to ring plates (Levin et al., 2007b) containing liquid CD + Met medium with pH 2.0 or 1M $MgSO_4$ + 1% glucose, respectively. In the former case, the pH of the CD + Met medium was adjusted with HCl. For carbon- and nitrogen starvation, colonies were transferred to CD + Met medium with 1.5% agar without glucose and methionine, or without sodium nitrate and methionine, respectively. For high pH and hypotonic conditions, colonies were transferred to CD + Met medium with 1.5% agar with a pH of 11.0 or to 1.5% agar + 1% glucose, respectively. In the former case, the pH of the CD + Met medium was adjusted with NaOH. To recover, colonies were transferred to fresh CD + Met medium with 1.5% agar and were grown for two days. Colony diameter was measured after 3, 6, and 8 days from the start of the experiment. ANOVA analysis was used with Dunnet's post-hoc correction when differences in growth were assessed between a control condition and stress conditions. An independent-samples T-test with a Levene's test was used when growth of

Table 3: Colony diameter (mm) of aspergilli when grown at different temperatures. Aspergilli were grown on CD + Met medium for four days.

Temperature		15 °C			25 °C			30 °C			37 °C		
Strain	Growth (days)	2	3	4	2	3	4	2	3	4	2	3	4
A. clavatus		0	2	4	7	12	17	9	15	21	3	6	9
A. niger		0	0	2	7	11	16	9	14	19	9	19	27
A. oryzae		0	0	0	10	15	24	12	20	34	12	21	31
A. terreus		0	0	0	3	6	13	7	14	24	14	25	38
N. fischeri		0	0	0	8	15	24	14	23	38	22	37	55
A. fumigatus		0	0	0	5	9	16	10	18	30	16	33	47

two strains was compared for one condition. In all cases, a difference was assumed significant when $p < 0.05$.

Results

Septal plugging occurs in growing vegetative hyphae of a variety of aspergilli

Wild-type strains of *A. oryzae*, *A. clavatus*, *A. terreus*, *A. fumigatus, N. fischeri* and the *A. niger* N402 strain were grown for 4 days on CD + Met medium at 15, 25, 30 and 37 °C. At 15 °C only *A. clavatus* grew, whereas all fungi were able to grow at 25-37 °C (Table 3). The optimum temperature for *A. niger*, *A. terreus*, *A. fumigatus* and *N. fischeri* was 37 °C. In contrast, *A. oryzae* and *A. clavatus* had an optimum growth temperature of 30 °C.

Septal plugging of intact growing vegetative hyphae was determined at different growth temperatures using CD + Met medium. Apical compartments of leading hyphae were injured with laser dissection. Bleeding of cytoplasm from the sub-apical, adjacent compartment through the septum of the damaged compartment was monitored to assess whether it was open (see Figure 4 of Chapter 3). The septum was scored as 'open' when cytoplasm was moving through the septum towards the ruptured compartment (see Supplemental Movies Chapter 3). In the case cytoplasmic streaming was not observed, the septum was scored as 'closed'. Using these criteria, it was found that the septal plugging incidence at the optimal growth temperature of the aspergilli ranged from 37.5-80% (Figure 1) but these differences were not significant. Differences in the incidence of plugging of the first septum at the different growth temperatures were also not significant in the case of the selected aspergilli except for *A. niger*. For example, 87.5% ± 3.5, 80% ± 0 and 72.5% ± 3.5 of the *A.*

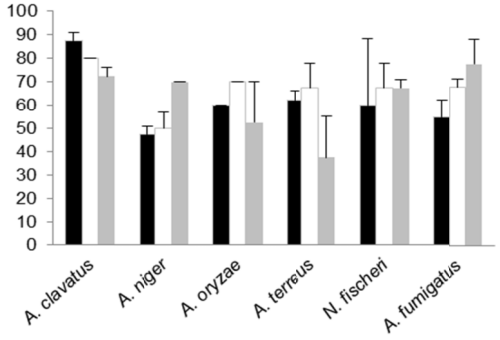

Figure 1: Percentage of open apical septa of leading hyphae of aspergilli that had been grown at different temperatures. Aspergilli were grown on CD + Met medium for three days. Black, white and grey shading indicate growth at 25, 30 or 37 °C, respectively. Error bars indicate standard deviations.

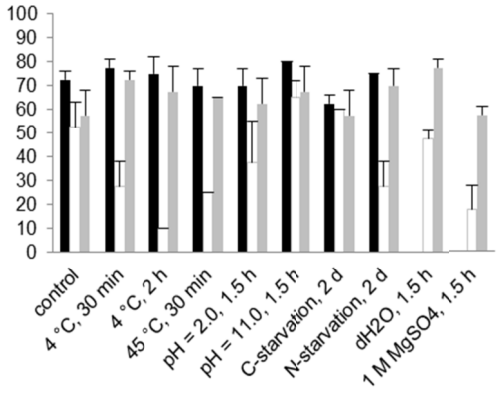

Figure 2: Percentage of open apical septa of leading hyphae of aspergilli that had either or not been subjected to stress. Aspergilli were grown for two days on CD + Met medium at 30 °C, after which they were either or not exposed to temperature stress, pH stress and hypo- or hypertonic conditions. Alternatively, strains were grown for two days with C- or N limitation. Black, white and grey shading represent *A. clavatus*, *A. niger* and *A. oryzae*, respectively. Error bars indicate standard deviations.

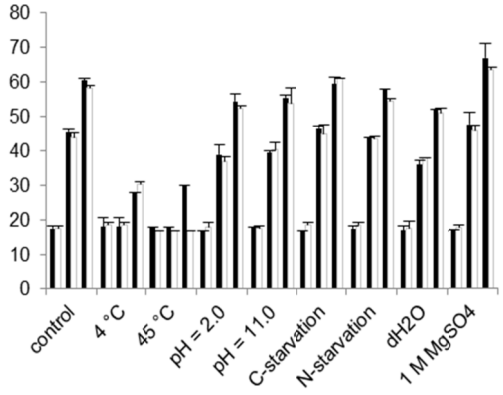

Figure 3: Colony diameter of *A. niger* strains N402 and N402Δ*Anhex1* after growth on CD + Met medium, after stress conditions and after recovery of stress. Strains N402 (black bars) and N402Δ*Anhex1* (white bars) were grown for three days on CD + Met medium at 30 °C (left set of bars). Subsequently, colonies were exposed to stress conditions for three days (middle set of bars) and then again exposed to standard growth conditions for two days (right set of bars). Colony diameter is shown in mm. Error bars indicate standard deviations.

clavatus septa were open at 25, 30 and 37 °C, respectively (Figure 1). In contrast, plugging in *A. niger* was significantly different after growth at 37 °C (70% ± 0) compared to 25 °C (47.5% ± 3.5) or 30 °C (50% ± 7.1).

The effect of other environmental conditions on septal plugging

A. clavatus, *A. niger* and *A. oryzae* were used to study the effect of stress on septal plugging. The aspergilli were grown on CD + Met medium for two days at 30 °C, after which they were subjected to several stresses. The apical compartments of leading hyphae of these colonies were injured with laser dissection to determine the incidence of septal plugging. No significant changes in plugging incidence were found in *A. clavatus* and *A. oryzae* when control growth conditions were compared to the stress conditions (Figure 2). In contrast, some of the environmental conditions affected the septal plugging incidence in *A. niger*.

In the control *A. niger* showed 52.5% ± 10.6 open septa (Figure 2). Incubation at 4 °C for 30 min showed no significant change (27.5% ± 10.6), while incubation for 2 h at 4 °C resulted in significant septal closing (10% ± 0). A trend for septal plugging was observed after a 30 min incubation at 45 °C. In this case only 25% ± 0 of the septa were still open ($p = 0.07$). Incubation for 2 h at 45 °C did not increase the plugging incidence (data not shown). Incubation with medium of low and high pH, and carbon- and nitrogen starvation did not result in an altered plugging incidence. For these conditions 37.5% ± 17.7, 65% ± 7.1,

60% ± 0 and 27.5% ± 10.6 of the septa were open. Incubation with H_2O for 1.5 hours (hypotonic conditions) showed 47.5% ± 3.5 open septa, while incubation with 1 M MgSO4 (hypertonic conditions) resulted in significant septal closing (17.5% ± 10.6; Figure 2).

Colony diameters of *A. niger* strains N402 and N402Δ*Anhex1* were similar (*i.e.* 17.4 ± 0.5 and 17.8 ± 0.6 mm, respectively) (Figure 3) after 3 days growth on CD + Met medium. At this time point, colonies were either or not subjected to stress conditions for three days. In all cases, colony diameters of N402 and N402Δ*Anhex1* were not significantly different (Figure 3). Radial growth of N402 and N402Δ*Anhex1* was not affected when grown with C- or N- limitation (biomass did decrease in both strains though) or during hypertonic conditions. In contrast, the diameter of the colonies had increased less, if at all, at low and high temperature, low and high pH and hypotonic conditions when compared to the control. Colonies of both strains continued or reinitiated growth after they were transferred from the stress condition to the standard growth condition. There was one exception. In contrast to N402, N402Δ*Anhex1* was not able to restart growth after incubation at 45 °C (Figure 3). This shows that Woronin bodies are essential to survive high temperature stress.

Heterogenic expression from the *glaA* promoter is abolished in a Δ*Aohex1* strain

Construct pAN52-10S65TGFPn/s (Siedenberg et al., 1999) encompassing the *sGFP* gene under control of the *glaA* promoter of *A. niger* was introduced in strains NSRKu70-1-1AS and NSRK-ΔHx5 of *A. oryzae.* Transformants were screened by fluorescence microscopy and two representative strains of each transformation were selected. These strains were called RB#140.1 and RB#140.2 (Δ*ku70* background) and RB#141.3 and RB141.4 (Δ*ku70* Δ*Aohex1* background). Hyphal heterogeneity of GFP expression in colonies of these strains was assessed by modelling fluorescence intensity distributions assuming the existence of two populations of hyphae (*i.e.* one highly and one lowly expressing GFP) (Figure 4). The confidence intervals of the populations lowly (μ_1) and highly (μ_2) expressing GFP were not overlapping in *A.*

Table 4: 95% CI of the mean after bootstrapping. N = sample size, CI ($\mu_{1\ or\ 2}$) = confidence interval lower- and upper limits of population 1 or 2. CI (pf_1) = confidence interval lower- and upper limits of participation fraction of population 1.

Strains	Background	N	CI (μ_1)	CI (μ_2)	CI (pf_1)
RB#140.1	Δ*ku70*	203	-0.10 ; 1.18	1.43 ; 2.30	0.078 ; 0.892
RB#140.2	Δ*ku70*	209	-0.18 ; 1.19	1.23 ; 2.71	0.041 ; 0.955
RB#141.3	Δ*ku70* Δ*Aohex1*	247	-2.00 ; 1.27	0.65 ; 3.04	0.005 ; 0.972
RB#141.4	Δ*ku70* Δ*Aohex1*	242	-2.21 ; 1.31	1.01 ; 2.73	0.007 ; 0.942

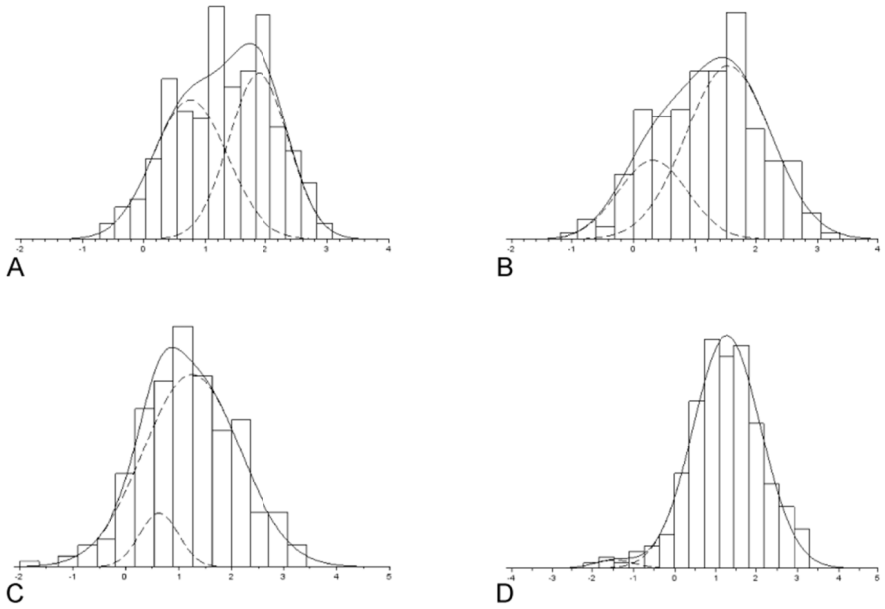

Figure 4: Fluorescence intensity distributions resulting from *glaA* driven GFP expression in *A. oryzae* strains with (A, B) or without Woronin bodies (*ΔAohex1*) (C, D). Data were fitted assuming the existence of bimodal distributions.

oryzae strains RB#140.1 and RB#140.2 that contain Woronin bodies (Table 4). This showed that *glaA* driven expression of GFP is heterogeneous in these strains. In contrast, the confidence intervals of the two populations of strains RB#141.3 and RB#141.4, both having the *Aohex1* deletion, did overlap. Thus, the assumption that GFP is heterogeneously expressed in these strains was falsified (Figure 4, Table 4). These data show that *glaA* of *A. niger* is heterogeneously expressed in hyphae of *A. oryzae* and that Woronin bodies maintain the heterogeneity in the *A. oryzae* mycelium.

Discussion

Previously, it has been shown that part of the septa in intact vegetative hyphae of the basidiomycete *Schizophyllum commune* and the ascomycete *A. oryzae* are closed (van Peer et al., 2009; Chapter 3). Here, it was shown that this was also the case for five other aspergilli. Septal plugging may thus be a general phenomenon in the higher fungi. As shown in this report, septal plugging causes hyphal heterogeneity and impacts survival when exposed to particular environmental conditions.

At least two populations of hyphae can be distinguished at the periphery of the *A. niger* colony based on their transcriptional and translational activity (Vinck et al., 2005, 2010). It was proposed that the transcriptional and translational activity in the "low-expressing" hyphal population is sufficient to support growth. The activity in

the "high-expressing" hyphal population would be needed to secrete high levels of protein. In this study it was shown that *glaA* of *A. niger* is also heterogeneously expressed in *A. oryzae.* This is the second filamentous fungus where the phenomenon of hyphal heterogeneity has been shown to occur. Hyphal heterogeneity conflicts the general accepted view that the cytoplasm of fungi is continuous due to the porosity of septa (Jennings et al., 1974, 1987). However, the fact that about 35% of the septa is closed in the case of aspergilli implies that the cytoplasm of two hyphal compartments have a change of about 7,5% to be in physical contract when they are separated by 6 septa. This would promote hyphal heterogeneity because of the strongly reduced cytoplasmic streaming. Indeed, two populations of hyphae could not been shown to exist in the Δ*Aohex1* mutant. By increasing or decreasing the incidence of septal plugging, fungi would be able to regulate hyphal heterogeneity. In Chapter 3 it was shown that this process is reversible. The fact that the incidence of septal plugging was not significantly different in 5 selected aspergilli during different environmental (stress) conditions suggests the absence of a regulatory mechanism to plug septa. However, the incidence of septal plugging was significantly increased in *A. niger* when this fungus was exposed to hypertonic conditions or to temperatures below the optimal growth temperature. Moreover, there was a trend towards closure of septa during exposure to 45 °C. Why *A. niger* and not the other aspergilli shows increased plugging of septa is subject of future studies. It is interesting to note that from the 6 aspergilli used in this study, *A. niger* can grow in the widest range of temperature, pH and water activity (Krijgsheld et al., 2012; Chapter 1). *A. niger* is not an exception in the fungal kingdom with respect to its ability to change the incidence of plugging as a response to environmental conditions. In *S. commune* it was shown that environmental conditions can induce septal plugging as well. For example, heat shock, hypertonic shock, incubation with an antibiotic or ethanol promoted septal plugging. Closure of septa after heat shock was shown to be reversible (van Peer et al., 2009).

So far, it is not clear why colonies send out exploring hyphae that are heterogenic with respect to transcriptional and translational activity. Possibly, this increases the chance that (some of the) hyphae survive when a colony is exposed to stress conditions like the presence of antibiotics, reactive oxygen species or high temperature. Deletion of a gene encoding the Woronin body associated transmembrane protein TmpL in *Alternaria brassicicola* and *A. fumigatus* results in hypersensitivity to oxidative stress (Kim et al., 2009). Moreover, a Δ*HEX1* strain of *M. grisea* is not able to survive nitrogen starvation (Soundararajan et al., 2004). Of interest, the *A. niger* Δ*Anhex1* strain died when exposed for three days to 45 °C, whereas the strain with Woronin bodies did survive this condition. This may be due to the phenomenon of hyphal heterogeneity, which promotes variation in physiology between hyphae. This variation may increase the chance that particular hyphae survive a particular stress and can proceed with extension of the mycelium.

Acknowledgments

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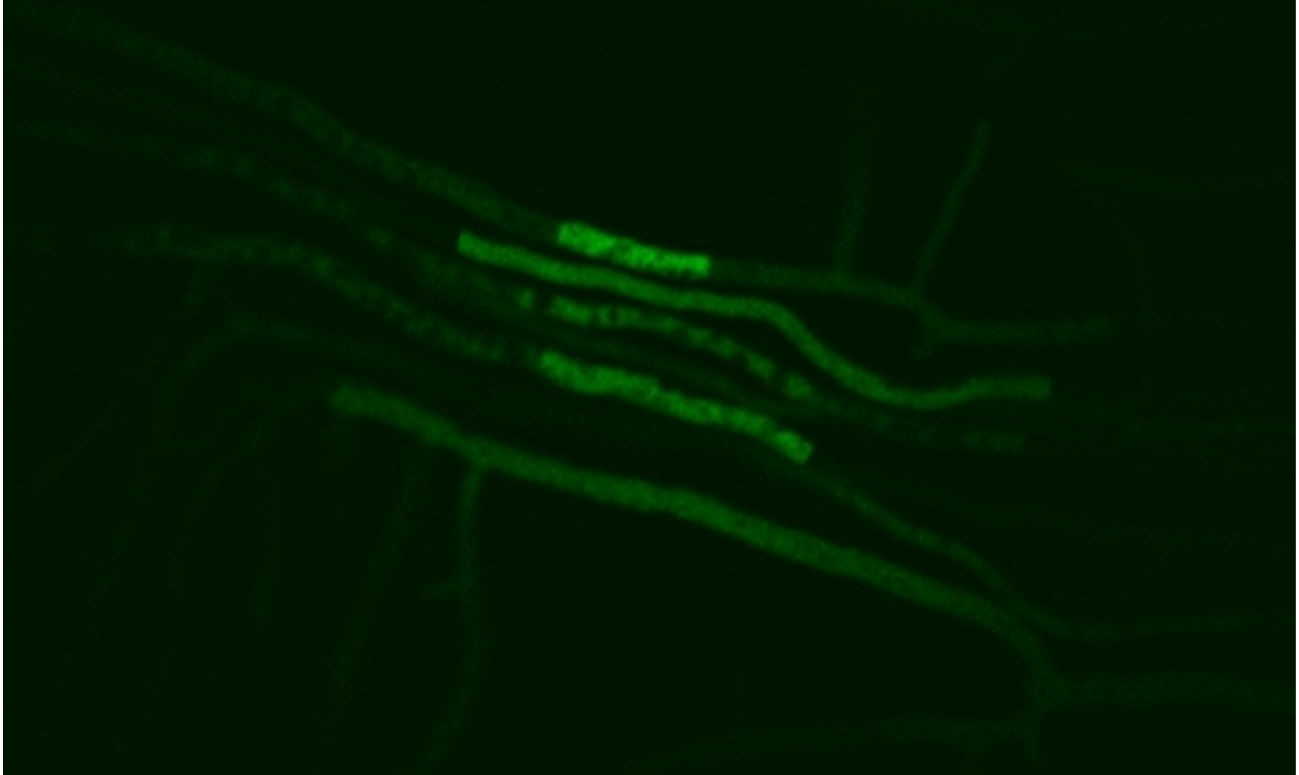
Chapter 5

Cytosolic protein streaming, but not sugar transport, depends on septal plugging in *Aspergillus*



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Parts of this chapter have been submitted.

Abstract
Hyphae in the mycelium of ascomycetes are compartmentalized by septa. The central pore in these septa allows for cytoplasmic streaming. Previously, it was shown that the central pore is closed by Woronin bodies in approximately 35% of the septa of intact hyphae of *Aspergillus*. This finding impacts the concept of cytoplasmic continuity of the fungal mycelium. In this study inter- and intra-cellular streaming of glucose and GFP was studied. It is shown that glucose and its inert analogue 3-OMG stream from the center to the periphery of the *Aspergillus* colony, but not vice versa. Streaming was identical in strains without Woronin bodies (Δ*HEX1*). This shows that long distance transport of glucose is not affected by septal plugging. Short distance intercellular streaming of glucose was also not affected by septal plugging. The fluorescent analogue of glucose 2-NBDG was still streaming to neighboring compartments under conditions that septa had plugged (*i.e.* after incubation for 2 h at 4 °C or 45 °C). In contrast, short distance streaming of photo-activatable GFP (PA-GFP) was affected by closure of septal pores. PA-GFP always streamed from the second to the most apical compartment. In contrast, inter-compartmental streaming was not observed in 70% of the cases when PA-GFP had been activated in the 10th compartment. Inter-compartmental streaming from the 2nd compartment was also rarely observed, if observed at all, when colonies had been exposed for 2 h at 4 °C or 45 °C.

Introduction
Filamentous fungi form mycelia consisting of branched and interconnected hyphae. These hyphae grow by apical extension. Hyphae are compartmentalized by invaginations of the cell wall, called septa. These septa allow cytoplasmic streaming since they contain a central pore of approximately 50-500 nm (Shatkin and Tatum, 1959; Moore and McAlear, 1962; Gull, 1978; Lew, 2005). However, in aspergilli approximately 35% of the septa of intact vegetative hyphae are plugged by Woronin bodies (Chapter 3 and 4). As a result, the cytoplasm of two hyphal compartments has a chance of less than 10% to be in physical contract when they are separated by 6 septa. Thus, cytoplasmic continuity in an *Aspergillus* mycelium is restricted. Consequently, hyphal heterogeneity is maintained (Chapter 4).

Here, inter- and intra-compartmental streaming of glucose and GFP was studied. It is shown that long and short distance flow of glucose is independent of septal plugging but that short distance streaming of GFP is affected by the plugging state of the septa.

Material and methods

Strains and growth conditions
Strains used in this study are listed in Table 1. *A. niger* N402 was derived as a short conidiophore mutant from *A. niger* strain NRRL 3 (Δ*cspA1*; Bos et al., 1988). The N402Δ*Anhex1* strain has an *Anhex1* deletion and was previously described (Chapter 4). AB4.1 (Δ*cspA1 pyrA*$^{-}$) (van Hartingsveldt et al., 1987) and N593 (Δ*cspA1 pyrA*$^{-}$) (Goosen et al., 1987) are derivatives of N402 that contain an inactive *pyrA* gene. The

Table 1: Strains used in this study.

Strain	Parental Strain	Genotype	Auxotrophy	Reporter plasmid	References
N402	NRRL 3	Δ*cspA1*			Bos et al., 1988
AB4.1	N402	Δ*cspA1*	*pyrA*$^{-}$		van Hartingsveldt et al., 1987
N593	N402	Δ*cspA1*	*pyrA*$^{-}$		Goosen et al., 1987
RB#7.1	AB4.1	Δ*cspA1*		pRB012 (P*mpdA*::*sGFP*)	This study
RB#13.1	AB4.1	Δ*cspA1*		pRB013 (P*gpdA*::*gpdA*::*PA-GFP*)	This study
RB#14.5	AB4.1	Δ*cspA1*		pRB014 (P*gpdA*::*PA-GFP*)	This study
RB#77.1	N593	Δ*cspA1*		pRB077 (P*hyp1*::*sGFP*)	This study
N402Δ*Anhex1*	N402	Δ*cspA1* Δ*Anhex1*			Chapter 4
NSRKu70-1-1AS	NSRku70-1-1A	Δ*ku70*	*niaD*$^{-}$		Chapter 3
NSRK-ΔHx5	NSRku70-1-1A	Δ*ku70* Δ*Aohex1*	*niaD*$^{-}$		Chapter 3

Δ*cspA1* strains RB#14.5, RB#13.1 and RB#7.1 are based on AB4.1. RB#14.5 expresses *PA-GFP* (Patterson and Lippincott-Schwartz, 2004) from the *gpdA* promoter of *A. nidulans.* RB#13.1 expresses the gene encoding the fusion protein of glyceraldehyde-3-phosphate dehydrogenase and PA-GFP (GPD-PA-GFP) from the *gpdA* promoter of *A. niger*. RB#7.1 expresses *sGFP* from the *mpdA* (mannitol-1-phosphate dehydrogenase) promoter of *A. niger*. Strain RB#77.1 is based on strain N593. It expresses *sGFP* from the promoter of the hydrophobin gene *hyp1* of *A. niger* (An07g03340; Pel et al., 2007; Jensen et al., 2010). *A. oryzae* strains NSRK-ΔHx5 and NSRKu70-1-1AS were previously described (Chapter 3). The former strain contains an *Aohex1* deletion and the latter is a control strain both having *niaD*- auxotrophy.

To obtain spores for inoculation, strains were grown on 3.7% PDA (Potato Dextrose Agar, Sigma Aldrich, www.sigmaaldrich.com). Spores were harvested in 0.9% NaCl (w/v) containing 0.05% (v/v) Tween-20 and were routinely diluted to a final concentration of $5*10^5$ spores ml^{-1}. In the case of sandwiched cultures that were used for long-distance glucose transport, spores were diluted to $1*10^8$ spores ml^{-1}. *A. niger* and *A. oryzae* strains were grown at 30 °C unless described otherwise.

Construction of plasmids

DNA fragments were amplified by PCR using Phusion® High-Fidelity DNA polymerase (Finnzymes; www.finnzymes.com), *A. niger* N402 chromosomal DNA or pRSETA-WEGFPH plasmid DNA (Patterson and Lippincott-Schwartz, 2002) as template, and oligonucleotides presented in Table 2. The blunt end PCR products of the promoters of *mpdA* and *hyp1*, the ORF of PA-GFP, and the promoter and ORF of *gpdA* were inserted in the *Sma*I site of pUC19. This resulted in plasmids pRB015, pRB075, pRB011 and pRB009, respectively. The cloned fragments were cut out of pRB015, pRB075, and pRB009 with *Not*I/*Nco*I. pRB009 was first partially digested with *Nco*I, since it contains an internal *Nco*I site. The fragments were ligated in p*GPD*GFP (Lagopodi et al., 2002) that had been digested with *Not*I and *Nco*I. This resulted in pRB012, pRB077 and pRB010, respectively. The ORF of PA-GFP was cut out of pRB011 with *Nco*I/*Hin*DIII and ligated in the respective sites of pRB010 and p*GPD*GFP, generating pRB013 and pRB014, respectively. These plasmids express the

Table 2: Primers used in this study. Restriction sites used for cloning are underlined.

Gene ID	Primer name	Sequence
mpdA (An02g05830)	PmpdA-FW-NotI	GCGGCCGCTAACAGTAGAATCTCTTTTG
	PmpdA-REV-NcoI	CCATGGGATGTAGTGGAAGTCTGATAGTAGAAGGGAG ATAGAGTGTAATTG
gpdA (An16g01830)	gpdA(full)-FW-NotI	GCGGCCGCTCCAGAAAGGAG
	gpdA(full)-REV-NcoI	CCATGGGGCATCAACCTTGG
hyp1 (An07g03340)	Phyp1-FW-NotI	GCGGCCGCGGCCCTCTAATAGCTAACAAGGAACTAGG G
	Phyp1-REV-NcoI	CCATGGTGTGACGGTGGGTTTGGAATTG
PA-GFP	PA-GFP-FW-NcoI	CCATGGTGAGCAAGGGCGAGG
	PA-GFP-REV-HindIII	AAGCTTACTTGTACAGCTCGTCCATGCCG

genes encoding PA-GFP-GPD and PA-GFP under the control of the *A. niger gpdA* and the *A. nidulans gpdA* promoters, respectively.

Transformation

Protoplasting of *A. niger* was performed according to de Bekker et al. (2009) and transformed using polyethylene glycol (Punt and van den Hondel, 1992). AB4.1 and N593 were co-transformed with pGW635 that contains *pyrA* as a selection marker (Goosen et al., 1989). Strains were selected on MMS medium (minimal medium pH 6.0, 0.95 M sucrose and 1.5% agar; de Bekker et al., 2009) based on *pyrA* prototrophy.

Monitoring Intra- and inter-compartmental streaming of GFP

RB#14.5 and RB#13.1 were grown as sandwiched cultures on minimal medium (0.6% $NaNO_3$, 0.15% KH_2PO_4, 0.05% KCl, 0.05% $MgSO_4.7H_2O$, 0.2 ml/l Vishniac (per liter: 10 g EDTA, 4.4 g $ZnSO_4$, 1.01 g $MnCl_2$, 0.32 g $CoCl_2$, 0.315 g $CuSO_4$, 0.22 g ammonium heptamolybdate, 1.47 g $CaCl_2$ and 1.0 g $FeSO_4$; Vishniac and Santer, 1957), pH 6.0; de Vries et al., 2004) containing 25 mM maltose and 3% agar. To this end, plates were inoculated in the middle with 2 µl spore suspension. After 24 h of growth, a polycarbonate membrane (76 mm; Profiltra, www.profiltra.nl) was placed on top of the colony and growth was prolonged for another 24 h. For microcopy, the membrane was removed and pieces of the agar medium (10 x 10 mm) with the mycelium on top of it were excised and placed up-side-down on a drop of 100 µl minimal medium with 25 mM maltose. Growth was prolonged for 1 h.

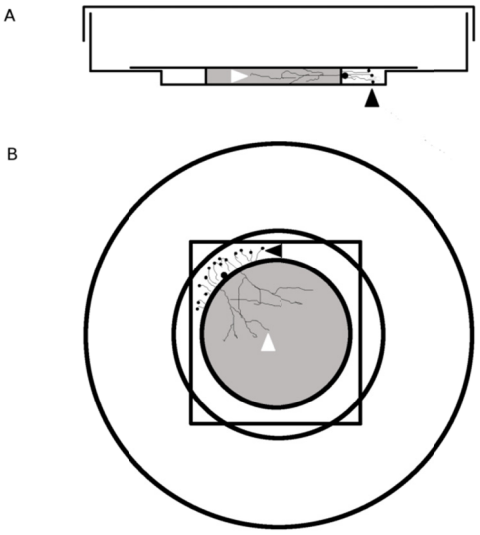

Figure 1: Schematic representation of the culture method to grow vegetative hyphae and conidiophores in a near two-dimensional way. Front view (A). Top view (B). Square represents cover glass (B). Gray indicates agarose medium. Black dot indicates point of inoculation. Conidiophores and vegetative hyphae are indicated by black- and white arrow heads, respectively.

RB#7.1 and RB#77.1 were grown for 3 days as sandwiched cultures under water saturated conditions. To this end, glass bottom dishes (MatTek, www.glass-bottom-dishes.com, P35G-1.5-20-C) were filled with 200 µl minimal medium (pre-warmed at 60 °C) containing 1% agarose and 25 mM maltose. On top of the medium, an 18 x 18 mm cover glass was placed. After the medium had solidified, 0.5 µl of spore suspension was placed next to the agarose medium (Figure 1). After three days, hyphae had grown in the agarose

medium and conidiophores had formed at the medium/air interface.

PA-GFP was activated in a region of 20 x 30 µm by a 5 sec exposure to a 405 nm laser line with 3.75 mW laser power and a pinhole of 3.06 airy units. An inverted Zeiss LSM 5 system was used for imaging in combination with a Plan-Neofluar 25x/0.8 Imm corr objective (Zeiss, www.zeiss.com) with oil immersion. Time lapse movies of PA-GFP fluorescence were made for 2 min using a 488 nm laser with 4.73 mW power, a pin hole of 3.29 airy units, a pixel dwell time of 3.20 µs and a LP 505 filter. Image resolution was 512 x 512 pixels. The number of hyphae measured in each experiment is indicated in the text.

To determine the rate of PA-GFP streaming, background fluorescence was measured for ten hyphae for each strain. Increase of fluorescence was monitored in time using the ROI tool from the PASCAL software (Zeiss). Rate of streaming was determined using the total distance of streaming and the time point at which fluorescence intensity was above twice the standard deviation of the background fluorescence. Data were statistically analyzed using an independent-samples T-test with a Levene's test. In all cases, a difference was assumed significant when $p < 0.05$.

Monitoring sugar up-take and short distance glucose transport

A. niger strains N402 and RB#14.5 were grown for 2 days as sandwiched cultures on minimal medium containing 3% agar and 25 mM maltose (de Bekker et al., 2011). A polycarbonate membrane (76 mm; Profiltra) was placed on top of the solidified medium and was inoculated with 2 µl spore suspension. After 24 h a Lumox membrane (20 x 20 mm, manually cut; Greiner Bio-One, www.greinerbioone.com) was placed on top of the culture with the hydrophobic site facing the colony. After 2 days the colony was transferred to solid minimal medium containing 10 µM 2-NBDG (2-(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)-2-deoxyglucose; Molecular Probes, www.invitrogen.com) without carbon source or with 25 mM maltose. This was either or not followed by incubation at 4 °C or 45 °C for 2 h. Glucose up-take was visualized by CLSM with an inverted Zeiss LSM 5 system using a Plan-Neofluar 25x/0.8 Imm corr objective (Zeiss) with oil immersion. A 488 nm laser was used for detection with 5 mW power, a pinhole of 1.1 airy units, a pixel dwelling time of 3.20 µs, and a LP530 filter. Images were taken with 512 x 512 pixels.

Recovery of fluorescence (FRAP) was monitored by CSLM making time-lapse movies of 2-5 min. Photo-bleaching was obtained by a 5 sec exposure to 12.5 mW 488 nm light using a pinhole of 1.1 airy units.

Monitoring long distance glucose transport

A. niger strains N402 and N402Δ*Anhex1*, and *A. oryzae* strains NSRKu70-1-1AS and NSRK-ΔHx5 were grown for 5 days as sandwiched cultures (Wösten et al., 1991) under water saturated conditions. The sandwiched colonies were grown on solidified (1.5% agar) minimal medium or CD + Met medium (0.3% $NaNO_3$, 0.2% KCl, 0.1%, KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.002%, $FeSO_4.7H_2O$, 1% glucose, 0.0015% methionine, pH 5.5; Maruyama et al., 2010) for *A. niger* and *A. oryzae* strains,

respectively, with 200 mM xylose as carbon source. Sandwiched cultures were inoculated with 1.5 µl spore solution. After 5 days of growth the cultures were transferred to fresh solid medium containing 25 mM maltose or to medium without carbon source. Colonies were labeled with 1 µCi of [^{14}C]D-maltose (specific activity, 2.04 gBq mmol^{-1}; MP Biomedicals, www.mpbio.com) or [^{14}C]3-O-methyl-D-glucose (specific activity, 2.04 gBq mmol^{-1}; MP Biomedicals) for 5 or 24 h. Label was absorbed to a piece of rice paper (diameter 20 mm) and placed on top of the sandwiched culture either at its center or periphery. After labeling, colonies were either or not fixed with 4% formaldehyde for 1 h at room temperature. Colonies were washed 3 times for 60 min with 100 mM D-maltose or 100 mM D-glucose at room temperature. After air-drying, colonies were exposed to X-OMAT films (Kodak, www.kodak.com).

Results

Long distance streaming of glucose

Sandwiched colonies of strain N402 were grown for 5 days on 200 mM xylose, after which they were transferred to medium containing 25 mM maltose or to medium without carbon source. After transfer, the center of the colonies was exposed for 5 or 24 h with ^{14}C-labeled maltose. D-maltose is extra-cellularly converted into glucose, which is taken up by the fungus and metabolized (van Kuyk et al., 2011). Autoradiography showed that the ^{14}C-glucose that had been taken up in the center had been transported towards the periphery of the colony (Figure 2A). Labeling was more intense when colonies had been labeled for 24 h instead of 5 h and when the medium did not contain carbon source. However, distribution patterns were similar

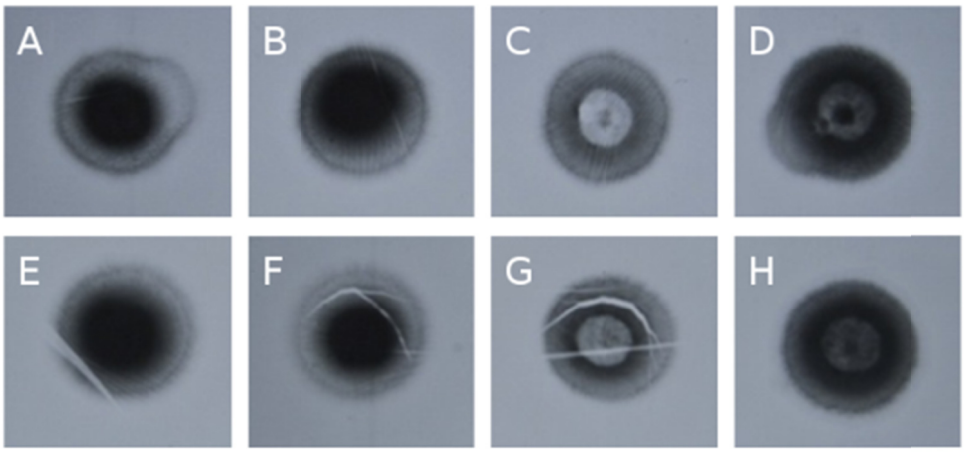

Figure 2: Fixed (A, C, E, G) and non-fixed (B, D, F, H) colonies of *A. niger* strains N402 (A-D) and N402Δ*Anhex1* (E-H) that had been labeled for 24 h at the center of the mycelium with ^{14}C maltose (A, B, E, F) or ^{14}C 3-O-methyl-D-glucose (C, D, G, H).

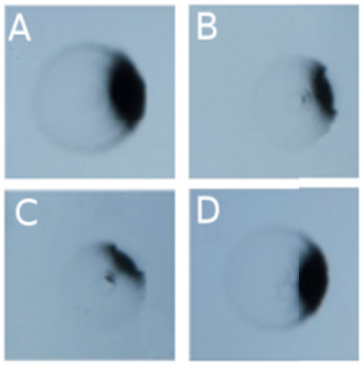

Figure 3: Wild-type N402 (A, C) and N402Δ*Anhex1* (B, D) colonies of *A. niger* that had been labeled for 24 h at the periphery of the mycelium with ^{14}C maltose (A, B) or ^{14}C 3-O-methyl-D-glucose (C, D).

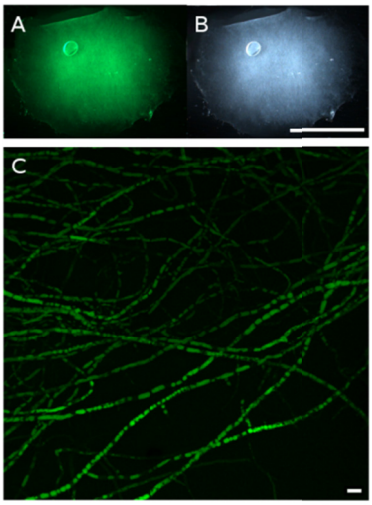

Figure 4: Glucose is taken up by the entire colony of *A. niger* and accumulates in vacuoles. Strain N402 was grown for two days as a sandwiched culture on minimal medium with 25 mM maltose. The colonies were transferred to minimal medium plates containing 10 µM 2-NBDG for 1 h. Distribution of 2-NBDG in a N402 colony (A). Bright field image (B). Distribution of 2-NBDG in peripheral hyphae of N402 as shown by a maximum intensity projection (C). Bars represent 5 mm (A, B) and 10 µm (C).

(data not shown). Labeling of N402Δ*Anhex1* was similar as strain N402 (Figure 2E). Apparently, absence of Woronin bodies does not affect transport of sugars through the colony.

By fixing colonies with formaldehyde and washing with non-labeled maltose (as in Figure 2A, E) only label is retained in the colony that has been incorporated in macro-molecules. Label in glucose or other small organic molecules is also being monitored by washing non-fixed colonies (Levin et al., 2007). No difference in the spatial distribution of label was observed between fixed and non-fixed colonies (Compare Figure 2A, E with Figure 2B, F). This shows that [^{14}C] glucose is readily incorporated into macro-molecules. The rapid metabolism of the sugar may impact its distribution through the mycelium. Therefore, colonies were labeled with the inert glucose analogue ^{14}C-3-*O*-methyl glucose (3-OMG). Distribution of 3-OMG in fixed wild-type and Δ*Anhex1* colonies was different from that of ^{14}C-glucose (compare Figure 2A, E with Figure 2C, G). In the case of 3-OMG, label was present at the periphery, but was absent at the place of up-take; *i.e.* the colony center. Apparently, 3-OMG quickly moves outwards after up-take. Non-fixed colonies that had been labeled with ^{14}C-3-OMG contained more label than fixed colonies (compare Figure 2C, G and Figure 2D, H). Apparently, most of the ^{14}C-3-OMG label is washed away in the fixed samples illustrating that most of the label is not incorporated into macro-molecules. The fact that fixed colonies do contain ^{14}C-label suggests that *A. niger* can metabolize this inert sugar analogue, albeit at a low level.

Glucose and 3-OMG did not move to the center of the colony when ^{14}C- maltose or ^{14}C-3-OMG was applied at the periphery of the N402 or N402Δ*Anhex1* mycelium (Figure 3). All labeling experiments described above were also performed with the *A. oryzae* strains NSRKu70-1-1AS and NSRK-ΔHx5. Similar results were obtained when compared to the *A. niger* strains N402 and

N402Δ*Anhex1* (data not shown). Taken together, the results show that glucose and 3-OMG stream from the center to the periphery of the *Aspergillus* colony, but not vice versa and that the presence of Woronin bodies does not affect the migration of these compounds in the mycelium.

Intra- and intercellular streaming of glucose

A. niger strains N402 and RB#14.5 (see below) were grown for 2 days as a sandwiched colony on minimal medium with 25 mM maltose as a carbon source, after which it was transferred to minimal medium containing 10 µM of the inert fluorescent analogue of glucose 2-NBDG (2-(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)-2-deoxyglucose). After 1 h, the entire colony had taken up 2-NBDG (Figure 4A). Almost no 2-NBDG was taken up when the medium also contained 25 mM maltose (data not shown). This can be explained by competition for up-take between glucose and 2-NBDG. A maximum intensity projection showed that some peripheral hyphae of N402 had taken up more 2-NBDG than others (Figure 4C). Most of the fluorescent analogue of glucose accumulated in vacuoles (Figure 4C). Vacuoles showed FRAP (Fluorescence Recovery After Photo-bleaching) within five minutes after photo-bleaching (data not shown), showing that 2-NBDG was taken-up from the cytosol. FRAP was also observed within two minutes in 10 out of 10 hyphae after photo-bleaching the second compartment (data not shown).

When the culture was incubated for 2 h at 4 °C (Figure 5A-D) or 45 °C (data not shown) we observed FRAP in 5 out of 5 hyphae for both conditions after photo-bleaching the second compartment. Moreover, when RB#14.5 (see below) was incubated with 2-NBDG and subsequently was exposed to 4 °C for 2 h a compartment showed FRAP after photo-bleaching (Figure 5 E, F). When in the same compartment PA-GFP was photo-activated, we did not observe streaming to neighboring compartments, showing that the septa were closed (Figure 5G-J). From these data it is concluded that the glucose analogue was intercellularly streaming and that septal plugging is not a barrier for this.

Intercellular streaming of GFP in *A. niger*

Genes *mpdA* and *hyp1* are up-regulated in the vegetative mycelium and in aerial structures, respectively (Chapter 2). Constructs encompassing the *GFP* gene under control of the *mpdA* or the *hyp1* promoter were introduced in the *A. niger* strain AB4.1. Strains RB#7.1 and RB#77.1 were selected as representative transformants, respectively, for further studies. These strains were grown for 3 days in glass bottom dishes (Figure 1) on minimal medium containing 25 mM maltose. Expression from the *mpdA* promoter resulted in similar, if not higher, fluorescence in the conidiophores when compared to the vegetative hyphae (Figure 6A, C). In contrast, *hyp1* driven expression of *GFP* in strain RB#77.1 resulted in fluorescent conidiophores only (Figures 6E, G).

PA-GFP is a photo-activatable form of GFP (Patterson and Lippincott-Schwartz, 2002). A construct encompassing the *PA-GFP* gene under control of the

constitutive *gpdA* promoter of *A. nidulans* was introduced in *A. niger* strain AB4.1. Strain RB#14.5 was selected as a representative transformant for further studies. PA-GFP was activated in vegetative hyphae that were in contact with the conidiophore stalk. Streaming of PA-GFP from the vegetative hyphae to the conidiophore was observed in approximately 25% of the cases (Figure 7, Supplementary Movie 1; see http://hdl.handle.net/10411/10107). In contrast, PA-GFP that had been activated at the base of conidiophore stalks did not stream into vegetative hyphae. Similarly, PA-GFP did not stream from conidiospores and/or the conidiophore vesicle towards the base of the conidiophore stalk (data not shown). Taken together, these data show that PA-GFP streams from the vegetative mycelium towards the conidiophores but not vice versa.

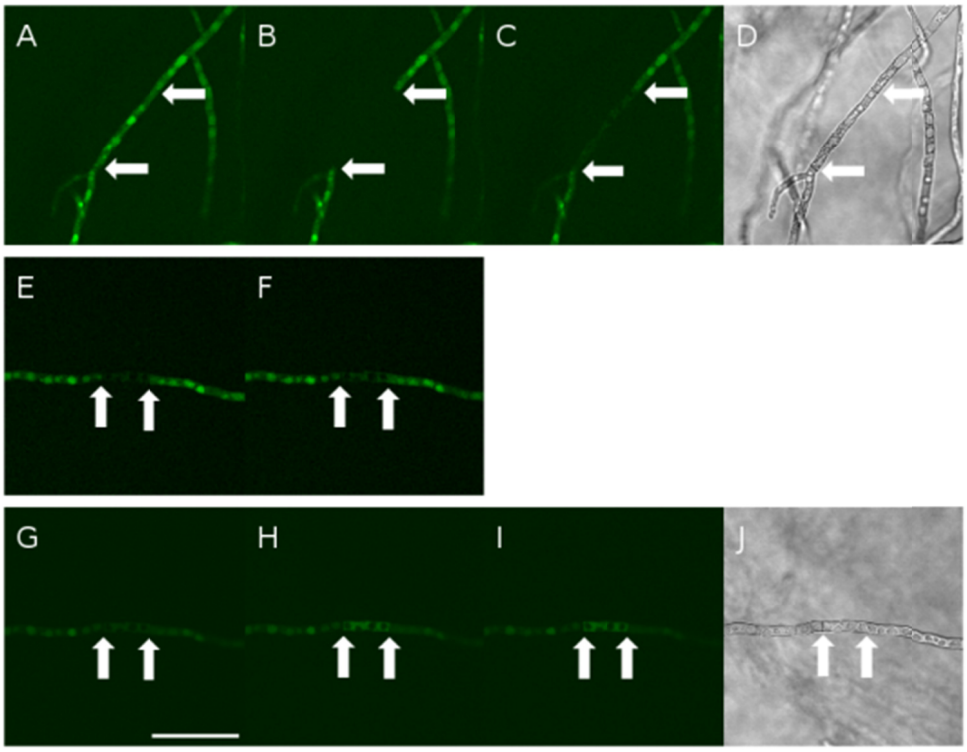

Figure 5: Septal plugging does not impede Intercellular glucose streaming but does impede intracellular protein streaming. *A. niger* strains N402 (A-D) and RB#14.5 (E-J) were grown for two days as sandwiched cultures on minimal medium with 25 mM maltose. The colonies were transferred to minimal medium plates containing 10 µM 2-NBDG for 1 h and were subsequently incubated at 4 °C for 2 h. The second compartment is shown before (A), directly after photo-bleaching (B, E) and after 2 min FRAP (C, F). The second compartment of a hypha (E-J) is shown before (G), directly after (H) and 2 min after (I) PA-GFP had been activated. (D) and (J) representing bright field images. Arrows indicate septa. Bar represents 50 µm.

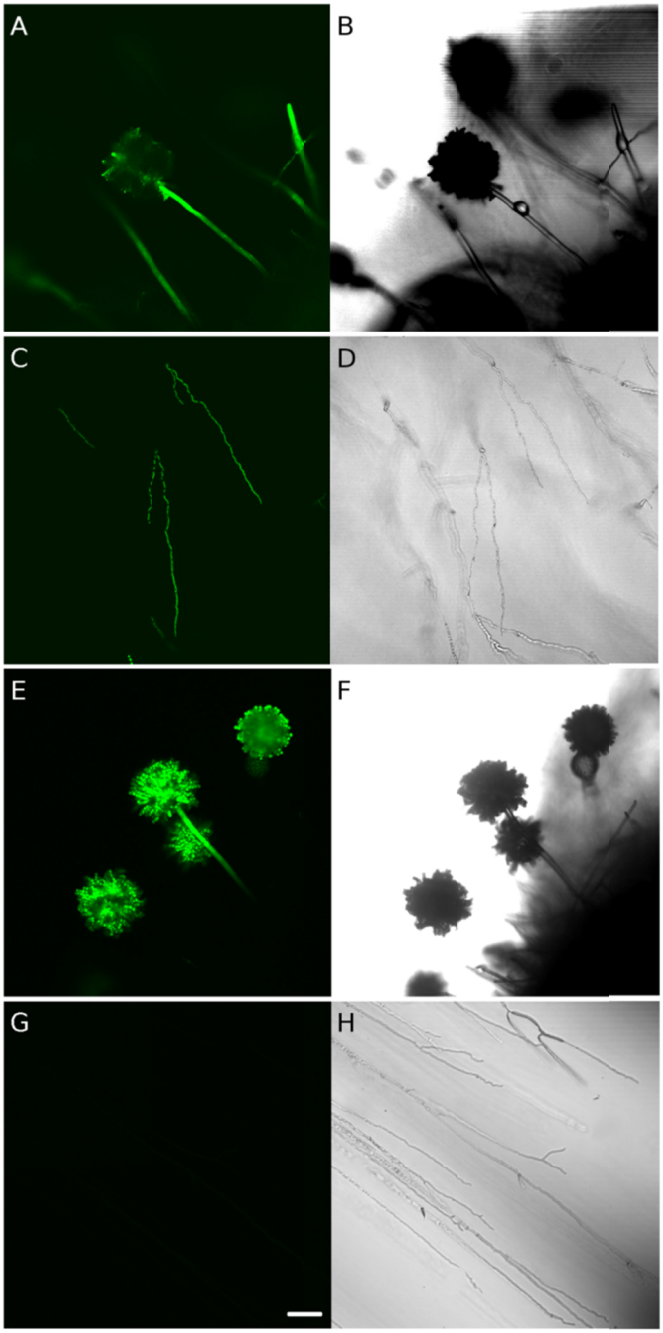

Figure 6: Localization of *mpdA*- (A, C) and *hyp1* (E, G) driven *GFP* expression in *A. niger.* A and E represent conidiophores; C and G represent vegetative hyphae. B, D, F, H represent bright field images. Bar represents 50 µm.

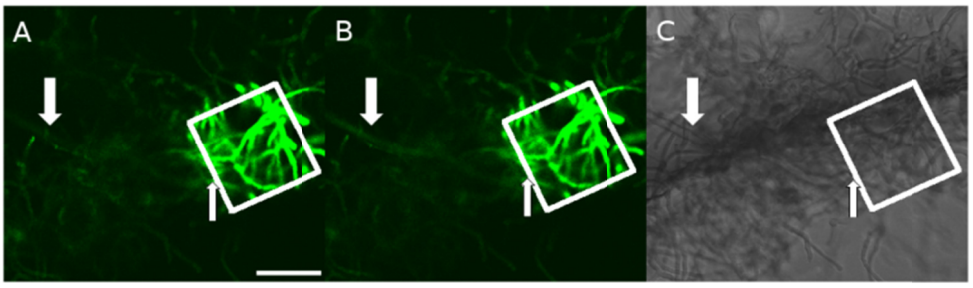

Figure 7: Streaming of GFP from vegetative hyphae to the conidiophore stalk. PA-GFP was photo-activated in vegetative hyphae (A). After 2 min, fluorescence intensity had increased in the conidiophore stalk (B). (C) represents bright field image. Small white arrow indicates location of septum in the conidiophore stalk. Large white arrow indicates conidiophore stalk. White box indicates activation window. Bar represents 50 µm.

Intracellular streaming of GFP in *A. niger*

PA-GFP was activated 100 µm from the tip or at the tip (Figure 8, Supplementary Movie 2 and 3). Streaming of PA-GFP towards the tip (Figure 8A and B) or to sub-apical regions (Figure 8A, B, D and E) occurred at rates that were not significantly different (11.2 ± 0 µm s^{-1} and 14.8 ± 7.6 µm s^{-1}, respectively). Rate of streaming of PA-GFP to the base of the conidiophore stalk or to the conidiophore vesicles (Figure 9, Supplementary Movies 4 and 5) was similar to that in vegetative hyphae (14.3 ± 0 µm s^{-1}).

In the next set of experiments, cytosolic streaming was studied using *A. niger* strain RB#13.1, which expresses a GPD-PA-GFP fusion protein under control of its own (*i.e. gpdA*) promoter. GPD-PA-GFP was activated 100 µm from the tip or at the apex. Cytosolic streaming towards the hyphal tip and towards sub-apical parts were not significantly different from each other (6.2 ± 1.3 µm s^{-1} and 4.1 ± 1.6 µm s^{-1}, respectively) but was different from streaming towards the apex of PA-GFP that was not fused to GPD.

Cytoplasmic continuity depends on environmental conditions and the position of the compartment

Strain RB#14.5 was grown for 2 days as a sandwiched culture on minimal medium containing 25 mM maltose. The colony was cut out and put upside down in 100 µl minimal medium with 25 mM maltose. PA-GFP was activated in the 2nd and 10th compartment of leading hyphae (Figure 10). In all cases, PA-GFP was streaming from the second to the first compartment. In contrast, PA-GFP activated in the 10th compartment was not streaming to neighboring compartments in 70% of the cases.

Cultures were either or not subjected to 4 °C or 45 °C for 2 h. Subsequently, PA-GFP was photo-activated in the second apical compartment of leading hyphae. After 2 h incubation at 4 °C or 45 °C, intercellular streaming of PA-GFP was observed in 0% and 10% of the cases, respectively. Septal plugging after incubation at 4 °C

could be reversed but not after incubation at 45 °C (Figure 10). Streaming of PA-GFP from the second to the first compartment was observed in 70% and 10% of the cases, respectively, after incubation of the cultures at 30 °C for 30 min after they had been exposed to 4 °C or 45 °C for 2 h. These data show that cytoplasmic continuity is reduced in basal parts of leading hyphae and that it is influenced by the temperature.

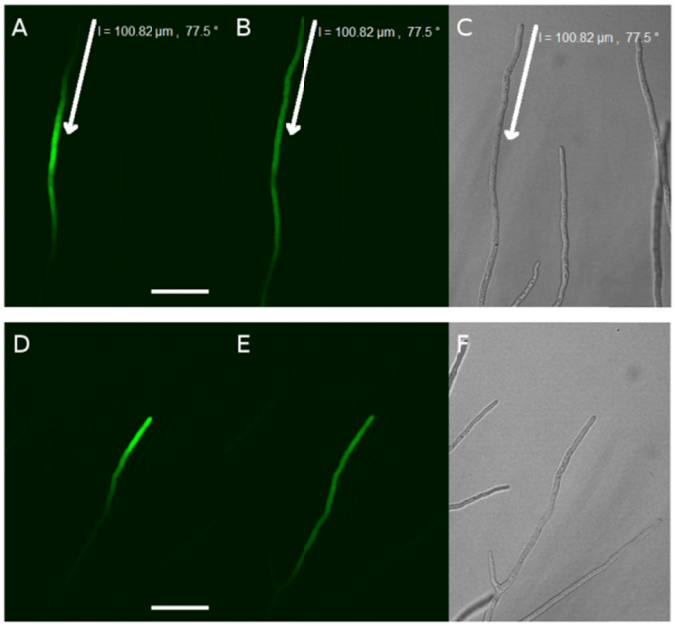

Figure 8: Streaming of PA-GFP in apical hyphal compartments. PA-GFP was activated 100 µm from the hyphal tip (A-C) or at the hyphal tip (D-F). Fluorescence was monitored directly (A, D) or 2 min (B, E) after activation; (C) and (F) representing bright field images. Arrow indicates distance from the hyphal tip (A-C). Bars represent 50 µm.

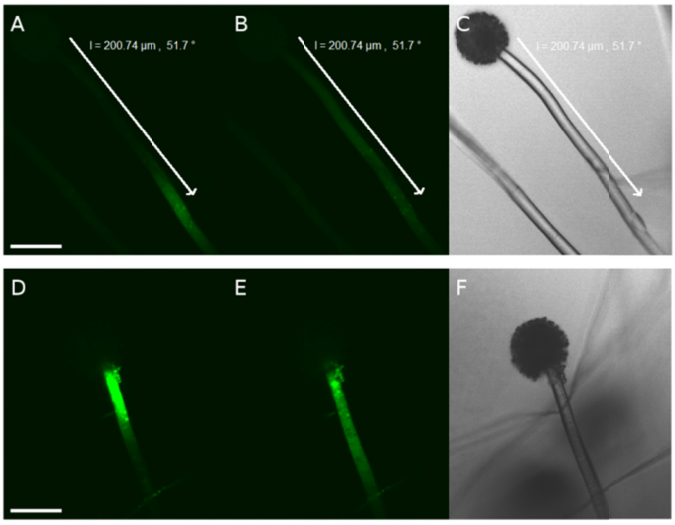

Figure 9: Intracellular streaming of PA-GFP in conidiophores. PA-GFP was photo-activated at the conidiophore base 200 µm from the conidiophore vesicle (A-C) or just under the conidiophore vesicle (D-F). Fluorescence was monitored directly (A, D) or 2 min (B, E) after activation. (C) and (F) represent bright field images. Arrow indicates distance from the conidiophore vesicle. Bars represent 50 µm.

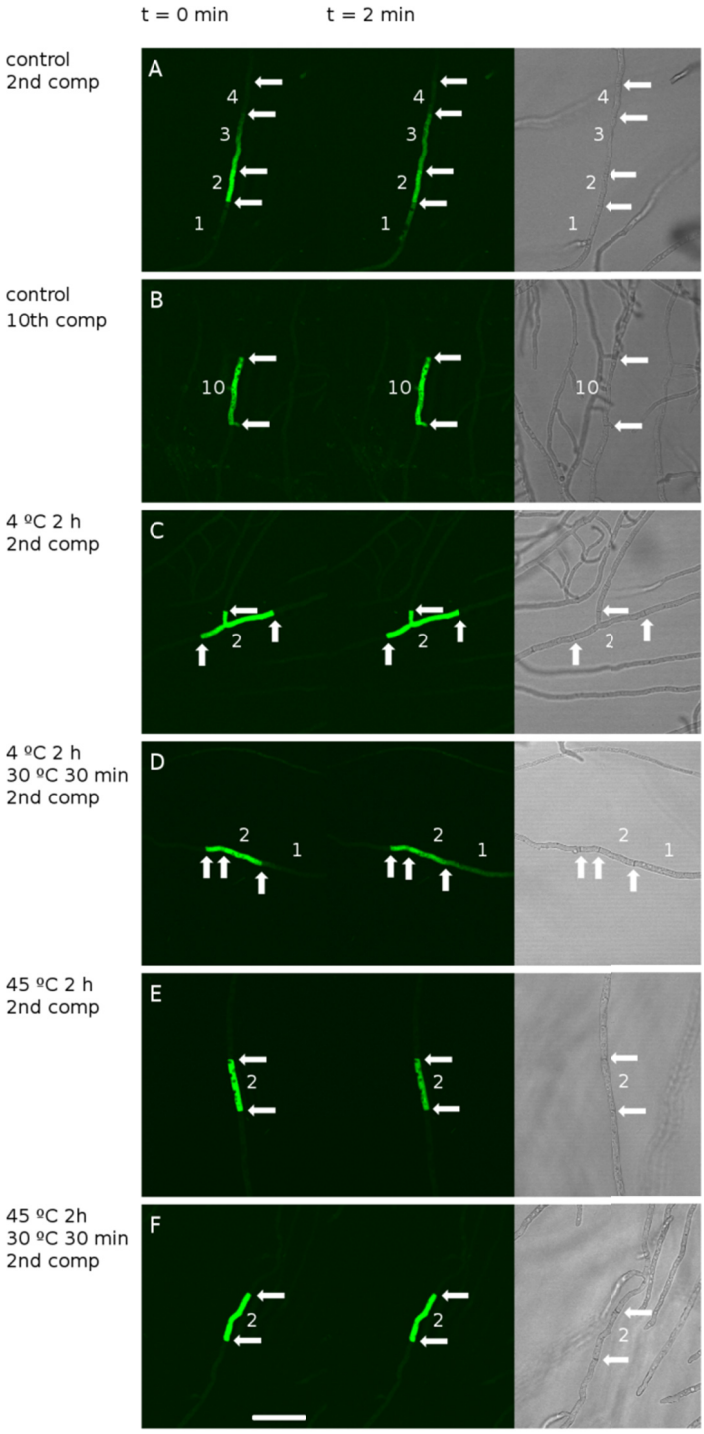

Figure 10: Cytoplasmic continuity. RB#14.5 expressing PA-GFP behind the *gpdA* promoter was grown for 2 days at 30 °C. PA-GFP was photo-activated in the second (A) or the tenth (B) compartment using UV laser light. Alternatively, PA-GFP was activated in the second compartment after subjecting the culture for 2 h to 4 °C (C) or 45 °C (E) and subsequently to 30 °C for 30 min (D and F, respectively). Time-lapse movies were made during 2 minutes directly after photo-activation. Bright field images are shown in the panels at the right. Number indicates compartment number as counted from the hyphal tip. Arrows indicate septa. Bar represents 50 µm.

Discussion

It has been shown that approximately 35% of the septa of intact vegetative hyphae of aspergilli are closed (Chapter 3 and 4). Plugging of septa is expected to affect short- and long-distance inter-compartmental and inter-hyphal streaming of monomeric and polymeric organic molecules. Indeed, it was shown in this chapter that streaming of GFP was affected by closure of septa. In contrast, an effect on streaming of glucose could not been shown.

PA-GFP streams to apical and sub-apical regions of hyphae of *A. niger*. (Vinck et al., 2005; this Chapter). This finding contrast results obtained in *Neuropora crassa.* Oil droplets that had been injected into hyphae only moved to the tip with an average speed of approximately 5 μm s^{-1} and a maximum speed of 60 μm s^{-1} (Lew, 2005). PA-GFP had a rate of streaming in *A. niger* of approximately 10-15 μm s^{-1}. The speed was similar in both directions and was similar in vegetative hyphae and conidiophores. The streaming rate of PA-GFP was decreased to about 4-6 μm s^{-1} when the reporter protein was fused to the glyceraldehyde-3-phosphate dehydrogenase (GPD) protein. Possibly, this is due to the fact that GPD is part of a large protein complex. The yeast GPD homologs Tdh1, Tdh2 and Tdh3 were found to be part of 17 unique protein complexes (Gavin et al., 2006). For instance, Tdh3 is the core of a complex that also includes Tdh1 and Tdh2 as well as the trans-membrane proteins Gpi17 and Ptm1. These membrane proteins may well decrease the streaming rate by immobilizing the complex at the membrane.

Cytoplasm was always streaming towards the 1st compartment when PA-GFP was activated in the 2nd compartment of *A. niger*. It was however expected that approximately 50-60% of the septa of *A. niger* would be open and thus would allow inter-compartmental streaming in about half of the cases (Chapter 4). Indeed, this percentage was also found with FRAP experiments using cytosolic GFP (unpublished data). The fact that streaming to the first compartment was observed in 100% of the cases when PA-GFP had been activated in the second compartment may just have been by chance. Previously, it was shown that the plugging incidence was similar between the first, second and third septum of *A. oryzae* (Chapter 3) and *A. niger* (unpublished data). Inter-compartmental streaming of PA-GFP was observed in only 30% of the cases when the reporter protein was activated in the 10th compartment. Future studies will be done to confirm that the plugging incidence of septa is higher in sub-apical parts of the hyphae. If so, cytoplasmic continuity would be even less than proposed in Chapters 3 and 4.

PA-GFP was shown to stream from vegetative hyphae into the conidiophores but the opposite was not observed. These findings were confirmed by expressing *GFP* from promoters that are differentially expressed in the vegetative mycelium and the aerial structures. Fluorescence of GFP was only observed in conidiophores when the gene encoding this reporter was expressed from the aerial structure specific promoters of *hyp1* (Figure 6) and *oxi* (data not shown). Fluorescence of GFP was similar in conidiophores and vegetative mycelium (and may have even be higher in the aerial structures) when the *mpdA* promoter was used to express *GFP.* This and the fact that

RNA levels of *mpdA* are higher in the vegetative mycelium (Chapter 2) indicates that GFP streams from the vegetative mycelium into conidiophores. Similar results were obtained using the *glaA* promoter (Vinck, 2007). GFP levels were much higher in conidiophores although the *glaA* promoter is higher expressed in the vegetative mycelium.

An effect of septal plugging on long-distance glucose transport could not be shown. Streaming of the sugar from the periphery to the center or vice versa was not affected in Δ*hex1* strains of *A. niger* and *A. oryzae* when compared to the wild-type strains. Inter-compartmental short-distance transport of glucose was also shown not to be affected by septal plugging. The glucose analogue 2-NBDG, but not PA-GFP, was still moving to neighboring compartments when cultures had been incubated for 2 h at 4 °C or 45 °C. These conditions induce septal plugging. Likely, septa contain sugar transporters. This is strengthened by the fact that 2-NBDG was accumulating in septa (data not shown).

Acknowledgments

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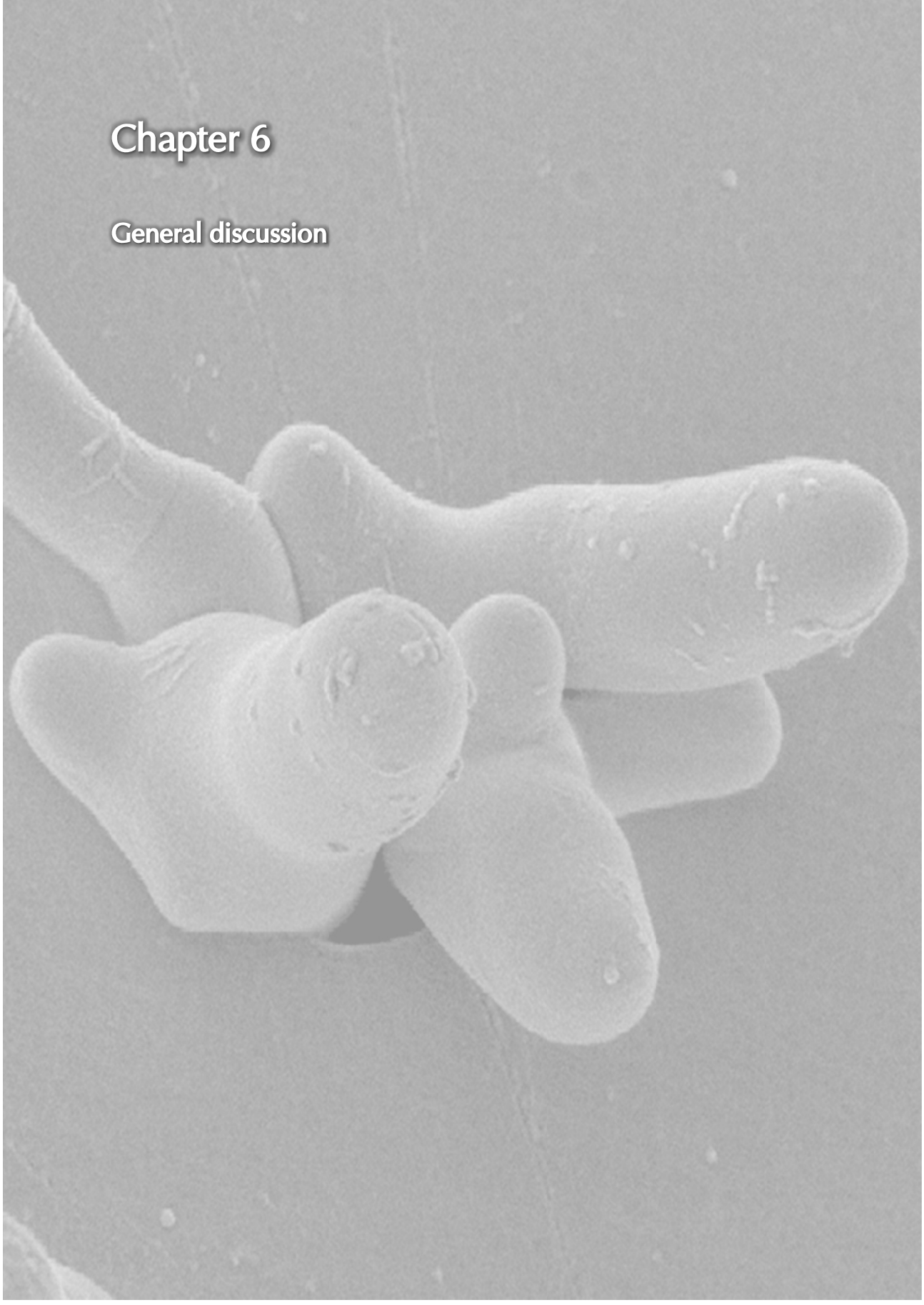
Chapter 6

General discussion



Aspergillus species belong to the most abundant fungi in the world. These ascomycetes feed on a large variety of substrates but are predominantly found on complex plant polymers. Aspergilli normally adopt a saprobic life style but they can also be pathogens of plants, animals and humans. Like other filamentous ascomycetes, aspergilli grow by means of hyphae that form an interconnected mycelium by sub-apical branching and apical extension. After a period of vegetative growth *Aspergillus* starts to grow into the air to form conidiophores that produce a-sexual conidiospores. These spores are dispersed by wind and can give rise to a new mycelium when the environmental conditions are favorable. The hyphae of ascomycetes are compartmentalized by septa that contain a central pore, which allows for cytoplasmic streaming (Shatkin and Tatum, 1959; Moore and McAlear, 1962; Gull, 1978; Lew, 2005). Upon mechanical damage, these septal pores quickly close by Woronin bodies, preventing excessive bleeding of cytoplasm (Shatkin and Tatum, 1959; Trinci and Collinge, 1974; Collinge and Markham, 1985; Jedd and Chua, 2000; Tenney et al., 2000; Soundararajan et al., 2004; Maruyama et al., 2005).

The mycelium of ascomycetes is heterogeneous with respect to growth, secretion and mRNA composition (Wösten et al. 1991; Teertstra et al., 2004; Vinck et al., 2005, 2011; Masai et al., 2006; Levin et al., 2007a, b; Kasuga and Glass, 2008; Etxebeste et al., 2009; de Bekker et al., 2011a, b). For instance, in *Aspergillus niger* 25% of the genes show a more than 2-fold differential mRNA accumulation at the center and the periphery of the colony (Levin et al., 2007a). Notably, even neighboring hyphae show heterogeneity with respect to gene expression (Vinck et al., 2005, 2011; de Bekker et al., 2011a) and secretion (Wösten et al., 1991). Two populations of hyphae can be distinguished at the outer part of an *A. niger* colony; those highly and those lowly expressing the glucoamylase gene *glaA* (Vinck et al., 2005). The hyphae that highly express *glaA* also highly express other genes encoding secreted enzymes and they have high rRNA and *gpdA* levels (Vinck et al., 2011).

The aim of this thesis was to study streaming of cytosol between compartments and between hyphae of *Aspergillus* and the effect of this on hyphal heterogeneity and a-sexual development.

Intracellular streaming

It has been shown that organelle movement along the cytoskeleton induces cytoplasmic streaming in plants (Esseling-Ozdoba et al., 2008; Verchot-Lubicz and Goldstein, 2010). This movement would result in a coordinated cytoplasmic streaming. In fungi cytoplasmic streaming is also observed. It is mediated by internal osmotic gradients and possibly also by organelle movement along the cytoskeleton via molecular motors (Lew, 2005). Cytoplasmic streaming is uni-directional in the ascomycete *Neuropora crassa*. The rate towards the apex is approximately 5 μm s^{-1} on average (Lew, 2005). In Chapter 5 it was shown that intracellular streaming of PA-GFP is bi-directional in vegetative hyphae and conidiophores of *A. niger*. Streaming towards the apex or to sub-apical regions occurred at a rate of about 10-15 μm s^{-1}. When PA-GFP was fused to the glyceraldehyde-3-phosphate dehydrogenase (GPD) protein, its streaming rate was reduced to 4-6 μm s^{-1}. Possibly, GPD of *A. niger* is part of a large protein complex that also consists of membrane proteins. The yeast GPD

homologs Tdh1, Tdh2 and Tdh3 are part of 17 unique protein complexes (Gavin et al., 2006). For instance, Tdh1, Tdh2 and Tdh3 are part of a complex containing the trans-membrane proteins Gpi17 and Ptm1. These proteins may immobilize the protein complex at the membrane thereby reducing the overall rate of streaming of GPD. The fact that streaming has a similar rate in vegetative hyphae and conidiophores of *A. niger* contrasts the situation in plants where the rate of cytoplasmic streaming depends on cell type and developmental stage (Verchot-Lubicz and Goldstein, 2010). In *Arabidopsis thaliana* streaming rates of 2, 3 and 8-14 μm s^{-1} are found in sub-apical parts of root hairs, cortical hypocotyl cells and the root hair shank, respectively (Emons, 1987; Miller et al., 1999; Sieber and Emons, 2000).

Intercellular streaming

Intercellular cytoplasmic connections have been identified in multicellular eukaryotic organisms belonging to the four classical kingdoms. The cytoplasm of plant and animal cells is connected via plasmodesmata and gap junctions, respectively. Plasmodesmata are highly regulated membrane tunnels. They can be closed to regulate plant development and defense (Xu and Jackson, 2010; Lee and Lu, 2011; Maule et al., 2011). The size exclusion limit for plasmodesmata is > 67 kDa in *A. thaliana* (Stadler et al., 2005) and thus allows for long-distance intercellular streaming of GFP (Imlau et al., 1999). Gap junctions are intercellular connections between animal cells and are formed by two unrelated proteins families, the pannexins and connexins (Meşe et al., 2007). The restricted pore size of the gap junctions (1.5 nm) in animals only allows streaming of small molecules (≤ 1000 Da) such as ions, second messengers and small metabolites (Meşe et al., 2007). For example, in hippocampal astrocytes sodium is diffusing via gap junctions to neighboring cells (Langer et al., 2011).

The lower fungi consisting of the Chytridiomycota, Zygomycota and Glomeromycota generally have coenocytic mycelia without septa. They only produce closed septa to isolate old or reproductive regions from the rest of the mycelium (Schüßler et al., 2001; Mirghani and Abdulla, 2004; Yassin et al., 2008). The higher fungi (*i.e.* the Ascomycota and the Basidiomycota) have septa that compartmentalize hyphae. These septa have a central pore of 50-500 nm that allows cytoplasmic streaming (Shatkin and Tatum, 1959; Moore and McAlear, 1962; Gull, 1978; Lew, 2005). Septa can be closed upon hyphal damage to prevent excessive loss of cytoplasm (Chapter 3, 4; Shatkin and Tatum, 1959; Trinci and Collinge, 1974; Collinge and Markham, 1985; Jedd and Chua, 2000; Tenney et al., 2000; Soundararajan et al., 2004; Maruyama et al., 2005). A specialized form of the ER, the septal pore cap, mediates septal plugging in the basidiomycete *Schizophyllum commune.* Deletion of *spc33* that encodes one of the two major septal pore cap proteins abolishes the capability to plug pores (van Peer et al., 2010). It is thought that plugging material is released by the septal pore cap (van Driel et al., 2008). Woronin bodies plug septal pores in the case of ascomycetes. The Woronin body core is composed of hexagonal crystals of the HEX1 protein. The importance of this protein is shown by the fact that hyphal injury results in severe cytoplasmic bleeding in *HEX1* deletion mutants (Jedd and Chua, 2000; Tenney et al., 2000; Soundararajan et al.,

2004; Maruyama et al., 2005). Hyphal damage by laser dissection always resulted in streaming of cytoplasm from adjacent compartments to damaged compartments in Δ*Aohex1* and Δ*Anhex1* strains of *A. oryzae* and *A. niger.* This showed that septa of these mutant strains were open and could not close to prevent excessive cytoplasmic bleeding (Chapter 3; my unpublished results). This was irrespective of growth conditions (low or high temperature; low or high pH; absence or presence of C- and N-source). Notably, cytoplasmic streaming from an adjacent compartment to a damaged compartment was not observed in 40% and 50% of the cases in wild type *A. oryzae* and *A. niger*, respectively (Chapter 3 and 4). This showed that the pores of these septa had already been plugged before damage took place. This finding implies that the cytoplasm of ascomycetes is not continuous, at least not to the extent that is generally assumed. The septal plugging incidence was similar in the first, second and third septum under normal growth conditions (Chapter 3). Moreover, it was shown that septal plugging was not related to the plugging state of neighboring septa. Live cell imaging showed that septal pore Woronin body localization in *A. oryzae* is dynamic. This implies that closure of septa is reversible and is possibly a stochastic process (Chapter 3). In Chapter 5 it was shown that the plugging incidence was higher (*i.e.* 70%) for septa of the 10th compartment of hyphae of *A. niger*. It may therefore be that the plugging incidence is higher for septa of old compartments.

Aspergillus clavatus, *A. niger* and *A. oryzae* were exposed to low or high temperature; absence or presence of C- and N-source; low and high pH; and/or hyper and hypotonic conditions to assess whether these conditions affect the incidence of septal plugging. Only *A. niger* showed a response to some of the conditions. 50% of the apical septa of *A. niger* were plugged in the control, whereas 82-90% of the septa were plugged during temperature stress (2 h at 4 °C) and during hypertonic conditions (1.5 h 1 M $MgSO_4$) (Chapter 4). Exposure to 45 °C also showed a trend of increased septal plugging. When PA-GFP was activated in the second compartment it was always streaming towards the apical compartment. However, when the culture was subjected to 4 °C or 45 °C for 2 h, PA-GFP was retained in the second compartment in 100 and 90% of the cases, respectively (Chapter 5). The plugging induced by cold shock was shown to be reversible within 30 minutes after placing the culture back at 30 °C (Chapter 5). Increased plugging was also observed for *S. commune* when subjected to heat shock, hypertonic shock, and incubation with an antibiotic or ethanol (van Peer et al., 2009). Plugging induced by heat shock was shown to be reversible. The apical septa re-opened within a period of 15-30 minutes when the culture was transferred back to the normal growth temperature. The question why *A. niger* plugs septa as a response to environmental conditions, whereas other aspergilli do not is a topic for future research. Interestingly, *A. niger* is known to grow under the widest range of temperature, pH and water activity conditions (Krijgsheld et al., 2012; Chapter 1). It is tempting to speculate that septal plugging contributes to the flexibility of *A. niger* to adapt to environmental conditions. Of interest, the *A. niger* Δ*Anhex1* strain but not the wild-type strain died after 3 days incubation at 45 °C. This may be due to the phenomenon of hyphal heterogeneity, which promotes variation in physiology between hyphae. This variation increases the chance that particular hyphae survive a particular stress.

As mentioned above, two populations of hyphae can be distinguished at the periphery of the *A. niger* colony based on their transcriptional and translational activity (Vinck et al., 2005, 2011). In Chapter 4 it was shown that *glaA* of *A. niger* is also heterogeneously expressed in *A. oryzae.* This is the second filamentous fungus where the phenomenon of hyphal heterogeneity has been shown to occur. Hyphal heterogeneity conflicts the accepted view that the cytoplasm of fungi is continuous due to the large pores in their septa. However, the fact that about 40% of the septa is closed in the case of *A. oryzae* implies that the cytoplasm of two hyphal compartments have a change of about 3% to be in physical contact when they are separated by 7 septa. It was hypothesized that this reduced cytoplasmic streaming is sufficient to maintain hyphal heterogeneity. Indeed, two populations of hyphae could not be shown to exist in the Δ*Aohex1* mutant, which showed no septal plugging (Chapter 4). The septal pores thus suppress hyphal heterogeneity when open. Notably, gap junctions have also been shown to suppress cellular heterogeneity (Benninger et al., 2011). By increasing or decreasing the incidence of septal plugging, fungi would be able to regulate hyphal heterogeneity. This may be an instrument to increase survival under stress conditions (see above).

It was shown that septal plugging affected inter-compartmental streaming of GFP (Chapter 5). In contrast, an effect on short and long-distance sugar transport was not observed. Wild type and Δ*Aohex1* and Δ*Anhex1* strains of *A. oryzae* and *A. niger* showed a similar spatial distribution of glucose when the center or the periphery had been exposed to ^{14}C glucose. Plugged septa seem not to be a barrier for sugars. Inter-compartmental streaming of the 2-NBDG fluorescent glucose analogue was still observed under conditions that septa had been induced to plug (Chapter 5). Possibly, the cell membrane lining the septa contains sugar transporters that allow for intercellular sugar transport. The fact that 2-NBDG also accumulated in septa, besides in the cytosol and vacuoles, strengthens the latter idea (my unpublished results).

Differential expression in vegetative and aerial structures of *A. niger*

The RNA composition of the vegetative mycelium and the aerial structures (*i.e.* aerial hyphae, conidiophores and conidiospores) of 7-day-old maltose-grown colonies of *A. niger* was analyzed (Chapter 2). In total, 6476 out of the 14259 genes were expressed, whereas 5095 and 5939 genes were expressed in the vegetative mycelium and the aerial structures, respectively. 2056 genes had a fold change ≥ 2, of which 1144 were up-regulated in the aerial structures. This finding agrees with the estimation that approximately 1300 genes are up-regulated during a-sexual spore formation in *A. nidulans* (Timberlake, 1980). BrlA, AbaA, and WetA play a central role in the formation of conidiophores. Their encoding genes were all ≥ 8-fold up-regulated in the aerial structures. In contrast, genes encoding polysaccharide degrading enzymes (*e.g.* the glucoamylase gene *glaA*, and the acid α-amylase *aamA*) and monosaccharide permeases were among the genes that were up-regulated in the vegetative mycelium.

Gene *GFP* was expressed from promoters that were up-regulated in the vegetative mycelium and in the aerial structures. This showed that GFP was streaming from the vegetative mycelium towards the aerial structures, but not vice versa

(Chapter 5). This uni-directional flow agrees with the fact that the aerial structures act as a sink for water and nutrients from the vegetative mycelium. The flow of GFP from the vegetative mycelium to the aerial structures also suggests that RNA can stream from the vegetative mycelium to the aerial structures. This was tested by comparing the micro-array data with a nuclear run-on transcription assay (Chapter 2). Micro-array analysis showed that *gpdA* was constitutively expressed but the run-on analysis showed higher expression in the vegetative mycelium. These results can be explained by streaming of *gpdA* transcripts from the vegetative mycelium to the aerial structures, thereby leveling out the differences of *gpdA* transcription. An alternative explanation would be that *gpdA* transcripts are less stable in the vegetative mycelium. Micro-array analysis showed that transcript levels of *mpdA* were higher in the vegetative mycelium than in the aerial structures. In contrast, they were found to be more abundant in the aerial structures in the run-on analysis. These results can be explained by lower transcript stability in the aerial structures or by streaming/transport of the transcripts from the aerial structures towards the vegetative mycelium. The latter is not very likely considering the fact that GFP did not stream from the aerial structures to the vegetative mycelium. Lower transcript stability in the aerial structures would also explain the differences in RNA ratios of the flavohemoprotein (flavo) obtained with the run-on analysis and the micro-array analysis (Chapter 2). The flavo gene was similarly expressed in the mycelium and the aerial structures according to the run-on analysis, but micro-array analysis showed higher mRNA levels in the vegetative mycelium.

Taken together, the results presented in Chapter 2 show that the vegetative mycelium and the aerial structures of *A. niger* are heterogenic with respect to RNA composition. This heterogeneity is the result of differential gene expression, as well as differential mRNA stability and/or mRNA streaming or even transport.

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Nederlandse samenvatting

Inleiding

Het geslacht *Aspergillus* herbergt de meest voorkomende schimmels ter wereld. Zij voeden zich met een breed scala aan dode organische substraten, maar groeien vooral op plantaardig materiaal. Sommige aspergilli kunnen ziekte veroorzaken bij mensen en dieren met een verminderd afweersysteem. Net zoals andere filamenteuze schimmels groeien aspergilli middels hyfen (schimmeldraden) die zich aan hun top verlengen. De hyfen worden in compartimenten verdeeld middels septa. Deze septa bevatten een grote porie, waardoor in principe cytoplasma (celmateriaal) van het ene compartiment naar het andere compartiment kan stromen. De schimmeldraden vormen een aaneengesloten mycelium door te vertakken en onderling te fuseren. Dit en de aanwezigheid van poreuze septa maakt dat men aanneemt dat al het cytoplasma binnen het mycelium met elkaar in contact staat.

Na een bepaalde tijd vegetatief te hebben gegroeid, ontsnapt *Aspergillus* aan het substraat en maakt voortplantingsstructuren in de lucht. Deze conidiophoren vormen a-sexuele sporen die door de wind worden meegenomen. Zij kunnen elders een nieuw mycelium vormen wanneer de milieu omstandigheden dit toelaten. Er zijn verschillen (heterogeniteit) in de samenstelling tussen de hyfen die het substraat doorgroeien en de hyfen die voortplantingsstructuren vormen. De mate waarin zij verschillen was nog niet bekend bij het begin van dit proefschrift. Zelfs binnen het mycelium dat het substraat doorgroeit zijn er verschillen als men kijkt naar groei, uitscheiding van eiwitten en mRNA samenstelling. Dit geldt zelfs voor naburige hyfen. Er worden twee typen schimmeldraden aan de rand van het mycelium van *Aspergillus niger* onderscheiden. Één type is actief met het produceren van uitgescheiden enzymen, het andere type is veel minder actief en bevat daartoe minder ribosomaal RNA en glyceraldehyde-3-fosfaat dehydrogenase (*gpdA*) mRNA.

In dit proefschrift wordt onderzoek beschreven naar de stroming van cytoplasma tussen compartimenten en tussen schimmeldraden van *Aspergillus* en wat dit voor effect heeft op heterogeniteit van schimmeldraden en a-sexuele ontwikkeling.

Stroming in compartimenten

Het is bekend dat cytoplasmatische stroming in cellen wordt veroorzaakt door bewegingen van organellen langs het celskelet. Verder speelt de osmotische balans van cellen een rol. In Hoofdstuk 5 wordt beschreven dat intracellulaire stroming van GFP (een fluorescent eiwit) in twee richtingen plaatsvindt in zowel vegetatieve schimmeldraden van *A. niger* als in conidiophoren. Stroming van en naar het uiteinde van de schimmeldraad gebeurt met een snelheid van 10-15 μm s^{-1}, terwijl de schimmeldraad zich zelf verlengt met een snelheid van 0,14 μm s^{-1}. Wanneer GFP aan het GPD eiwit werd gekoppeld verlaagde de stromingssnelheid van het fluorescente eiwit tot 4-6 μm s^{-1}. GPD vormt waarschijnlijk een complex met andere eiwitten, waarvan een aantal in de celmembraan zitten verankerd. Dit zou de lagere snelheid van GPD kunnen verklaren.

Stroming tussen compartimenten en schimmeldraden

Aspergillus en andere ascomyceten bezitten septa, die de schimmeldraden opdelen in compartimenten. Deze septa hebben een porie van 100-500 nm, die stroming van cytoplasma toelaat. Septa kunnen worden afgesloten met Woronin bodies om bloeding van cytoplasma te voorkomen als schimmeldraden worden beschadigd. Woronin bodies zijn opgebouwd uit een centrale kern van gekristalliseerd Hex1 eiwit. Als men het *hex1* gen uitschakelt worden er geen Woronin bodies meer gemaakt. Wanneer schimmeldraden van een dergelijke Δ*hex1* stam van *A. oryzae* werden beschadigd door laser dissectie dan was er altijd stroming waar te nemen naar naburige compartimenten (Hoofdstuk 3). Dit toonde aan dat septa van deze mutant altijd open stonden en niet meer dicht konden gaan om cytoplasmatische bloeding te voorkomen. Dit was onafhankelijk van de groeiomstandigheden (lage- of hoge temperatuur, lage- of hoge zuurgraad, aan- of afwezigheid van een koolstof- of stikstofbron) (Hoofdstuk 3). Opmerkelijk was dat stroming van cytoplasma niet te zien was in 40-50% van de gevallen in stammen van *A. oryzae* en *A. niger* waarin het *hex1* gen nog intact was (wildtype). Dit toonde aan dat septa al dicht waren voordat de schimmeldraden beschadigd werden. Deze vinding impliceert dat het cytoplasma van deze ascomyceten niet aaneengesloten is, tenminste niet zoals algemeen wordt aangenomen. Het percentage open septa was gelijk voor het eerste, tweede en derde septum tijdens normale groeicondities. Door filmpjes te maken werd onder de microscoop waargenomen dat lokalisatie van Woronin bodies bij de porie van het septum dynamisch is. Dit impliceert dat het sluiten van septa een omkeerbaar proces is. In Hoofdstuk 5 werd getoond dat septa bij het tiende compartiment vaker dicht staan in *A. niger*. Dit impliceert dat septa vaker gesloten zijn in oude compartimenten.

In Hoofdstuk 4 werden verschillende aspergilli blootgesteld aan lage- of hoge temperatuur, de aan- of afwezigheid van een koolstof- of stikstofbron, lage- of hoge zuurgraad, en/of hyper- en hypotone condities om te kijken of deze condities het dichtgaan van septa beïnvloeden. *A. niger*, maar niet *Aspergillus clavatus* en *A. oryzae*, liet een respons zien bij sommige condities. 50% van de apicale septa van *A. niger* waren dicht in de controle, terwijl 82-90% van deze septa dicht waren tijdens temperatuur stress (2 uur bij 4 °C) en hypertone condities (1,5 uur 1 M $MgSO_4$). Blootstelling aan 45 °C liet een trend zien dat meer septa dicht gaan. Wanneer PA-GFP (een eiwit dat alleen fluorescent wordt als het beschenen wordt met licht met een golflengte van 405 nm) werd geactiveerd in het tweede compartiment, stroomde het altijd naar het apicale compartiment. Alleen wanneer de cultuur werd blootgesteld aan 4 °C of 45 °C gedurende 2 uur, bleef het PA-GFP in het tweede compartiment in respectievelijk 100% en 90% van de gevallen (Hoofdstuk 5). Het door koude gestimuleerde sluiten van septa was omkeerbaar door de cultuur 30 minuten terug te plaatsen bij de normale groeitemperatuur van 30 °C. De vraag waarom alleen *A. niger* reageert op veranderde milieu omstandigheden wat betreft het sluiten van septa is onderwerp voor toekomstige studies. Mogelijk geeft het sluiten van septa *A. niger* de mogelijkheid om zich beter aan te passen aan verschillende milieu omstandigheden. Hierbij is het interessant om op te merken dat de Δ*hex1* stam dood ging na 3 dagen bij 45 °C te zijn geplaatst. Dit gebeurde niet bij het wildtype. Dit zou veroorzaakt kunnen worden door het fenomeen van heterogeniteit, wat

verschillen in fysiologie tussen schimmeldraden veroorzaakt. Deze fysiologische verschillen zouden de kans kunnen vergroten dat bepaalde schimmeldraden overleven en anderen niet.

In Hoofdstuk 4 werd aangetoond dat het *glaA* gen van *A. niger* dat codeert voor het glucoamylase eiwit (dat zetmeel omzet in glucose) heterogeen tot expressie komt in *A. oryzae*. Dit is naast *A. niger* de tweede schimmel waarin heterogeniteit van schimmeldraden is aangetoond wat betreft genexpressie tussen naburige hyfen. Heterogeniteit van schimmeldraden conflicteert met het algemeen geaccepteerde idee dat het cytoplasma van schimmels aaneengesloten is door de poriën in de septa. Ik heb echter aangetoond dat ongeveer 40% van de septa gesloten zijn in *A. niger*. Dit betekent dat het cytoplasma van twee schimmeldraden een kans heeft van slechts 3% om met elkaar in contact te staan wanneer deze door 7 septa gescheiden zijn. Dit zou voldoende kunnen zijn om heterogeniteit tussen schimmeldraden te behouden. Inderdaad werd er gevonden dat er geen twee populaties schimmeldraden meer waren in de Δ*hex1* stam, die alleen maar open septa heeft (Hoofdstuk 4). Door het sluiten van septa te reguleren zouden schimmeldraden heterogeniteit kunnen beïnvloeden. Daarmee zou dit een instrument kunnen zijn voor schimmeldraden om overleving tijdens stress condities te bevorderen (zie hierboven).

Opmerkelijk was de vinding dat het sluiten van septa geen effect had op de stroming van suikers op korte- en lange afstand. Wildtype en Δ*hex1* stammen van *A. oryzae* en *A. niger* lieten dezelfde verdeling van glucose zien, nadat het centrum of de buitenkant van de kolonie was blootgesteld aan ^{14}C-glucose. Gesloten septa lijken derhalve geen barrière te vormen voor suikers. Inderdaad bleek inter-compartimentale stroming van 2-NBDG, een fluorescent analoog van glucose, ook nog steeds plaats te vinden onder condities waarbij septa gesloten waren (Hoofdstuk 5). Mogelijk bevat het celmembraan dat de septa omringt transportkanalen voor suikers die intercellulair transport mogelijk maken. Het feit dat 2-NBDG accumuleerde in septa ondersteunt dit idee.

Differentiële expressie in vegetatieve- en luchtstructuren van *A. niger*

De RNA samenstelling van vegetatief mycelium en luchtstructuren (het totaal van luchthyfen, conidiophoren en sporen) van 7 dagen oude kolonies, die op maltose waren gegroeid, werd geanalyseerd (Hoofdstuk 2). In totaal kwamen 6476 van de 14259 genen tot expressie. Daarvan kwamen 5095 genen in het mycelium en 5939 genen in de luchtstructuren tot expressie. 2056 genen kwamen meer dan tweevoud differentieel tot expressie, waarvan er 1144 hoger aanstonden in de luchtstructuren.

Het *GFP* gen werd tot expressie gebracht door deze te plaatsen achter een aantal promotors van genen die actiever zijn in het mycelium of de luchtstructuren. Dit toonde aan dat GFP alleen van het mycelium naar de luchtstructuren kan stromen, maar niet andersom (Hoofdstuk 5). De stroming van GFP naar de luchtstructuren suggereert dat mRNA mogelijk ook vanuit het vegetatieve mycelium naar de luchtstructuren kan stromen. Om dit te onderzoeken werden de micro-array data vergeleken met een nuclear run-on transcription assay (Hoofdstuk 2). Micro-array analyse liet zien dat *gpdA* altijd aan stond, maar de run-on analyse liet juist hogere expressie zien in het vegetatieve mycelium. Deze resultaten kunnen worden

verklaard door stroming van *gpdA* transcripten vanuit het mycelium naar de luchtstructuren, waardoor de verschillen in *gpdA* transcriptie worden uitgemiddeld. Een andere verklaring zou kunnen zijn dat *gpdA* transcripten minder stabiel zijn in het vegetatieve mycelium. Micro-array analyse toonde aan dat er meer *mpdA* transcripten in het mycelium waren dan in de luchtstructuren. Echter volgens de run-on analyse waren ze meer aanwezig in de luchtstructuren. Dit kan verklaard worden door lagere stabiliteit van *mpdA* transcripten in de luchtstructuren of door stroming/transport van transcripten vanuit de luchtstructuren naar het mycelium. Dit laatste is niet heel erg waarschijnlijk, omdat GFP niet bleek te stromen vanuit de luchtstructuren naar het mycelium (Hoofdstuk 5). Een lagere transcript stabiliteit zou ook de verschillen in mRNA niveaus van het flavohemo eiwit coderende gen (flavo) verklaren die gevonden zijn met de run-on analyse en de micro-array analyse (Hoofdstuk 2).

Samenvattend laten de resultaten uit Hoofdstuk 2 zien dat het mycelium en de luchtstructuren van *A. niger* heterogeen zijn wat betreft de samenstelling van hun RNA. De heterogeniteit is niet alleen het resultaat van differentiële genexpressie, maar ook van differentiële mRNA stabiliteit en/of mRNA stroming of zelfs transport.

Curriculum vitae

Robert-Jan Bleichrodt was born on December 30th, 1983 in Gouda, The Netherlands. He followed his secondary education at the Goudse Waarden in Gouda, The Netherlands and graduated in 2002 with an Atheneum-level diploma. In September of the same year he began his coursework in Biology at Utrecht University. As part of his studies he did an internship in the Cell biology group of the Department of Biology at Utrecht University under the supervision of Drs. M.J.A. Moes, followed by a second internship in the Molecular Microbiology group of the Department of Biology at Utrecht University under the supervision of Dr. R.P. de Vries. Robert-Jan obtained his MSc diploma in September 2007. In October of the same year he started his PhD with the Molecular Microbiology group of the Department of Biology at Utrecht University under the supervision of Prof. Dr. H.A.B. Wösten. Research on intercompartmental streaming in *Aspergillus* was financially supported during this period by the Netherlands Organisation for Scientific Research (NWO) and is described in this thesis. In October 2011 Robert-Jan continued working in the Molecular Microbiology group of the Department of Biology at Utrecht University, The Netherlands, were he studies development in *Aspergillus niger*.

Abstracts and presentations

Bleichrodt R, Rector B, Han A.B. Wösten. Intercellular streaming in *Aspergillus niger*. Mycology day, November 26, 2010. Utrecht, the Netherlands (oral presentation).

Bleichrodt R, Brand Rector, Han A.B. Wösten. Intercellular streaming in *Aspergillus niger*. 3rd Kluyver Centre Programme Meeting Day, September 23, 2010. Wageningen, the Netherlands (oral presentation).

Bleichrodt R, van Peer AF, Recter B, Muller WH, Boekhout T, Maruyama J, Kitamoto K, Lugones LG and Wösten HAB. Septal plugging is dynamically regulated during vegetative growth and stress conditions. 26th Fungal Genetics Conference Asilomar, March 15-20, 2011. Monterey, USA (oral presentation).

Bleichrodt R, van Veluw GJ, Recter B, Maruyama J, Kitamoto K, Wösten HAB. Hyphal heterogeneity in fungi is the result of dynamic interruption of cytoplasmic continuity. Kluyver Centre Symposium, January 24-26, 2012. Noordwijkerhout, the Netherlands (oral and poster presentation).

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