Hyphal differentiation in the fungal mycelium

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Differentiatie van hyfen in het mycelium van schimmels

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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Contents

Chapter 1

General Introduction

The fungal kingdom

The fungal kingdom represents a diverse and widespread group of unicellular and multicellular eukaryotic micro-organisms. It has been estimated that it encompasses 1.5 million species, which are divided in the chitridiomycetes, the zygomycetes, the glomeromycetes, the ascomycetes and the basidiomycetes. These groups differ significantly at the molecular, cellular and organismal level. Originally classified as plants, fungi are now placed together with animals and coanoflaggelates in the same group, collectively classified as the opisthokonts (Porter, 2004). Often inconspicuous, fungi grow in virtually all environments, where they fulfil essential roles. They can establish mutual beneficial and parasitic interactions and can be saprotrophs. Many fungi are opportunists by having the capacity to grow saprotrophically and to establish a mutual beneficial and/or parasitic interaction.

Fungi can form a mutualistic symbiosis with a variety of organisms. Fungi belonging to the chitridiomycetes help to digest plant material such as cellulose, lignin, hemicellulose and pectin in the rumen of ruminants (e.g. cow, sheep and goat). Fungi can also be found in the digestive track of insects where they fulfil a similar role. Many fungi form a mutualistic symbiosis with plants, known as mycorrhiza (meaning *fungus-roots*). In a mycorrhizal interaction the roots of the plant are closely associated with fungal hyphae. It has been estimated that over 95% of the plant species are mycorrhizal (Davidson & Christensen, 1977; St. John, 1980) and fossil records (Nicholson, 1975) show that intimate relationships between the fungi and plants date back at least to the Devonian when land plants arose. In the mycorrhizal interaction, the plant donates carbohydrates. In fact, as much as 20% of the total carbon assimilated by plants may be transferred to the fungal partner (Johnson *et al.*, 1997). In return, the extended hyphal network of the fungus aids the plant with the uptake of water and minerals, especially phosphorous (a limiting nutrient in many soils), from the soil. Rousseau *et al.* (1994) found that mycorrhizal hyphae contribute to 80% of the absorbing surface area of pine seedlings. The mycorrhizal fungi also protect the plant against diseases, pathogens and the effects of drought. Lichens represent the interaction of fungi with algae and/or cyanobacteria. This interaction appeared some 600 million years ago (Yuan *et al.*, 2005) and is very similar to that of mycorrhizae with the algae and/or cyanobacteria representing the photobiont.

Contrary to a mutualistic symbiosis, parasitic interactions are harmful to the host and can lead to diseases or even death. Fungi can form parasitic symbioses with numerous organisms (e.g.

plants, animals, humans and other fungi). The parasitic interactions with plants are especially devastating. Virtually every plant has fungal pathogens and every agricultural crop can be infected by a number of pathogens. In general, crop losses may be up to 15% in developed countries while they may even exceed 50% in the developing world (Bowyer, 1999). Although less dramatic compared to plants, fungi also infect humans. About 50 species can infect immuno-competent humans. Among these pathogens are the dermatophytes that infect the nails, hair shafts and the outer layer of the skin (Hosking, 1999). The opportunistic pathogens infect immuno-compromised patients or patients that have received prolonged treatment with broad spectrum antibiotics or treatment with corticosteroids (Hosking, 1999). At the moment about 350 of these species are known (de Hoog *et al.*, 2000), among which *Candida, Cryptococcus* and *Aspergillus* species.

Saprophytic fungi are the primary decomposers of dead organic matter (mostly plant material) and recycle about 85% of the carbon with bacteria and animals mineralizing the remaining 15%. The importance of fungi is illustrated by the fact that they are the only organisms (together with some actinomycetes) that effectively degrade lignin, which represents one of the 3 major constituents of wood (the other two being cellulose and hemicellulose) into carbon dioxide and water. The atmosphere contains about 700×10^9 tons of carbon in the form of carbon dioxide, of which 4-8% is fixed annually by terrestrial plants, most of it going into the synthesis of cell walls. Without fungi dead plant material would accumulate and eventually terrestrial plant life would cease to exist due to depletion of atmospheric carbon (Wessels, 1993a). The fungi are thus indispensable for the global carbon cycling.

The life cycle of mycelial fungi

The life cycle of filamentous fungi begins with the germination of a spore. This results in the formation of hyphae that grow by apical extension and that branch subapically. Subsequent hyphal fusion results in a network of interconnected cells known as the mycelium. Fungal mycelia can colonize distinct patches of substrate but can cover immense areas as well. For instance, genetic individuals of *Armillaria* have been identified that had colonized up to 1000 hectares of forest (Smith & Anderson, 1992; Ferguson *et al.*, 2003). In most natural habitats (e.g. forest soil) the supply of nutrients is not uniform. Filamentous fungi circumvent this spatial heterogeneity by translocation of nutrients through the mycelium (Jennings, 1984; 1987). This is facilitated by porous septa that divide the hyphae in compartments. The

cytoplasm within a fungal mycelium is thus a continuous system. Translocation of nutrients also enables fungi to colonise substrates with low initial resource availability and to actively increase the resource availability in this part of the substrate (Lindahl & Olsson, 2004).

After a sufficient amount of vegetative mycelium has been established the fungal colony initiates the formation of aerial structures that may further differentiate into asexual or sexual spore forming structures of which the mushrooms are the most conspicuous. Growth of these aerial hyphae depends on the translocation of nutrients and water from the vegetative mycelium (Jennings, 1984; Wösten & Wessels, 2006). In this phase of the life-cycle the vegetative mycelium may even become subject to degradation to support the development of the aerial structures (Wessels & Sietsma, 1979; Ruiters & Wessels, 1989; Wessels, 1992).

In the next two sections I will introduce the life cycle of the ascomycete *Aspergillus niger* and the basidiomycete *Schizophyllum commune*, which were used as model systems in this Thesis. Their role in nature, horticulture and the industry is also briefly discussed.

The life cycle of *A. niger* and its role in nature and for the industry

The ecological niche of A. niger and its role in the industry

A. niger is part of the genus *Aspergillus*, which comprises around 175 species that are among the most abundant fungi worldwide. The natural habitat of the Aspergilli is soil and decaying organic matter and they are considered common food spoilage fungi (Pitt & Hocking, 1997). *A. niger* mainly exhibits a saprophytic life style but is also a pathogen of animals and humans with a compromised immune system (Pitt, 1994; Brakhage, 2005). It causes, among others, aspergillosis, which comprises of three principal manifestations; invasive aspergillosis (involving several organ systems, particularly pulmonary disease), non-invasive pulmonary aspergilloma and allergic bronchopulmonary aspergillosis (Stevens *et al.*, 2000).

A. niger secretes a wide variety and large amounts of enzymes that degrade polymers within the substrate into small molecules that can be taken up to serve as nutrients. For instance, glucoamylase is secreted to degrade starch, xylanases to degrade xylan (a constituent of hemicellulose) and feruloyl esterase to degrade lignocellulose within plant material. Apart from these enzymes, it secretes high amounts of organic acids such as citric acid. The capacity to secrete large amounts of proteins and organic acids in combination with the established fermentation technology and molecular biology makes *A. niger* (and other aspergilli)

attractive cell factories. Moreover, the fermentation products of *A. niger* are generally recognized as safe. Nowadays, strains of *A. niger* produce more than 30 grams per liter of glucoamylase (Finkelstein *et al.*, 1989). This enzyme, which is used for the production of glucose syrups, is only one of the examples of enzymes from this *Aspergillus* that is used in the food and feed industry (see Wösten *et al.*, 2007). In addition to its use as a cell factory for its own enzymes, *A. niger* is also used to produce heterologous proteins (Punt *et al.*, 2002).

The life cycle of A. niger

Aspergillus nidulans is the best studied member of the genus *Aspergillus*. It reproduces by releasing both sexual and asexual spores. In contrast, *A. niger* reproduces only asexually. Analysis of the genomic sequence did not reveal why *A. niger* would be incapable of sexual reproduction (Pel *et al.*, 2007). It was therefore suggested that *A. niger*, like *A. fumigatus* and *A. oryzae*, have sexual potential (Galagan *et al.*, 2005; Paoletti *et al.*, 2005; Pel *et al.*, 2007). Asexual reproduction in Aspergilli is mediated by the formation of conidiophores that form spores called conidia. Conidiophore development has been extensively studied in *A. nidulans* (Adams, 1998). The genomic sequence of *A. niger* suggests that the mechanisms underlying asexual reproduction is highly similar, if not identical, to that in *A. nidulans* (Pel *et al.*, 2007).

Conidiophore development in *A. nidulans* begins with the formation of a stalk that elongates by apical extension of an aerial branch (Figure 1A). The conidiophore stalk differs from vegetative hyphae in at least three ways (Mims *et al.*, 1988; Timberlake, 1990). First, the stalk cell extends from a specialized thick-walled cell, termed foot cell, which anchors the stalk to the growth substratum. Second, the conidiophore stalk has a 4 to 5 μm diameter as opposed to the 2 to 3 μm diameter more typical of vegetative hyphae. Finally, unlike vegetative hyphae that can grow indefinitely and are capable of branching, conidiophore stalks rarely branch and their length is relatively determinate. After apical extension of the conidiophore stalk comes to an end, the tip begins to swell (Figure 1B). This structure, the conidiophore vesicle, is not separated by a septum from the stalk. Therefore, the stalk, vesicle and foot cell comprise a single unit. In biserate species, e.g. *A. nidulans* and *A. niger*, the vesicle surface buds producing a layer of primary sterigmata termed metulae (Figure 1C). The metulae in turn bud twice to produce a layer of uninucleate sterigmata termed phialides (Figure 1D). Although phenotypically like metulae in size and shape, phialides differ from metulae in that they give rise to chains of uninucleate conidiospores (Figure 1E). Moreover, phialides form products

that end up and function in the spore (Timberlake, 1990; Stringer *et al.*, 1991; Mayorga & Timberlake, 1990).

It has been estimated that about 1200 genes are up-regulated during a-sexual spore formation of *A. nidulans* (Timberlake, 1980). This high number of genes can be explained by the fact that the environmental conditions and the morphology of the aerial spore-forming conidiophores are totally different from those of the hyphae in the substrate mycelium. It is still unclear if substrate-specific genes, e.g. genes encoding enzymes involved in degradation of the substratum, are expressed by the aerial mycelium. This was studied in **Chapter 4**.

The life-cycle of *S. commune* and its role in nature and for the industry

The ecological niche of S. commune and its role as a model for mushroom formation

S. commune is characterized by its split gills (hence the genus name *Schizo-phyllum*) (Figure 2D). It can be found all over the world (hence the species name *commune*) on fallen branches and logs of especially hardwood (e.g. birch, beech, oak). At least 150 genera of woody plants have shown to be substrates for *S. commune* (Cooke, 1961). Belonging to the white rot fungi, *S. commune* is able to degrade cellulose as well as lignin. It should be noted however, that its lignin-degrading activity is low (Boyle *et al.*, 1992; Boyle, 1998). Although *S. commune* is considered a saprothroph, it also has some pathogenic potential. It has been found to infect apple and apricot trees (Latham, 1970; Lacok, 1986) as well as beech (W.R. Teertstra, unpublished). Moreover, humans have been reported to be infected by this basidiomycete. In fact, the incidence of sinusitis and invasive disease caused by *S. commune* in both immuno-competent and immuno-compromised hosts is increasing (Unno *et al.*, 2005; Sigler *et al.*, 1999).

S. commune completes its life-cycle within a week on synthetic media. Moreover, it forms uninucleate spores, which facilitates both classical and molecular genetics. These properties make *S. commune* an attractive model system to study mushroom formation. This is exemplified by the life-cycle of *Agaricus bisporus* (the white button mushroom). Mycelia formed by this fungus grow slowly on synthetic medium and mushrooms are only produced when the mycelium is grown on compost (Van Griensven, 1988). Moreover, spores formed by *A. bisporus* contain two nuclei with a different mating type. Germination of these spores thus results in a self-fertile heterokaryotic mycelium, containing a variable number of both

nuclear types. Apart from its role as a model system, *S. commune* is also used by the industry to produce fumaric acid (Nout, 2000) and has attracted interest from the cosmetic and pharmaceutical industry because of its production of schizophyllan.

The life cycle of S. commune

The life-cycle of *S. commune* starts with the germination of a basidiospore, which results in a sterile monokaryotic (homokaryotic) mycelium. This mycelium contains identical haploid nuclei with one nucleus in each hyphal compartment. Initially, the mycelium grows submerged. However, after the mycelium has reached a certain biomass, aerial hyphae are formed (for an example of aerial hyphae, see Figure 2A) that have a role in colonization of new substrates (de Jong, 2006).

Monokaryotic mycelia will fuse when they are confronted with each other. Fusion is independent of the mating-type loci *MAT*A and *MAT*B. However, the alleles of these loci should differ for the formation of a fertile dikaryon. The genes in the *MAT*A locus encode homeodomain proteins, which form active heterodimers with homeodomain proteins encoded in other alleles of this locus. The *MAT*B locus encodes pheromones and receptors. These receptors bind pheromones encoded by other alleles of this locus (see Kothe, 1996; Casselton & Olesnicky, 1998).

The fertile dikaryon forms fruiting bodies under appropriate environmental conditions and after a certain biomass is formed. A short exposure to light induces fruiting body formation (Perkins, 1969), while a high concentration of carbon dioxide (Niederpruem, 1963; Sietsma *et al.*, 1977) and high temperatures (30-37 ^{o}C) (Wessels, 1965) are inhibitory. Fruiting body formation starts with the formation of aerial hyphae. These, dikaryotic aerial hyphae leave the substrate and grow as aggregates into the air to form fruiting body primordia (Figure 2). Aerial hyphae of the primordium grow inward and subsequently downward, forming a cup-shaped structure in which the hymenium develops (van der Valk & Marchant, 1978; Raudaskoski & Vauras, 1982). After formation of the gills, basidia develop. In each basidium, karyogamy takes place, which is followed by meiosis. The resulting haploid basidiospores are disseminated and can form a new monokaryotic mycelium in a fresh substrate.

Differentiation within a fungal mycelium

Mycelial growth changes the spatial nutrient availability in the substrate. Nutrients are partly or even completely exhausted in the centre of the colony while they are still available at the

periphery. The spatial distribution and concentration of the phosphor, sulphur and nitrogen source were hardly affected when *A. niger* colonies were grown for 7-days on minimal medium with 25 mM maltose or xylose as a carbon source. In contrast, the carbon source was exhausted in the medium underlying the central and middle parts of the colony, while it was still available at the periphery (Levin, 2007). One may expect that such differences in carbon source availability would affect gene expression in different parts of the colonies. To study this, expression profiles were determined from 5 concentric zones (i.e. from the centre to the periphery) of 7-days-old colonies (Levin, 2007). Global gene expression in the different zones was indeed shown to be dependent on the nature and the availability of the carbon source. However, medium independent differentiation also plays a major role in temporal and spatial expression in the *A. niger* colony (Levin, 2007).

Approximately 50% of the *A. niger* genes were expressed in colonies that had been grown on xylose and/or maltose containing minimal medium. Of these, about 60% were expressed on both carbon sources in all 5 concentric zones of the colony. Thus, 40% of the genes were expressed in particular zones or on a specific carbon source. 3% of the genes were uniquely expressed in a single zone (e.g. the outer peripheral zone or the most central zone) on one of the carbon sources. To study the influence of the spatial distribution of nutrients in more detail, expression profiles were analyzed of 7-day-old xylose grown colonies that had either or not been transferred for 24 h to a fresh medium (Levin, 2007). Statistical analysis indicated that about 20% of the variability could be attributed to the medium composition, most probably to the concentration of the carbon source. The xylanolytic genes strongly responded to the medium composition. Before transfer, most of these genes were highest expressed at the periphery. However, expression was more evenly distributed when the colony had been transferred to a fresh medium. A similar response was observed for genes related to protein secretion and genes encoding putative proteases.

Interestingly, expression profiles at the periphery of the colony changed in time despite the fact that this zone continuously extended in an unexplored medium (i.e. medium composition remains constant in time for this zone of the colony) (Levin, 2007). Expression of approximately 9% of the genes, belonging to different functional gene classes, changed at the periphery between day 3 and 10 when colonies were grown on maltose. Among these genes were half of the xylanolytic genes. Based on these results it was proposed that the periphery goes through a developmental program that is regulated by the age of the colony. Apart from temporal changes in the expression profiles, also spatial changes were observed that were independent of the medium composition. In fact, analyses of the mRNA pools of the

transferred and non-transferred colonies (see above) indicated that the spatial position and not the nutrient availability is the main determinant of the variability in gene expression (Levin, 2007). The gene cluster involved in the conversion of extracellular nitrate to intracellular ammonium was the most remarkable example of the positional differentiation. These genes are only expressed at the periphery, irrespective of the fact that the nitrate concentration was not limited in the central zone of the colony. Unfortunately, it is not known which mechanisms underly the nutrient-independent temporal and spatial expression in the colony. It is clear, however, that the cytoplasmic continuity of the *A. niger* mycelium in combination with cytoplasmic streaming does not result in a homogenous mRNA composition throughout the mycelium. It may well be that mRNA and proteins stream less efficient, if at all, through the mycelium when compared to water and nutrients.

Other studies also implicated differentiation in the fungal vegetative mycelium. Two growth zones were identified in colonies of *A. niger* and *Phanerochaete chrysosporium* (Wösten *et al.*, 1991; Moukha *et al.*, 1993). Secretion of proteins was confined to these growth zones that are located at the periphery and the centre of the colony. Interestingly, differentiation also seems to take place within a particular zone of the colony. The secreting hyphae in the central zone of *P. chrysosporium* were thin compared to the hyphae that grow at the periphery (Moukha *et al.*, 1993). Similarly, fusion-competent substrate hyphae of the ascomycete *Neurospora crassa* are morphologically distinct from those hyphae that are fusion-incompetent (Hickey *et al.*, 2002). Although hyphae at the periphery of *A. niger* colonies were similar in morphology, they also seem to differentiate. At least, immuno-labelling indicated that only part of the growing hyphae within this zone of the colony secreted glucoamylase (Wösten *et al.*, 1991). This phenomenon was further studied in **Chapter 2** and **3**.

Lighting up fungal biology; use of GFP and dsRed as reporter proteins

The fluorescent proteins isolated from marine organisms (e.g., *Aequorea victoria* GFP and *Discosoma striata* DsRed) have revolutionized cellular biology. These proteins offer the ability to visualize, track, and quantify molecules and events in living cells with high spatial and temporal resolution. A broad palette of emission colours ranging from blue to red have been developed to permit co-localization of proteins, co-localization of gene activity, and to create pairs of donors and acceptors for fluorescence resonance energy transfer (FRET). Here, I discuss the properties of selected fluorescent protein variants derived from *A. victoria* GFP and *D. striata* DsRed. These proteins have their own unique properties, while sharing

common structural, biochemical and photophysical characteristics. In addition, I discuss their applications in monitoring gene expression, protein localization, protein secretion and organelle dynamics in living fungal cells.

Structural, biochemical and photophysical biology of GFP and DsRed

GFP and DsRed are relatively small molecules, 27 and 28 kDa, respectively. Among the most important features of these proteins is that the entire native peptide structure is essential and sufficient for the development and maintenance of its fluorescence. Thus, the fluorescence mechanism is self-contained, requiring no co-factors, apart form molecular oxygen. Despite the low level of overall amino acid sequence identity (25%) GFP and DsRed monomers adopt virtually the same three-dimensional structure. Both proteins consist of an 11-stranded β-barrel with a coaxial central α-helix and α-helical caps on the barrel ends. This motif is called the β-can and provides the proper environment for the chromophore to fluoresce (Yang *et al.*, 1996; Yarbrough *et al.*, 2001). The packing of amino acids inside the β-can is very stable, which results in relatively high fluorescence quantum yields (up to 80 percent) and resistance to changes in pH, temperature, and common denaturants, such as urea. The chromophore of GFP and DsRed consists of a tripeptide residing on the central α-helix in the core of the proteins and results from the spontaneous cyclization and oxidation of this sequence (GFP Ser65-Tyr66-Gly67; DsRed Gln66-Tyr67-Gly68) (Yang *et al.*, 1996; Gross *et al.*, 2000). In DsRed an additional oxidation step occurs, that results in its red fluorescence (Gross *et al.*, 2000). Two of the most crucial residues contributing to the chromophore of GFP, Tyr66 and Gly67, and some of the important polar residues contacting the chromophore like Arg96 and Glu222 are conserved in DsRed. GFP has a major excitation peak at 395 nm that is about three times higher in amplitude than a minor peak at 475 nm. Excitation at these wave lengths gives emission peaks at 508 and 503 nm, respectively (Heim *et al.*, 1994). The major excitation and emission peaks of DsRed are 558 and 583 nm. Derivatives of GFP and DsRed have been produced to improve the use of these proteins as *in vivo* reporters. Mutations resulted in GFP and DsRed variants with higher solubility, brighter emission and more rapid chromophore maturation. In addition, codon-optimized versions have been created for efficient translation in various host cells.

Derivatives of GFP

Mutagenesis studies of wild-type GFP (wtGFP) yielded many different variants. A single amino acid substitution, Ser65 to Thr65 (S65T) was found to have profound effects (Heim *et*

al., 1995). This mutation results in a GFP in which the major and minor absorbance peaks of wtGFP are converted to a single absorbance peak at 489 nm and a slightly red-shifted emmision maximum of 510 nm. The argon-ion laser used in most FACS machines and confocal scanning laser microscopes emits at 488 nm, so excitation of this red-shifted GFP variant is much more efficient than excitation of wtGFP. The S65T substitution also accelerates the speed of fluorophore formation (from a time constant of 2 hours to 0.45 hour) and makes the protein less susceptible to photobleaching. Several other mutations located throughout the protein were found to help the molecule fold correctly at 37° C (Tsien, 1998). GFP dimerization at high concentrations was overcome by the mutations Ala206 to Lys206, Leu221 to Lys221, or Phe223 to Arg223 (Zacharias *et al.*, 2002). Furthermore, protein expression was improved by the removal of cryptic intron sites (Haseloff *et al.*, 1997), converting wtGFP codons to forms more efficiently used by the host organisms (Chiu *et al.*, 1996; Fuhrmann *et al.*, 1999) and by changing upstream sequences flanking the coding regions to a Kozak consensus translation initiation site (Kozak, 1992). Several of these improved characteristics are combined in the GFP variant known as enhanced GFP (EGFP). It contains the S65T mutation, the Phe64 to Leu64 mutation that alleviates GFP's temperature sensitivity (Cormack *et al.*, 1996) and has its codons optimised for expression in human cells. This results in an exceptionally bright GFP derivative that fluoresces about 35-fold more intensely than wtGFP when excited at 488 nm.

A photo-activable GFP variant (T203H, PA-GFP) was developed that displays up to 100-fold increased fluorescence excitation at 488 nm when illuminated with 413 nm light (Patterson & Lippincott-Schwartz, 2002). PA-GFP can be used to highlight distinct pools of molecules within the cell and because only photoactivated molecules exhibit noticeable fluorescence, their lifetime and behaviour can be studied independently of other newly synthesized proteins (Patterson & Lippincott-Schwartz, 2002).

Further developments of GFP provided variants differing in their absorbance and emission spectra; blue (BFP), cyan (CFP) and yellow (YFP) fluorescent protein (Heim *et al.*, 1994; Ormö *et al.*, 1996). This allows for the simultaneous visualization of distinct GFP variants in a cell.

The pairing of CFP-YFP has been pivotal in the study of protein-protein interactions using FRET experiments and in the development of FRET-based fluorescent protein biosensors (see van Roessel *et al.*, 2002; Zhang *et al.*, 2002). Their use in the study of multi-protein trafficking has been equally important because they permit the simultaneous temporal and

spatial behaviour of different molecules to be analyzed (see Lippincot-Schwartz *et al.*, 2000). Efforts to further red-shift *A. victoria* fluorescent protein spectra had little success. However, discoveries from other marine organisms have extended available fluorescent proteins to the red wavelength range.

Derivatives of DsRed

The cloning of the red fluorescent protein DsRed (Matz *et al.*, 1999) initially held great promise for cell biology. Its red fluorescence would provide a distinct label for multicolour tracking of fusion proteins, and together with GFP (or a suitable variant) would result in a FRET donor-acceptor pair that should be superior to the currently preferred cyan-yellow pair. However, DsRed has several critical draw-backs including slow (T½ ~11 h) maturation and poor solubility (Baird *et al.*, 2000; Jakobs *et al.*, 2002). Moreover, maturation of DsRed involves tetramerization and an intermediate with fluorescence properties similar to that of wtGFP (Baird *et al.*, 2000). The former is especially problematic as it results in unwanted association of proteins to which DsRed is covalently linked (Lauf *et al.*, 2001). An engineered variant, known as DsRed.T1 (Bevis *et al.*, 2002), effectively solved the problem of the slow maturation (T½ < 1 h). The protein also displays enhanced solubility after decreasing the net charge near the N-terminus and is codon-optimized for expression in mammalian systems (Bevis *et al.*, 2002). DsRed.T1 was used as starting point for the directed stepwise evolution to first a dimer and then to a monomer, mRFP1 (monomeric red fluorescent protein). The subunit interfaces were disrupted by insertion of arginines, which initially crippled the resulting protein, but red fluorescence could be rescued by random and directed mutagenesis, cumulatively requiring 33 substitutions (Campbell *et al.*, 2002). Besides being a monomer and maturing rapidly, the excitation and emission peaks of mRFP1, 584 and 607 nm, are ~25 nm red-shifted from DsRed, which confers greater tissue penetration and spectral separation from autofluorescence and other fluorescent proteins. Albeit, at the expense of its fluorescence quantum yield, extinction coefficient and photostability which are lower than DsRed (Campbell *et al.*, 2002).

The latest generation of monomers derived from mRFP1 (Shaner *et al.*, 2004) include several new colours (yellow-orange to red-orange) with improved extinction coefficients, quantum yields, photostabilities and displaying minimal emission when excited at wavelengths optimal for GFP. Furthermore, tolerance to N and C-terminal fusions was increased by adding GFP-type termini.

The use of GFP and DsRed and their derivatives in filamentous fungi

The development of GFP and DsRed variants with altered spectral properties and preferential codon composition has allowed for efficient expression in a broad range of fungal species. The reporters have been employed for the analysis of diverse fungal cellular processes ranging from the control of the cell cycle, organelle movement and protein secretion to fungus-host interactions. GFP and DsRed derivatives have been extensively expressed in ascomycetes to tag organelles like the nucleus (Toews *et al.*, 2004), mitochondria (Toews *et al.*, 2004), ER (Gordon *et al.*, 2000b) and vacuoles (Shoji *et al.*, 2006). In *A. niger* and *A. oryzae* GFP was used to examine protein secretion *in vivo* (Gordon *et al.*, 2000ab; Masai *et al.*, 2003) enforcing the hypothesis that proteins are secreted at growing hyphal tips (Wösten *et al.*, 1991). Promoter fusions to GFP in *Magnaporthe grisea* showed that expression of the hydrophobin gene *mgp1* is restricted to conidial development and early infection events prior to plant penetration (Kershaw *et al.*, 1998). DsRed has been used to monitor RNA interference in *Acremonium chrysogenum* (Janus *et al.*, 2006). To date, expression of GFP and DsRed derivatives in homobasidiomycetes has been less successful. There are no reports describing DsRed expression, while GFP expression is limited to *P. chrysosporium* (Ma *et al.*, 2001), *Ganoderma lucidum* (Sun *et al.*, 2001), *S. commune* (Lugones *et al.*, 1999), *Agaricus bisporus* and *Coprinus cinereus* (Burns *et al.*, 2005). In these cases GFP was fused to strong promoter sequences and has not yet been used to tag specific organelles or proteins. The advance of the fluorescent protein technology will undoubtedly lead to the development of new techniques to study fungal biology in a broader range of species.

Outline of the Thesis

The aim of this PhD Thesis was to study differences in gene expression between and within zones of fungal colonies, in particular of genes encoding secreted enzymes. It was also demonstrated that proteins, at least GFP, can stream through the mycelium. Protein streaming may affect differentiation of the fungal mycelium.

Chapter 2 describes the expression of *GFP* in 5-day-old colonies of *A. niger* when placed under regulation of the glucoamylase *glaA* and the glyceraldehyde-3-phosphate dehydrogenase *gpdA* promoter. The *gpdA* gene was expressed throughout the colony while the *glaA* gene was expressed at the periphery of mycelium that had been transferred from a

repressing to an inducing medium. Within this zone the exploring hyphae differentiate with respect to gene expression; some strongly express the *glaA* gene, while others hardly express it at all. The phenomenon of hyphal differentiation indicates that a fungal mycelium is highly differentiated.

Chapter 3 shows that the α-glucuronidase gene *aguA* of *A. niger* is quite evenly expressed in 5-day-old colonies upon transfer to an inducing medium. However, both glucoamylase GlaA and α-glucuronidase AguA are only secreted at the periphery of the mycelium. Similar to *glaA*, *aguA* is subject to hyphal differentiation within the exploring zone.

No evidence was found for differential *glaA* and *aguA* expression in 24-h-old pellets grown in liquid shaken medium. However, the feruloyl esterase gene *faeA* was differentially expressed under this condition. These results show that the phenomenon of hyphal differentiation depends on the growth conditions and that it is a potential target to improve protein production in industrial fermentations.

Chapter 4 presents evidence that cytosolic proteins can be translocated from the vegetative mycelium into the conidiophore. This was concluded from the fact that both the vegetative mycelium and the conidiophores were fluorescent when a cytosolic GFP was expressed from the *gpdA* or *glaA* promoter. In contrast, only nuclei of the vegetative hyphae turned fluorescent when a nuclear targeting signal was fused to the GFP. Quantification of fluorescence of cytosolic GFP indicates that the composition of the conidiospore partially depends on the expression profile of the vegetative mycelium that underlies the conidiophore.

Chapter 5 describes the expression of the fluorescent reporters GFP and DsRed in *S. commune*. Steady state mRNA levels of *GFP* variants expressed from the *SC3* promoter were much lower than that of endogenous *SC3*. High mRNA accumulation of *GFP* was obtained when the reporter was fused to the N and/or C-terminal part of the SC3 and SC15 proteins. Immuno-labelling showed that these fusion proteins were secreted. However, no fluorescence was observed with the SC3 fusions while the SC15 fusion showed strong fluorescence in the cytoplasm. A GFP variant codon-optimized for expression in *S. commune* was not active in this basidiomycete. In contrast, the *DsRed2* gene was successfully expressed. In fact, the absence of red autofluorescence of *S. commune* hyphae favors the use of this reporter instead of GFP.

The results are summarized and discussed in **Chapter 6.**

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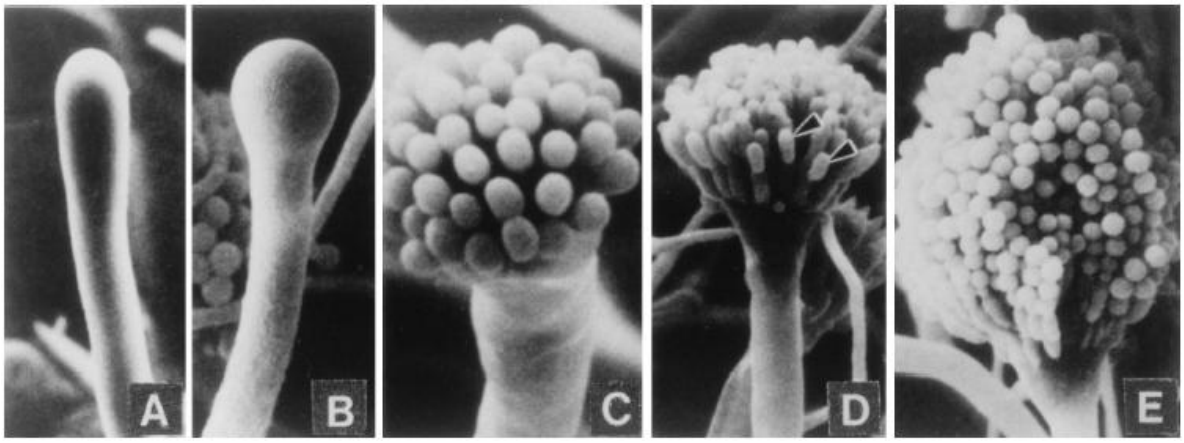

Figure 1. Asexual conidiation in *A. nidulans* (see text for details). Taken from Adams *et al.*, 1998.

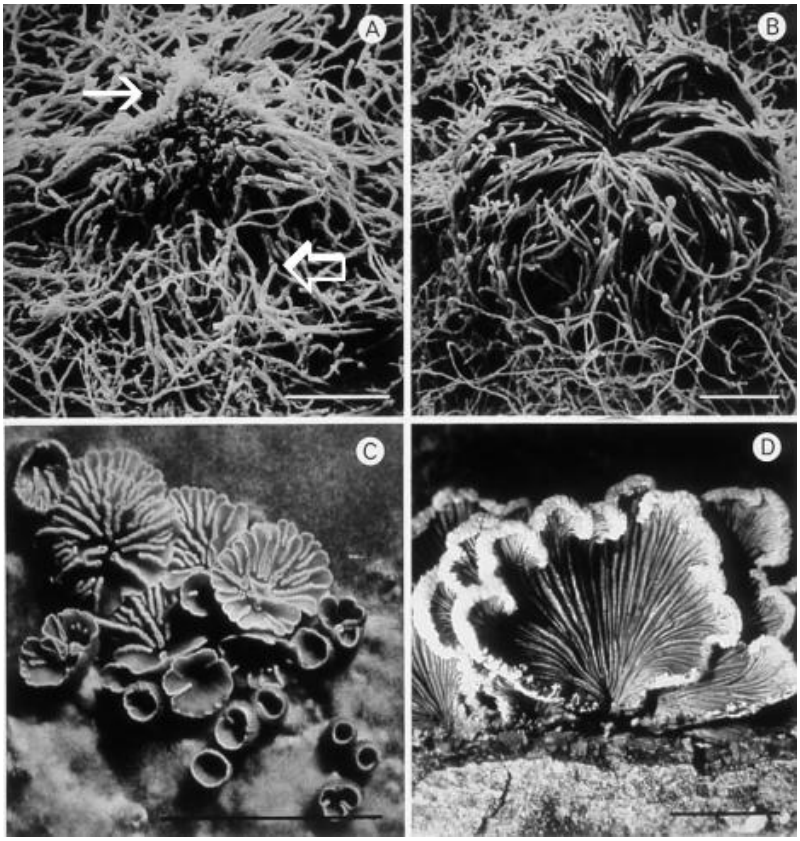

Figure 2. Formation of fruiting bodies is initiated with the formation of aerial hyphae (**A**). In the dikaryon, but not in the monokaryon, these aerial hyphae aggregate to form a primordium (**B**). The primordium develops into a simple cup (**C**) with the hymenium lining the entire inner surface. The split gills arise by marginal proliferation of the cup. As a geotropic response this marginal proliferation becomes unilateral in vent-shaped fruiting bodies (**D**). Open arrow indicates individual aerial hyphae, closed arrow indicates aggregated aerial hyphae (Taken from Wessels, 1993b).

Chapter 2

Hyphal differentiation in the exploring mycelium of *Aspergillus niger*; high and low glucoamylase expressers

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Abstract

Mycelial fungi play a central role in element cycling by degrading dead organic material such as wood. Moreover, they are devastating pathogens. Fungal colonization of a substrate starts with the invasion of exploring hyphae. These hyphae secrete enzymes that convert the organic material into small molecules that can be taken up by the fungus to serve as nutrients. Using GFP as a reporter, we show that exploring hyphae of *Aspergillus niger* differentiate with respect to enzyme secretion; some strongly express the glucoamylase gene, while others hardly express it at all. When a cytoplasmic GFP was used, 27% of the hyphae belonged to the low expressing hyphae. However, by fusing GFP to glucoamylase and by introducing an ER retention signal, this number increased to 50%. This difference is probably explained by cytoplasmic streaming of the reporter in the former case, as was shown by using a photoactivatable GFP. Our findings show that differentiation in a fungal mycelium is more advanced than generally assumed, especially when taking into account that hyphae in the exploring zone were exposed to the same nutritional conditions.

Introduction

Mycelial fungi use the hyphae growing at their apices to colonize dead organic material such as wood. These hyphae form an interconnected network called a mycelium, which can cover large areas. In fact, a genetic individual of *Armillaria* has been identified that had colonized up to 1000 hectares of forest (Smith *et al.*, 1992; Ferguson *et al.*, 2003). The substrate is explored by hyphae that grow at the periphery of the mycelium. The hyphae behind this 'exploration' zone branch intensely and thus expand colonization of the substrate. Growing hyphae in both the 'exploration' and the 'branching' zones secrete a wide variety of enzymes into the environment. These enzymes that are released at the hyphal tips (Wösten *et al.*, 1991) convert the polymers in the substrate into small products that can be taken up to serve as nutrients.

Proteins are not only secreted at the periphery of the mycelium (i.e. the 'exploration' and the 'branching' zones) but also in the centre (Wösten *et al.*, 1991; Moukha *et al.*, 1993). Here, secondary growing hyphae are formed. These hyphae secrete proteins different from those secreted at the periphery. For instance, glucoamylase is secreted at the periphery of the mycelium of *Aspergillus niger* (Wösten *et al.*, 1991) while manganese peroxidase is secreted

in the centre of the mycelium of *Phanerochaete chrysosporium* (Moukha *et al.*, 1993). Such differential expression can be explained by differences in the nutritional conditions in each zone of the mycelium. Here, we show that the glucoamylase gene *glaA* of *A. niger* is differentially expressed in the exploration zone of the mycelium, despite the fact that each hypha in this zone experienced identical nutritional conditions. Differentiation in fungi appears therefore to be more advanced than generally assumed.

Materials and Methods

Strains and plasmids

Aspergillus niger AB4.1 and its derivatives (Table 1) were used in this study. AB4.1 is derived from strain N402 (Bos *et al.*, 1988) and contains a mutation in *pyrG*1 (van Hartingsveldt *et al.*, 1987). *Escherichia coli* DH5α was used for cloning purposes.

Table 1. Strains used in this study.

Strain	Transforming Construct	Description of Construct
AR9#2	P*glaA*_sGFP **(a)**	Plasmid containing *sGFP* under regulation of the *glaA* promoter of *A. niger* (Siedenberg *et al.*, 1999)
AR19#1	P*gpdA*_sGFP	As **(a)** but with the *gpdA* promoter of *A. nidulans* (Lagopodi *et al.*, 2002)
AV10 #9	P*glaA*_Gla_{514}::sGFPhdel **(b)**	Plasmid containing a chimaeric gene consisting of the promoter and part of the coding sequence of *glaA* of *A. niger* followed by the coding sequence of *sGFP* and an ER retention signal (Gordon *et al.*, 2000)
XW2.3.1	P*gpdA*_Gla_{514}::sGFPhdel	As **(b)** but with the *gpdA* promoter of *A. nidulans* (This study)
AV13#1	P*glaA*_d2EGFP	As **(a)** but containing *d2EGFP* (Clontech) in stead of *sGFP* (This study)
P*glaA*_$PAGFP_{25}$	PAN52-10-PAGFP	As **(a)** but containing *PA-GFP* in stead of *sGFP* (This study)

Growth conditions

E. coli was grown at 37 ºC in Luria-Bertani (LB) medium either or not supplemented with ampicillin (50 μg ml^{-1}) and agar (15 g l^{-1}). Liquid cultures were shaken at 200 rpm. *A. niger* was grown at 30 °C in minimal medium (Pontecorvo *et al.*, 1953) containing trace elements according to Vishniac and Santer (Vishniac and Santer 1957) and 10 mM uridine in case of strain AB4.1. Either 3% D-xylose (w/v) or 3% D-maltose (w/v) was used as carbon source. GFP reporter studies were done in static liquid medium or using the sandwiched culturing technique (Wösten *et al.*, 1991). In the latter case, *A. niger* was grown in a 0.2 mm thin layer of 1.25% agarose (D-1 agarose, type low EEO, Hispanagar, Spain) in between two perforated polycarbonate membranes (diameter 76 mm, pore size 0.1 μm; Osmonics, GE Water Technologies, Trevose, PA, USA) placed on top of solidified (1.5% agar) minimal medium. 5-day-old colonies were grown from mycelial plugs, while 1-day-old germlings were grown from spores.

Construction of the GFP reporter constructs

PA-GFP (Patterson and Lippincott-Schwartz 2002) was cloned as an *Nco*I-*Bam*HI (blunted) fragment in pAN52-10Not digested with *Nco*I and *Eco*RV. This resulted in plasmid pAN52-10-PAGFP. P*gpdA*_Gla_{514}::sGFPhdel (Table 1) was constructed by replacing the *Not*I/*Nco*I promoter fragment of P*glaA*_Gla_{514}::sGFPhdel (Gordon *et al.*, 2000) for that of the *gpdA* promoter of *A. nidulans* derived from construct p*GPD*GFP (Lagopodi *et al.*, 2002). The internal *Nco*I-site of *d2EGFP* was removed by site-directed mutagenesis. To this end plasmid pd2EGFP (Clontech) was used as a template and Fd2EGFPMUT (5' gTACAAgAAgCTTAgTCAT-ggCTTCCCgCC 3') and Rd2EGFPMUT (5' ggCgggAAgCCATgACTAAgCTTCTTgTAC 3') as sense and anti-sense primers, respectively. The modified *d2EGFP* fragment was subsequently amplified with primers FGFPNco (5' CCATggTgAgCAAgggCgAggAgC 3') and Rd2EGFPEcoRV (5' gATATCCTACACATTgATCCTAgCAgAAgC 3') containing *Nco*I and *Eco*RV linkers, respectively. The fragment was cloned in the respective sites of pAN52-10Not. This resulted in plasmid P*glaA*_d2EGFP. PCR-products were verified by sequencing.

Transformation of *A. niger*

Protoplasts were prepared as described (Kusters-van Someren *et al.*, 1991) and transformed using polyethylene glycol (Punt and van den Hondel 1992). Transformants were selected by

co-transforming with pAB4.1 (containing the *A. niger pyrA* gene) and purified by repeated streaking of conidia.

Microscopy

GFP fluorescence at the colony level was monitored using a Leica MZFLIII binocular equipped with a HBO 100 W mercury lamp and a Photometrics Cool SNAP camera (1392 x 1024 pixels). GFP fluorescence at the hyphal level was studied by confocal scanning laser microscopy. Laser intensity was kept to a minimum to reduce photobleaching and phototoxic effects. Images were captured as *z*-series of optical sections. An inverted Leica TCS SPII system equipped with a PL FLUOTAR16x / 0.5 IMM plan apochromatic objective lens was used to image fluorescence of sGFP. The protein was excited with the 488 nm laser line and fluorescence was detected at 500-550 nm bandpass. The data sets were displayed as maximum intensity projections (1024 x 1024 pixels) using Leica LSM software. The fluorescence of the ER-targetted Gla_{514}::sGFP fusion and PA-GFP were monitored with an inverted Zeiss LSM5 system using a PLAN-Neofluar 20x /0.50 or a C-Apochromat 63x/1.2 W corr objective lens, respectively. These GFP variants were excited with the 488 nm laser line and fluorescence was detected at 505-530 nm bandpass. Fluorescence of PA-GFP was activated (~1 second) with the 405 nm laser line (2.5 mW). The data sets of the ER-targetted Gla_{514}::sGFP fusion were displayed as maximum intensity projections (1024 x 1024 pixels) using Zeiss software.

Image analysis

The intensity of fluorescence was quantified by measuring the mean pixel values of hyphae in geometrically calibrated images using a program based on KS400 software (Version 3.0; Carl Zeiss Vision, Oberkochen, Germany). Hyphae were selected by thresholding on the basis of grey value. The average grey value of the background was determined by the inverse of a dilated hyphal image. Fluorescence was quantified as the sum grey value in the hypha minus the sum background value in an equivalent area. Data was subjected to descriptive statistics using SPSS software.

To examine whether fluorescence intensity distributions can be explained by a mixture of two normally distributed components, the data was modelled in the probability distribution $\varphi : \varphi(x) = pN(x;\mu_1,\sigma_1) + (1-p)N(x;\mu_2,\sigma_2)$, where $0 < p < 1$ and $x \mapsto N(x;\mu,\sigma)$ is the probability density of the normal distribution with parameters μ and σ. This model will be

referred to as a mixture of a $N(\mu_1;\sigma_1)$-distribution and a $N(\mu_2;\ \sigma_2)$-distribution in which the degree of participation of the component $N(\mu_1;\ \sigma_1)$ is *p*. Note that there are five parameters in the model: $p;\ \mu_1;\ \sigma_1;\ \mu_2;\ \sigma_2$. In the statistical analysis these five parameters were fit to the empirical data by means of the maximum likelihood principle. Interval estimates for the parameters were obtained by means of bootstrapping (1000 replicates). The fit procedures were implemented in the Scilab (free) software package. The scripts of the Scilab functions used are available at http://www.bio.uu.nl/~microbio/Microbiology/Tools.htm.

Results

glaA is differentially expressed in the exploring zone of colonies

Expression of the *glaA* gene in the mycelium of *A. niger* was monitored by expressing the green fluorescent protein gene *sGFP* behind the *glaA* promoter (see Material and Methods). This promoter is repressed on D-xylose but induced on D-maltose (Fowler *et al.*, 1990). As a control, *sGFP* was placed under control of the constitutive *gpdA* promoter. 5-day-old sandwiched colonies of strain AR19#1 expressing *sGFP* behind the *gpdA* promoter were fluorescent throughout the mycelium when grown on D-xylose or D-maltose medium (Figure 1DF). Similar results were obtained when colonies of AR9#2 expressing *sGFP* behind the *glaA* promoter were grown for 5 days on D-maltose (Figure 1C). In contrast, almost no fluorescence was observed when cultures were grown for 5 days on the repressing carbon source D-xylose (Figure 1A). However, these colonies became fluorescent at the periphery when they were transferred to D-maltose containing medium for 8 h (Figure 1B). The fluorescence of colonies of strain AR19#1 was not affected by the transfer (Figure 1E). From these experiments it is concluded that the *glaA* gene is most active at the periphery of the colony, including the exploring zone. GFP fluorescence in the center of 5-day-old colonies grown continuously on D-maltose is probably the result of reporter molecules that were formed at the moment the center was the periphery of the colony. Fluorescence in the center may also be due to cytoplasmic streaming of the reporter through the colony (see below).

In the next set of experiments GFP fluorescence was studied at the hyphal level. CLSM showed that all hyphae in the exploring zone of 5-day-old D-maltose grown colonies of strains AR9#2 and AR19#1 were fluorescent (Figure 2AC). The distribution of fluorescence

intensity of individual hyphae suggested a normal distribution in both cases (Figure 2GI). Non-significant deviation from normality was verified by the Kolmogorov–Smirnov test (with the Lilliefors significance correction) ($p > 0.200$; Table 2). In contrast, the distribution of fluorescence intensity did not follow a normal distribution after inducing *glaA* expression for 8 h by transferring 5-day-old D-xylose grown colonies to D-maltose containing medium ($p < 0.01$). In contrast to *gpdA* driven expression (Figure 2J), the plot of induced *glaA* expression is skewed to the left (Figure 2H). Bootstrap analysis (Efron, 1979) fitted the distribution as a weighted mix of two normal distributions (Figure 3C; Table 2). This suggests that two groups of *glaA* expressing hyphae exist within the periphery of the *A. niger* mycelium; hyphae highly and hyphae lowly expressing the *glaA* gene. 73% of the hyphae belonged to the former group, 27% to the latter. Similar results were obtained when 16-h-old germlings of AR9#2 were transferred for 8 h to D-maltose containing medium or when these germlings were grown for 24 h on inducing medium ($p < 0.01$) (Figure 3AB, Figure 4, Table 2). In these cases 48 and 58% of the hyphae belonged to the high expressers, respectively. Variation in hyphal fluorescence of strain AR19#1 was normally distributed under these conditions ($p > 0.200$).

Cytoplasmic streaming interferes with localization of expression

Cytoplasmic streaming is a well-known phenomenon in filamentous fungi (Jennings, 1994). We here studied streaming of GFP in the cytoplasm of hyphae of *A. niger* using a photo-activatable variant of GFP (PA-GFP; Patterson and Lippincott-Schwartz, 2002). Strain P*glaA*_PAGFP$_{25}$ expressing PA-GFP under control of the *glaA* promoter was grown for 2 days in static liquid medium containing D-maltose. Activated PA-GFP in subapical parts of hyphae streamed to adjacent regions within seconds (Figure 5). PA-GFP was also streaming into branches (data not shown). These data show that cytoplasmic streaming interferes with localization studies at the colony and hyphal level by leveling out differences in GFP fluorescence.

A fusion between GlaA and GFP, containing a HDEL sequence, is differentially expressed in the exploring zone

The use of d2EGFP (Li *et al.*, 1998) was explored to reduce the effect of cytoplasmic streaming. This destabilized GFP has a half-life of 2 instead of 24 h when expressed in mice due to the presence of a PEST sequence (Rogers *et al.*, 1986). The d2EGFP protein has been succesfully expressed in the filamentous ascomycete *Magnaporthe grisea* (Soanes *et al.*,

2002). Construct P*glaA*_d2EGFP encompassing the *d2EGFP* gene under control of the *glaA* promoter was introduced in *A. niger*. Transformants were grown for 5 days on inducing medium and then transferred for 24 h to medium containing D-xylose to repress *glaA* expression. No difference in fluorescence intensity between sGFP and d2EGFP expressing transformants was observed before and after transfer even after prolonged incubation (48 h) (data not shown). From this it is concluded that the destabilized GFP variant is stable in *A. niger* under these growth conditions.

As an alternative to reduce the effect of cytoplasmic streaming, GFP was targeted to the endoplasmic reticulum (ER). To this end, a fusion protein was expressed consisting of the first 514 amino acids of glucoamylase followed by sGFP and an ER retention signal (HDEL). The latter was included to prevent the fusion protein from being secreted into the medium. The encoding gene was expressed from the *glaA* and the *gpdA* promoter. Fluorescence at the colony level was similar in strains expressing the cytoplasmic or the ER-located version of GFP (data not shown). In case of *gpdA* driven expression in strain XW2.3.1, a granular fluorescence was observed in all hyphae in the exploring zone (Figure 6) and their intensity followed a normal distribution ($p > 0.200$) (Table 2). In contrast, *glaA* driven expression of the ER-retained GFP in strain AV10#9 did not result in a normal distribution ($p < 0.005$) when 5-day-old D-xylose grown colonies were transferred to inducing medium for 8 h. Bootstrap analysis showed that in this case two types of hyphae can be distinguished; hyphae highly and hyphae lowly expressing the fusion protein. Both groups represented about 50% of the population (Table 2). Interestingly, in all cases when the ER-targeted GFP was expressed tips of hyphae were more fluorescent than the subapical parts. This was especially the case in the high expressing hyphae after induction of *glaA* (Figure 6).

Discussion

Differential expression of genes in filamentous fungi has been reported during asexual and sexual reproduction. For instance, it was estimated that about 1200 genes are up-regulated during asexual spore formation of *Aspergillus nidulans* (Timberlake, 1980). This high number of genes can be explained by differences in environmental conditions and morphology of the spore-forming conidiophores and the hyphae in the substrate. Until now hardly anything is known about differential expression of genes in the substrate mycelium. Interestingly,

glucoamylase secretion could only be detected at tips of part of the hyphae in the exploration zone of the sandwiched colony (Wösten *et al*., 1991). The absence of an immuno-labelling was at that time attributed to insensitivity of the method. However, we here showed that the *glaA* gene, encoding glucoamylase, is differentially expressed in this zone of the colony. This differentiation is remarkable considering the fact that each hypha in the exploration zone experiences similar nutritional conditions.

Table 2. Descriptive statistics of the fluorescence intensities of hyphae in the exploring mycelium of *A. niger* grown as sandwiched colonies or between cover glasses. AR9#2 and AV10#9 express *GFP* under the control of the *glaA* promoter, whereas AR19#1 and and XW2.3.1 express the reporter from the *gpdA* promoter. N = sample size; KS = *p*-value of Kolmogorov-Smirnov-test; $\mu_{1.2}$ = mean of component 1 and 2, respectively; $SD_{1.2}$ = standard deviation of component 1 and 2, respectively; pf = participation frequency of component 1; CI = 95% confidence interval of pf.

Strain & growth condition	N	SD	KS	μ_1	μ_2	SD_1	SD_2	pf	CI	CI
AR9#2 5d D-maltose	215	24.94	> 0.200*	100.01	95.93	20.85	40.84	0.85	0.01	1.00
AR9#2 8h D-maltose	508	36.48	> 0.006	65.15	112.19	16.11	33.53	0.27	0.12	0.57
AR19#1 5d D-maltose	115	19.62	> 0.200*	78.96	106.45	9.64	17.16	0.25	0.07	0.95
AR19#1 8h D-maltose	285	22.36	> 0.200*	96.54	142.80	19.78	9.90	0.94	0.06	0.97
AR9#2 24 h D-maltose	207	22.14	> 0.001	87.67	116.47	20.60	8.40	0.58	0.33	0.85
AR9#2 16 h xy 8h maltose	301	27.37	> 0.004	76.69	121.32	15.13	16.56	0.48	0.30	0.66
AR19#1 24 h D-maltose	199	19.05	> 0.200*	94.13	118.25	17.73	8.85	0.78	0.01	0.93
AR19#1 16 h xy 8h mal	192	17.60	> 0.200*	98.95	154.94	16.88	3.47	0.99	0.04	0.99
AR9#2 24 h D-maltose (glass cover)	537	24.56	> 0.076	93.69	127.94	20.82	21.28	0.83	0.09	0.99
AR19#1 24 h D-maltose (glass cover)	325	17.40	> 0.200*	96.50	102.30	13.35	20.03	0.48	0.04	1.00
AV10#9 8h D-maltose	239	37.15	> 0.005	78.17	124.84	20.62	36.52	0.54	0.18	0.84
AV10#9 5d D-maltose	107	32.03	> 0.200*	96.04	161.72	28.61	21.66	0.94	0.09	0.99
XW2.3.1. 8h D-maltose	94	24.54	> 0.200*	98.47	199.00	22.26	0.00	0.99	0.09	0.99

* This is a lower bound of the true significance.

Expression of the *glaA* gene was studied using GFP as a reporter. 5-day-old D-xylose grown colonies that had been transferred for 8 hrs on inducing D-maltose medium were highly fluorescent at the periphery of the colony. This agrees with the high accumulation of *glaA* mRNA and the abundant secretion of glucoamylase at the periphery of such colonies (Wösten *et al.*, 1991; Wösten, 1994). From this it can be concluded that the reporter construct that was used in this study gives a good representation of *glaA* expression.

Differential expression of *glaA* could not be unambiguously shown in case of 1-day-old germlings grown in the absence of a covering membrane or cover slide but was observed in sandwiched cultures. Possibly, hyphal differentiation depends on the growth rate since hyphal

extension was observed to be less in sandwiched colonies. Differential expression of *glaA* was shown in sandwiched cultures of 1-day-old germlings grown on D-maltose and in germlings and 5-day-old colonies that had been grown on D-xylose and transferred to inducing medium for 8 hours. Using the cytoplasmic version of GFP, about 40-50% of the germlings belonged to the low *glaA* expressing group, while 27% of the exploring hyphae of 5-day-old induced colonies belong to this group. The difference in the number of low expressers between the germlings and the 5-day-old colonies can be explained by cytoplasmic streaming of the reporter, as was shown using photo-activatable GFP. Indeed, the number of low expressing hyphae increased to 50% when an ER-retained GFP was used. Cytoplasmic streaming also explains why differential expression of *glaA* was not observed in colonies grown for 5 days on inducing medium.

How can we explain the phenomenon of differential expression of the glaA gene? Possibly, the low expressers have a reduced capacity to take up the inducer from the environment. These hyphae could for instance lack a sugar transporter. Alternatively, differential expression is due to the absence or presence of an activator or repressor. Stochastic processes may cause this. It has been shown that organisms use stochastic fluctuations to introduce diversity in a population, as occurs with the lysis-lysogeny bifurcation in phage λ (Arkin *et al.*, 1998) and during competence development in *Bacillus subtilis* (Smits *et al.*, 2005). An important source of stochasticity is the random noise in transcription and translation. This can result in different rates of the production of particular proteins in genetically identical cells that are exposed to identical environments (Elowitz *et al.*, 2002; Ozbudaki *et al.*, 2002; Blake *et al.*, 2003). This may be especially the case for transcription factors that are lowly abundant in the cell. When the transcription factor stimulates its own expression, the effect of noise can be amplified (Ferrell, 2002). As a result two populations of cells emerge, i.e. those with and those without a significant number of molecules of a particular transcription factor. Target genes are thus only activated in part of the cells.

This study has opened a new field in fungal cell biology. Future research should establish whether other genes are differentially expressed and how many hyphal types exist within the substrate mycelium. Moreover, it should be established whether this differentiation also operates during fungal pathogenesis or during protein production in industrial fermentations.

Acknowledgements

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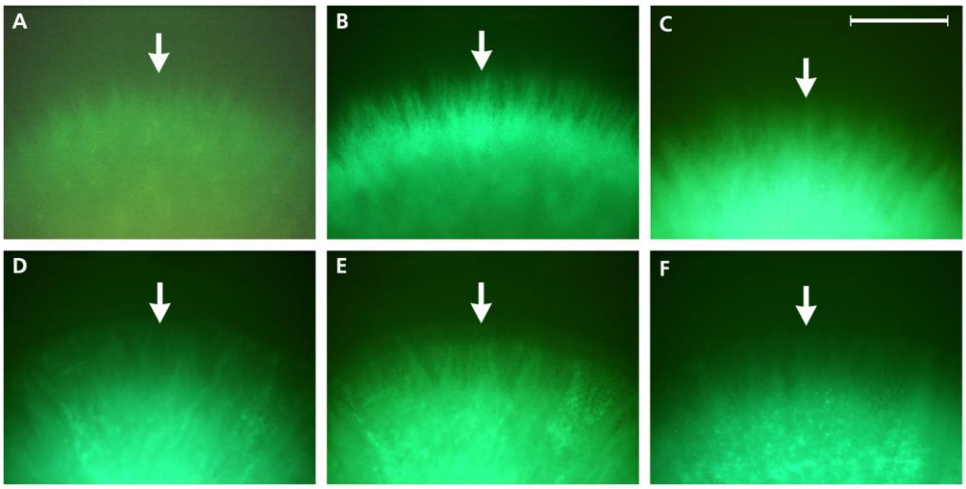

Figure 1. GFP fluorescence at the colony level of recombinant strains AR9#2 (**A-C**) and AR19#1 (**D-F**) expressing the reporter from the *glaA* and *gpdA* promoters, respectively. Colonies were grown for 5 days on D-xylose (**A, D**) and transferred for 8 hrs to D-maltose containing medium (**B, E**). Alternatively, colonies were grown for 5 days on D-maltose (**C, F**). Bar represents 1cm.

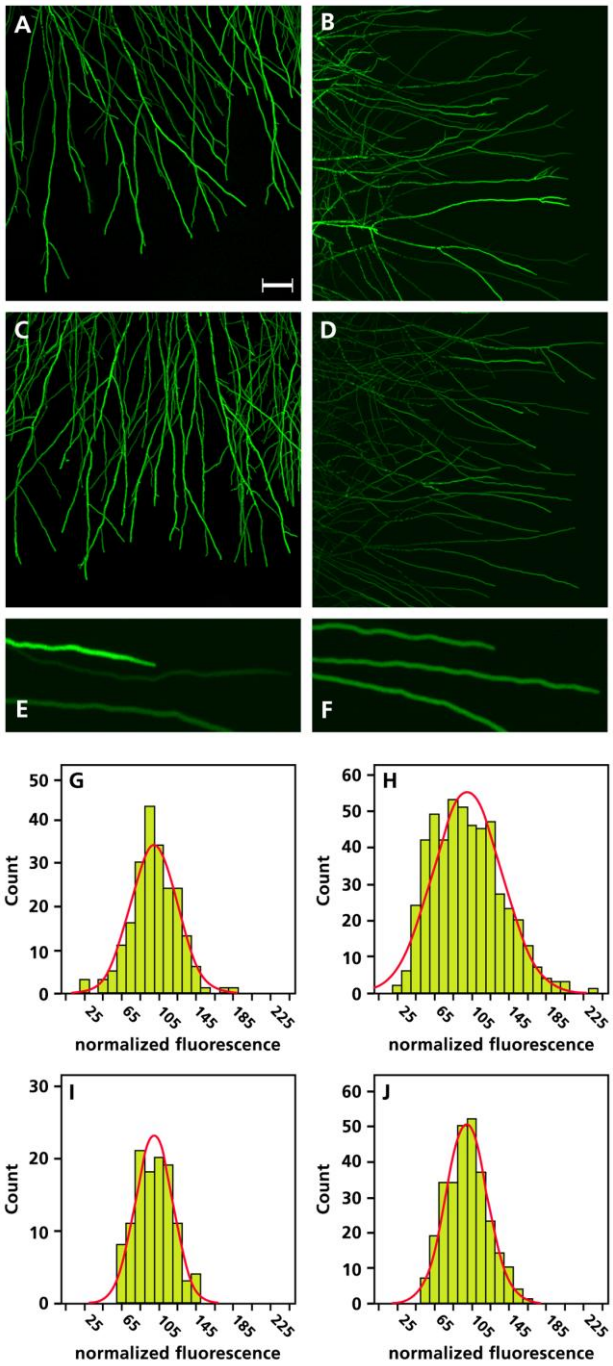

Figure 2. CLSM images (**A-D**) and the respective fluorescence intensity distribution (**G-J**) of hyphae in the exploration zone of colonies of strains AR9#2 (**A, B, G, H**) and AR19#1 (**C, D, I, J**). These strains express the reporter behind the *glaA* and *gpdA* promoter, respectively. Colonies were grown for 5 days on maltose (**A, C, G, I**) or for 5 days on xylose followed by 8 hrs on maltose (**B, D, H, J**). Images in **E** and **F** represent a magnification of selected hyphae shown in **B** and **D**, respectively. Bar represents 40 µm (**A-D**).

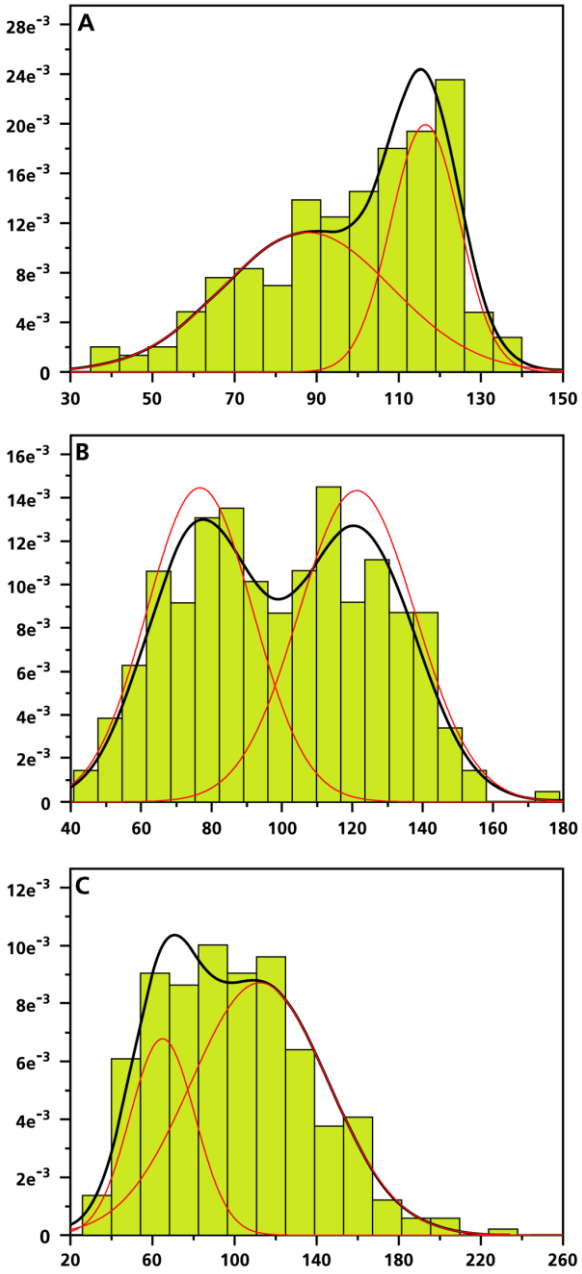

Figure 3. The optimal fit of the distribution of fluorescence of individual hyphae of strain AR9#2 expressing *sGFP* behind the *glaA* promoter can be described as a weighted mix of two normal distributions. Mycelium was grown for 24 h on D-maltose containing medium (**A**) or for 16 h (**B**) or 5 days (**C**) on D-xylose containing medium followed by a transfer for 8 h to medium containing D-maltose as carbon source.

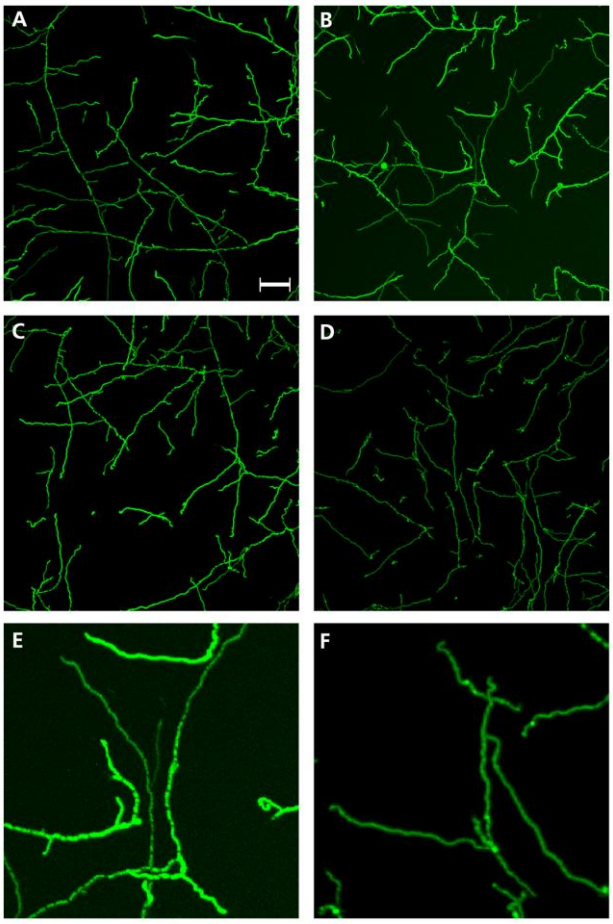

Figure 4. CLSM images of germlings of strains AR9#2 (**A, B, E**) and AR19#1 (**C, D, F**). Strains AR9#2 and AR19#1 express sGFP behind the *glaA* and *gpdA* promoter, respectively. Spores were germinated for 24 hrs on maltose containing medium (**A, C**) or for 16 hrs on xylose containing medium followed by a transfer for 8 hrs to medium containing maltose as carbon source (**B, D**). Images in **E** and **F** represent a magnification of selected hyphae shown in **B** and **D**, respectively.

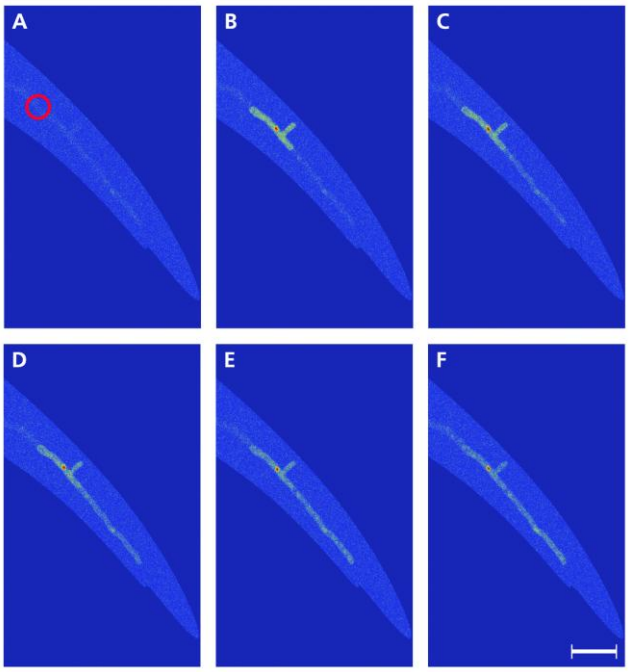

Figure 5. Cytoplasmic streaming of photo-activated PA-GFP through a hypha of strain P*glaA*_PAGFP$_{25}$. Photo-activation at the target area (as indicated by the red circle) (**A**) resulted in bright fluorescence (**B**). After 1 (**C**), 2 (**D**), 5 (**E**) and 7, 5 (**F**) minutes PA-GFP streamed towards the hyphal tip, into the branch and eventually towards subapical compartments. Images, as indicated in false colour, were cropped (as indicated by the light blue area surrounding the hypha) to increase the scan speed. Bar represents 20 µm.

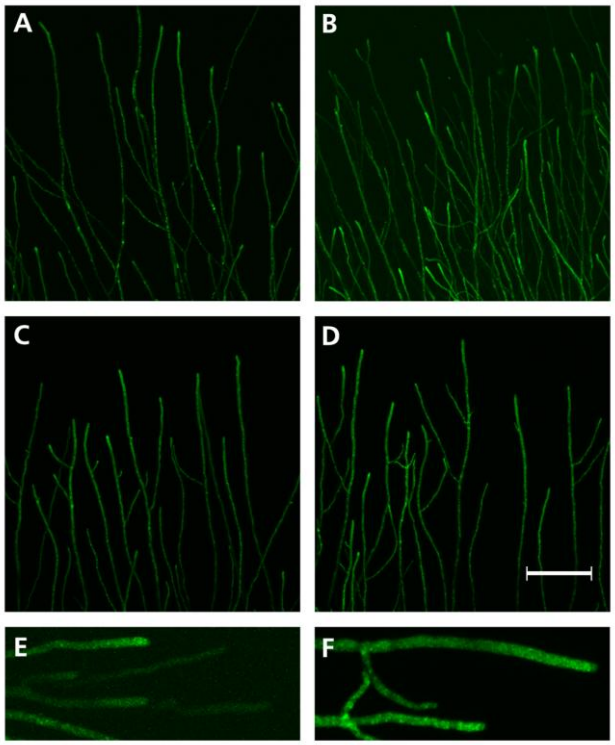

Figure 6. CLSM images of hyphae in the exploration zone of colonies of strains AV10#9 (**A, B**) and XW2.2.1. (**C, D**) expressing a gene encoding an ER-retained sGFP regulated by the *glaA* and *gpdA* promoter, respectively. Colonies were grown for 5 days on medium containing maltose as carbon source (**A, C**) or were grown for 5 days on xylose-containing medium followed by a transfer for 8 hrs to maltose containing medium (**B, D**). Images in **E** and **F** represent a magnification of selected hyphae shown in **B** and **D**, respectively. Bar represents 100 µm (**A**-**D**).

Chapter 3

Differential expression of *glaA*, *faeA* and *aguA* at the periphery of the mycelium in solid and liquid cultures of *Aspergillus niger*

Arman Vinck, Ronald P de Vries & Han AB Wösten

Abstract

The glucoamylase gene *glaA* is differentially expressed at the periphery of *Aspergillus niger* colonies; part of the hyphae highly express *glaA*, whereas others express it only lowly. This was an unexpected finding since all hyphae were exposed to identical environmental conditions. We here show that the α-glucuronidase gene *aguA* of *A. niger* is also subject to hyphal differentiation; 6-75% of the hyphal population highly expressed *aguA* at the periphery of the colony. In contrast, *glaA* and *aguA* were not differentially expressed at the periphery of 24-h-old pellets grown in liquid shaken medium. On the other hand, the feruloyl esterase gene *faeA* was differentially expressed under this condition. About 50% of the hyphae highly expressed *faeA* at the outer part of pellets. Our results indicate that at least a variety of *A. niger* genes encoding secreted proteins are subject to hyphal differentiation and that this regulatory process depends on the growth conditions.

Introduction

Substrate colonization by filamentous fungi starts with the invasion of the leading hyphae that grow from the advancing edges of the mycelium. Behind this exploration zone the hyphae branch intensely thereby expanding colonization of the substrate. Growing hyphae in both the exploration and the branching zone secrete enzymes into the environment. These enzymes convert the polymers in the substrate into small products that can be taken up to serve as nutrients. Proteins are not only secreted at the periphery of the mycelium (i.e. in the exploration and the branching zones) but also in the centre (Wösten *et al.*, 1991; Moukha *et al.*, 1993). Here, secondary growing hyphae are formed that secrete proteins different from those secreted at the periphery. For instance, the enzyme glucoamylase (GlaA) is secreted at the periphery of the mycelium of *Aspergillus niger*, but not in the colony centre (Wösten *et al.*, 1991).

Remarkably, not all growing hyphae at the periphery of an *A. niger* colony secrete GlaA (Wösten *et al.*, 1991), a phenomenon which we call hyphal or secretion differentiation. This was explained by differences in the *glaA* expression levels (Vinck *et al.*, 2005; **Chapter 2**). Two types of hyphae were distinguished; those that highly and those that lowly express *glaA*, each making up about 50% of the hyphal population. We here studied whether the α-glucuronidase A gene *aguA* (de Vries *et al.*, 2002) and the feruloyl esterase A gene *faeA* (de

Vries *et al.*, 1997) are also differentially expressed at the colony and hyphal level in *A. niger*. Moreover, we assessed whether differential expression of *glaA*, *aguA*, and *faeA* occurs at the periphery of the mycelium in liquid shaken cultures during logarithmic growth.

Materials and Methods

Strains and plasmids

GFP reporter constructs (Table 1) were introduced in *A. niger* strains AB4.1 (*pyrG cspA1*) (van Hartingsveldt *et al.*, 1987) and NW249 (Δ*argB pyrA6 nicA1 leuA1 cspA1*) (P.J.I. van de Vondervoort and Y. Muller, unpublished data). Both strains are derived from strain N402 (*cspA1*) (Bos *et al.*, 1988).

Table 1. Strains used in this study.

Strain	Parental strain	Transforming construct	Description of construct
AR9#2	AB4.1	P*glaA*_sGFP **(a)**	Plasmid containing *sGFP* under regulation of the *glaA* promoter of *A. niger* (Siedenberg *et al.*, 1999).
AR19#1	AB4.1	P*gpdA*_sGFP	As **(a)** but with the *gpdA* promoter of *A. nidulans* (Lagopodi *et al.*, 2002).
AV11#3 AV11#4	AB4.1	P*aguA*_sGFP	As **(a)** but with the *aguA* promoter of *A. niger* (This work).
UU-A005.4 UU-A005.6	NW249	P*faeA*_sGFP	As **(a)** but with the *faeA* promoter and terminator of *A. niger* (This work).

Growth conditions

A. niger was grown in sandwiched cultures or as liquid shaken cultures at 30 °C in minimal medium (MM) (de Vries *et al.*, 2004). To repress the *glaA* gene colonies were grown on 200 mM xylose, whereas the gene was induced using 25 mM D-maltose. The *aguA* and the *faeA* genes were repressed and induced, respectively, by growing on 50 mM D-glucose and 25 mM D-xylose. UU-A005.4 and UU-A005.6 were grown in the presence of leucine (0.2 mg ml^{-1}), arginine (0.2 mg ml^{-1}) and nicotinamide (0.001 mg ml^{-1}). 250 ml erlenmeyer flasks with 100 ml MM were used for liquid cultures. The medium was inoculated with 10^6 spores ml^{-1} and cultures were shaken at 250 rpm. Colonies of *A. niger* were grown from mycelial plugs as

sandwiched cultures (Wösten *et al.*, 1991). To this end, *A. niger* was grown in a 0.2 mm thin layer of 1.25% agarose (D-1 agarose, type low EEO, Hispanagar, Spain) in between two perforated polycarbonate membranes (diameter 76 mm, pore size 0.1 μm; Osmonics, GE Water Technologies, Trevose, PA, USA) placed on top of solidified (1.5% agar) MM.

Construction of plasmids for transformation

The *A. niger aguA* promoter was amplified by PCR from plasmid pIM3243 (de Vries *et al.*, 2002) with primers FPraguABam (5' ggATCCAATATATCgATACTTCTTgCC 3') and RPraguANco (5' CCATggTggCgggTTCCTTCTgg 3'). The resulting 550 bp fragment with *Bam*HI and *Nco*I linkers at the 5' and 3' ends, respectively, was cloned in pAN52-10S65TGFPn/s (Siedenberg *et al.*, 1999). To this end, pAN52-10S65TGFPn/s was partially digested with *Not*I and the overhangs were blunted with Klenow. This was followed by digestion with *Nco*I to remove the 4.1 kb *glaA* promoter. The 550 bp *Bam*HI/*Nco*I *aguA* promoter of which the *Bam*HI-site was blunted with Klenow was then inserted. This resulted in construct P*aguA*_sGFP that contains the *sGFP* gene under the regulation of the *aguA* promoter of *A. niger*.

A 3.5 kb genomic *Sma*I fragment from pIM3207 (de Vries *et al.*, 1997) containing the *faeA* open reading frame and 2000 and 300 bp 5' and 3' flanking sequences, respectively, was cloned in pGEM7 resulting in construct pRV421. The construct pHB65-16 was derived from pRV421 by removing the open reading frame of *faeA* by outward PCR using primers pFAEANcoNot (5' gggggCggCCgCCCATggTgAAATATgTgCgACAgTgAgTg 3') and tFAEANotPst (5' ggggCggCCgCCTgCAgATTATACAAAgAAgAATAAACCTC 3') thus introducing *Nco*I and *Not*I sites. A *Nco*I/*Not*I fragment containing the coding sequence of *sGFP* was introduced in the respective sites of pHB65-16, resulting in construct pHB68-28 (P*faeA*_sGFP).

Transformation of *A. niger*

Protoplast preparation and transformation were performed as previously described (Kusters-van Someren *et al.*, 1991). Transformants were selected by co-transforming with pAB4.1 (containing the *A. niger pyrA* gene) and purified by repeated streaking of conidia.

Immunolocalization at the colony level

For localization of secretion of α-glucuronidase AguA and glucoamylase GlaA, sandwiched colonies were transferred from a repressing medium to an inducing medium for 8 h.

Subsequently, a polyvinylidenedifluoride (PVDF) membrane was placed underneath the sandwiched colony for 5 h to trap the secreted proteins. AguA and GlaA were detected at the PVDF membrane using 5000- and 3000-fold diluted polyclonal antisera, respectively, as described (Wösten *et al.*, 1991).

Microscopy

GFP fluorescence at the colony level was monitored using a Leica MZFLIII binocular equipped with a HBO 100 W mercury lamp, an FITC filter and a Photometrics Cool SNAP camera (1392 x 1024 pixels). GFP fluorescence at the hyphal level was studied by confocal laser scanning microscopy (CLSM). An inverted Leica TCS SPII system equipped with a PL FLUOTAR16x / 0.5 IMM plan apochromatic objective lens was used for imaging. GFP was excited with the 488 nm laser line and fluorescence was detected at 500-550 nm bandpass. The laser intensity was kept to a minimum to reduce photobleaching and phototoxic effects. Images were captured as *z*-series of optical sections (~1 μm). The data sets were displayed as maximum intensity projections (1024 x 1024 pixels) using Leica LSM software.

Image analysis

The intensity of fluorescence was quantified as described (Vinck *et al.*, 2005; **Chapter 2**). Data was subjected to descriptive statistics using SPSS software. To examine whether fluorescence intensity distributions can be explained by a mixture of two normally distributed components, data was modeled in the probability distribution $\varphi : \varphi(x) = pN(x;\mu_1,\sigma_1) + (1-p)N(x;\mu_2,\sigma_2)$, where $0 < p < 1$ and $x \mapsto N(x;\mu,\sigma)$ is the probability density of the normal distribution with parameters μ and σ. This model will be referred to as a mix of a $N(\mu_1;\sigma_1)$-distribution and a $N(\mu_2;\sigma_2)$-distribution in which the degree of participation of the component $N(\mu_1;\sigma_1)$ is p. Note that there are five parameters in the model: p; μ_1; σ_1; μ_2; σ_2. In the statistical analysis these five parameters were fit to the empirical data by means of the maximum likelihood principle. Interval estimates for the parameters were obtained by means of bootstrapping (1000 replicates). The fit procedures were implemented in the Scilab (free) software package. The scripts of the Scilab functions used are available at http://www.bio.uu.nl/~microbio/Microbiology/Tools.htm.

Results

A. niger strains expressing *GFP* from the *glaA, gpdA, aguA* and *faeA* promoter

A. niger was transformed with constructs P*aguA*_sGFP and P*faeA*_sGFP that encompass the *sGFP* gene under control of the *aguA* and *faeA* promoter, respectively. These promoters are repressed by glucose and induced by xylose (de Vries *et al.*, 1999). Colonies of 8 transformants each were grown for 5 days on the repressing carbon source and induced on xylose for 8 hrs. Strains AV11#3 and AV11#4 were shown to be representative for the P*aguA*_sGFP transformants, while transformants UU-A005.4 and UU-A005.6 were representative for strains transformed with P*faeA*_sGFP. Strains AR9#2 and AR19#1 that express *sGFP* under control of the *glaA* and *gpdA* promoter, respectively, have been described previously (Lagopodi *et al.*, 2002; Siedenberg *et al.*, 1999). The *gpdA* promoter is constitutively expressed, while the *glaA* promoter is expressed on maltose but repressed on xylose (Fowler *et al.*, 1990).

Expression of *glaA, gpdA, and aguA* at the periphery of colonies of *A. niger*

Expression of *sGFP* under control of the *gpdA, glaA, aguA* or *faeA* promoter was monitored in 5 day-old sandwiched colonies (see Experimental procedures). As expected (Vinck *et al.*, 2005; **Chapter 2**), colonies of AR19#1 expressing *sGFP* behind the constitutive *gpdA* promoter were fluorescent throughout the mycelium when grown on xylose or glucose (data not shown). Similar results were obtained when colonies of AR9#2, AV11#3 and AV11#4, were grown for 5 days on inducing medium, while faint fluorescence was observed on the repressing carbon source (data not shown). Fluorescence was weak or even absent when strains UU-A005.4 and UU-A005.6 were grown either on repressing or inducing medium.

Strong fluorescence was observed at the periphery of colonies of AR9#2 when 5-day-old xylose grown colonies were transferred for 8 h to inducing medium containing 85 mM (Vinck *et al.*, 2005; **Chapter 2**) or 25 mM maltose (Figure 1A). In contrast, fluorescence was more evenly distributed in 5-day-old colonies of AV11#3 and AV11#4 that had been transferred from the repressing to the inducing carbon source (Figure 1B). Notably, fluorescence was lower in a zone just behind the periphery of the colony. Despite the difference in expression, both glucoamylase GlaA (Figure 1C; Wösten *et al.*, 1991) and α-glucuronidase AguA were primarily secreted at the periphery of the colony (Figure 1D).

Hyphae in the exploration zone of strains AR9#2 and AR19#1 were all fluorescent when 5-day-old cultures were induced for 8 h on 25 mM maltose (data not shown). The Kolmogorov–

Smirnov (KS) test (with the Lilliefors significance correction) showed that the variation in fluorescence of hyphae at the periphery of AR19#1 colonies followed a normal distribution (Table 2). In contrast, variation in fluorescence intensity did deviate from normality in case of the AR9#2 strain ($p \leq 0.05$). Bootstrap analysis (Efron, 1979) fitted the distribution of fluorescence intensities of AR9#2 hyphae as a weighted mixture of two normal components with high expressers representing 18% of the population. Fluorescence intensity of hyphae at the periphery of colonies also did not follow a normal distribution when cultures were grown for 5 days on 25 mM maltose (Table 2). In this case 9% of the hyphae highly expressed the *glaA* gene.

Hyphae in the exploration zone of 5-day-old AV11#3 and AV11#4 cultures were also all fluorescent when colonies had grown continuously or for 8 h on xylose (Figure 2A-D). The Kolmogorov–Smirnov (KS) test showed that the variation in fluorescence of AV11#3 and AV11#4 hyphae (Figure 2E-H) did not follow a normal distribution ($p \leq 0.05$; Table 2), even when the colonies had been grown for 5 days on the inducing carbon source. Bootstrap analysis fitted the distribution of fluorescence intensities as a weighted mixture of two normal components for strain AV11#3 and AV11#4 (Table 2). 6% and 75% of the hyphal population, respectively, highly expressed the *GFP* gene when cultures of the former and the latter strain had been grown for 8 h on the inducing carbon source. Two normally distributed components were also observed when strain AV11#4 had grown continuously on xylose. This was not the case for strain AV11#3.

Expression of *gpdA*, *glaA*, *aguA* and *faeA* in pellets of *A. niger*

Expression of *sGFP* under control of the *gpdA, glaA, aguA* and *faeA* promoter was monitored in liquid shaken cultures of *A. niger* under inducing conditions. Strains AR19#1 and AR9#2 were grown in maltose whereas strains AV11#3, AV11#4, UU-A005.4 and UU-A005.6 were grown in xylose. In all cases, GFP fluorescence was observed in the liquid cultures; i.e. also in the case of strains UU-A005.4 and UU-A005.6 that express *GFP* under the control of the *faeA* promoter. After 24 h of growth, a fraction of the hyphae was freely dispersed but most hyphae were part of a loose pellet. Prolonged incubation (up to 48 h) led to the formation of highly compact pellets that were not suitable for quantification of GFP fluorescence at the hyphal level.

Fluorescence intensity of individual hyphae at the outer part of 24 h old pellets (Figure 3A-D) was quantified and plotted as a frequency distribution (Figure 3E-H). The KS-test showed that

hyphal fluorescence did not deviate from normality except for UU-A005.4 and UU-A005.6 (Table 3). The high *faeA* expressers represented 47% and 56% of the hyphae, respectively.

Table 2. Descriptive statistics of the fluorescence intensities of hyphae in the exploring mycelium of *A. niger*. AV11#3 and AV11#4 express *GFP* from the *aguA* promoter, while AR9#2 and AR19#1 express the reporter gene from the *glaA* and the *gpdA* promoter, respectively. N = sample size; KS = *p*-value of Kolmogorov-Smirnov-test; $\mu_{1,2}$ = mean of component 1 and 2, respectively; $SD_{1,2}$ = standard deviation of component 1 and 2, respectively; pf = participation frequency of component 1; CI = 95% confidence interval of pf.

Strain & growth condition	N	SD	KS	μ_1	μ_2	SD_1	SD_2	pf	CI	CI
AV11#3; 5d xylose	245	23.41	> 0.009	89.47	115.35	15.58	24.81	0.61	0.28	0.98
AV11#3; 8h xylose	417	25.73	> 0.007	95.97	155.12	21.90	15.00	0.94	0.55	0.97
AV11#4; 5d xylose	347	22.31	> 0.042	84.83	110.14	14.68	20.75	0.42	0.11	0.97
AV11#4; 8h xylose	274	26.72	> 0.050	72.91	108.42	11.36	24.35	0.25	0.10	0.88
AR9#2; 5d maltose	191	34.05	> 0.0005	92.19	173.10	25.22	20.77	0.91	0.63	0.96
AR9#2; 8h maltose	156	30.54	> 0.013	88.98	146.45	21.57	16.99	0.82	0.23	0.92
AR19#1; 8h maltose	235	19.39	> 0.200*	87.15	114.48	14.51	12.74	0.55	0.05	0.92

* This is a lower bound of the true significance.

Table 3. Statistics of the fluorescence intensities of hyphae in pellets of *A. niger* grown in liquid medium. AR9#2 and AR19#1 express *GFP* under the control of the *glaA* and the *gpdA* promoter, respectively, whereas AV11#3 and AV11#4 express the reporter gene from the *aguA* promoter. In case of strains UU-A005.4 and UU-A005.6 *GFP* is placed under the control of the *faeA* promoter. N = sample size; KS = *p*-value of Kolmogorov-Smirnov-test; $\mu_{1,2}$ = mean of component 1 and 2, respectively; $SD_{1,2}$ = standard deviation of component 1 and 2, respectively; pf = participation frequency of component 1; CI = 95% confidence interval of pf.

Strain	**N**	**SD**	**KS**	**μ_1**	**μ_2**	**SD_1**	**SD_2**	**pf**	**CI**	**CI**
AR19#1	250	18.61	> 0.200*	54.02	101.07	9.88	16.72	0.03	0.01	1.00
AR9#2	256	20.11	> 0.200*	98.11	112.97	19.65	19.33	0.91	0.04	1.00
AV11#3	181	17.07	> 0.200*	82.26	101.32	3.39	16.86	0.01	0.07	0.99
AV11#4	262	16.77	> 0.200*	87.51	100.86	4.35	17.07	0.10	0.08	1.00
UU-A005.4	210	31.41	> 0.002	81.61	119.76	17.92	30.91	0.53	0.33	0.92
UU-A005.6	279	24.72	> 0.001	82.33	113.16	12.43	23.49	0.44	0.16	0.75

* This is a lower bound of the true significance.

Discussion

Using GFP as a reporter, the glucoamylase gene *glaA* was shown to be expressed at the periphery of 5-day-old colonies of *A. niger* that had been transferred for 8 h from a repressing to an inducing medium (Vinck *et al.*, 2005; **Chapter 2**). Using the same methodology we were not able to visualize expression of *faeA* in colonies of *A. niger*. A yellow autofluorescent pigment that hampered GFP localization accumulated faster than the reporter upon transfer to inducing medium. Expression of the α-glucuronidase A gene *aguA*, however, could be localized. Compared to *glaA*, expression of *aguA* was more evenly distributed in *A. niger* colonies. The difference in the expression pattern of *glaA* and *aguA* can be explained by the spatial expression of the activators of these genes; *amyR* (Petersen *et al.*, 1999) and *xlnR* (van Peij *et al.*, 1998), respectively. Gene *amyR* is expressed at the periphery of maltose-grown colonies. In contrast, *xlnR* is expressed constitutively throughout the colony (RP de Vries, AM Levin and HAB Wösten, unpublished results). The latter regulator is imported in the nucleus under inducing conditions (Hasper *et al.*, 2004). We have no explanation why expression of *aguA* is lower just behind the periphery of the colony. Possibly, different signals cause nuclear targeting of *xlnR* at the periphery and the central parts of the colony.

Despite the difference in the spatial expression of *glaA* and *aguA*, both the glucoamylase (Wösten *et al.*, 1991) and the α-glucuronidase were shown to be mainly secreted at the periphery of the colony. This can be explained by the fact that only growing hyphae secrete proteins. These hyphae can be mainly found at the outer zone of the mycelium (Wösten *et al.*, 1991). However, not all growing hyphae at the periphery of colonies express *glaA* and *aguA* at a similar level. Recently, it was shown that two types of hyphae can be distinguished at the outer part of the colony; those that highly and those that lowly express the glucoamylase gene (Vinck *et al.*, 2005; **Chapter 2**). By using an ER-targetted GFP it was shown that 50% of the hyphal population lowly expressed the *GFP* gene in 5-day-old sandwiched colonies that had been induced for 8 h. However, when a cytosolic GFP was used only 25% of the hyphae belonged to this group. This difference was shown to be due to cytoplasmic streaming of GFP from one hypha to the other, thus leveling out differences in fluorescence intensity. Since *aguA* and *faeA* are less highly expressed compared to *glaA*, quantification studies could only be done with the cytosolic version of the reporter. Thus, the fraction of the hyphae highly expressing the *aguA* and *faeA* promoters (see below) may be overrated.

Like *glaA*, *aguA* was shown to be differentially expressed at the periphery of the colony. Intensity of hyphal fluorescence deviated from normality at the periphery of colonies of strains AV11#3 and AV11#4 grown continuously on xylose or for 8 h at the inducing carbon source. Bootstrap analysis could fit the distribution of induced colonies of strains AV11#3 and AV11#4 as a weighted mixture of two normal distributions. 94% and 25% of the exploring hyphae, respectively, were low *aguA*-expressers, while 6% and 75% highly expressed the gene. It is not yet clear why the percentages are so different between both transformants.

Hyphal differentiation was also observed in liquid shaken cultures of strains expressing *sGFP* under control of the *faeA* promoter. About 50% of the hyphal population at the outer part of 24-h-old pellets were highly expressing the *faeA* gene. In contrast, hyphal differentiation was not observed in the outer zone of 24-h-old pellets of strains expressing *sGFP* under control of the *gpdA*, *glaA*, or *aguA* promoter. Although fluorescence intensity was quite variable in all cases, it followed a normal distribution. It thus seems that differential expression of *glaA* and *aguA* depends on the growth condition. Indeed, *glaA* is differentially expressed in 1-day-old sandwiched-grown germlings but differential expression could not be unequivocally shown in case germlings were grown in the absence of a covering membrane (Vinck *et al.*, 2005; **Chapter 2**). Moreover, hyphal differentiation was not observed in case of *glaA* driven *GFP* expression when sandwiched colonies where grown for 5 days on 85 mM maltose (Vinck *et al.*, 2005; **Chapter 2**). In contrast, hyphal differentiation did occur at 25 mM of the carbon source.

Differential expression of genes in the exploration zone of sandwiched colonies or at the outside of pellets in liquid shaken cultures is not obvious since each hypha experiences similar nutritional conditions. At this moment it is not yet clear which factors activate differential expression. We have previously shown that gradients in oxygen or carbon dioxide do not influence differential expression of *glaA* at the hyphal level (Vinck *et al.*, 2005). It was proposed that the low expressers have a reduced capacity for uptake of the inducer from the environment. These hyphae could for instance lack a sugar transporter. As an alternative, it was proposed that differential expression is caused by the absence or presence of an activator or repressor due to stochastic processes (Vinck *et al.*, 2005; **Chapter 2**). Whatever the underlying mechanism may be, it seems to be a promising target to improve protein production in industrial fermentations of *A. niger*.

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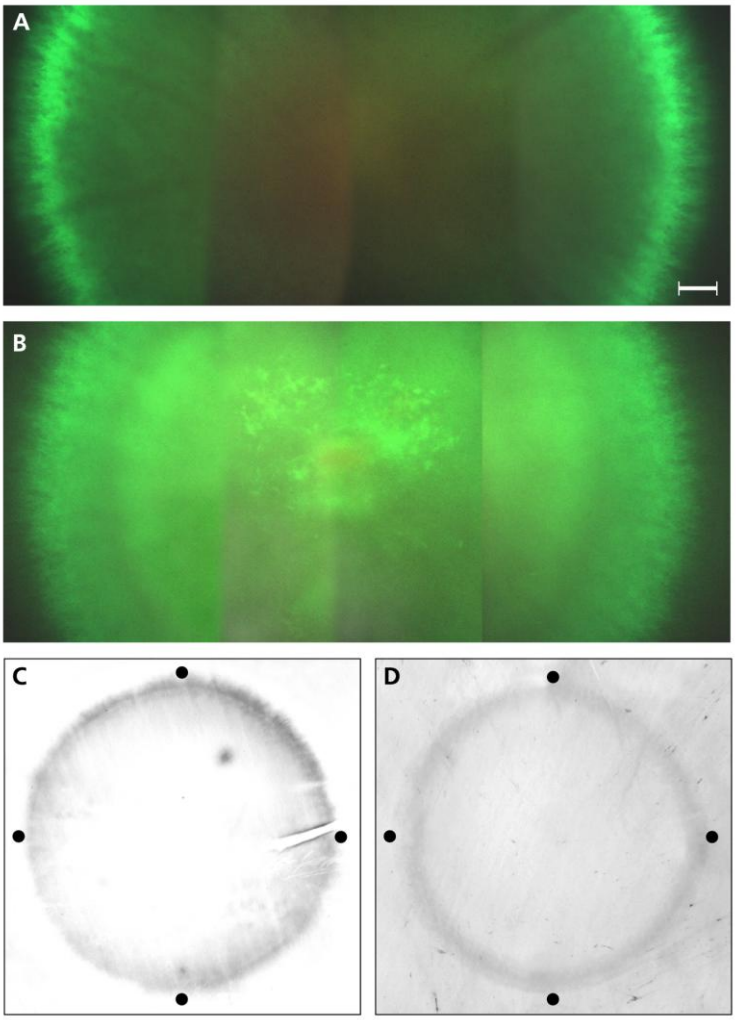

Figure 1. *glaA*- (**A**) and *aguA*-driven (**B**) GFP expression of strains AR9#2 and AV11#4, respectively, and the corresponding secretion of glucoamylase GlaA (**C**) and α-glucuronidase AguA (**D**) in 5-day-old sandwiched colonies that had been transferred from a repressing to an inducing medium for 8 h. Secretion of GlaA and AguA were monitored by immuno-detection on PVDF membranes that had been placed under the sandwiched cultures. The edge of panels (**A**) and (**B**) and the dots in panels (**C**) and (**D**) represent the periphery of the colony. Bar represents 5 mm.

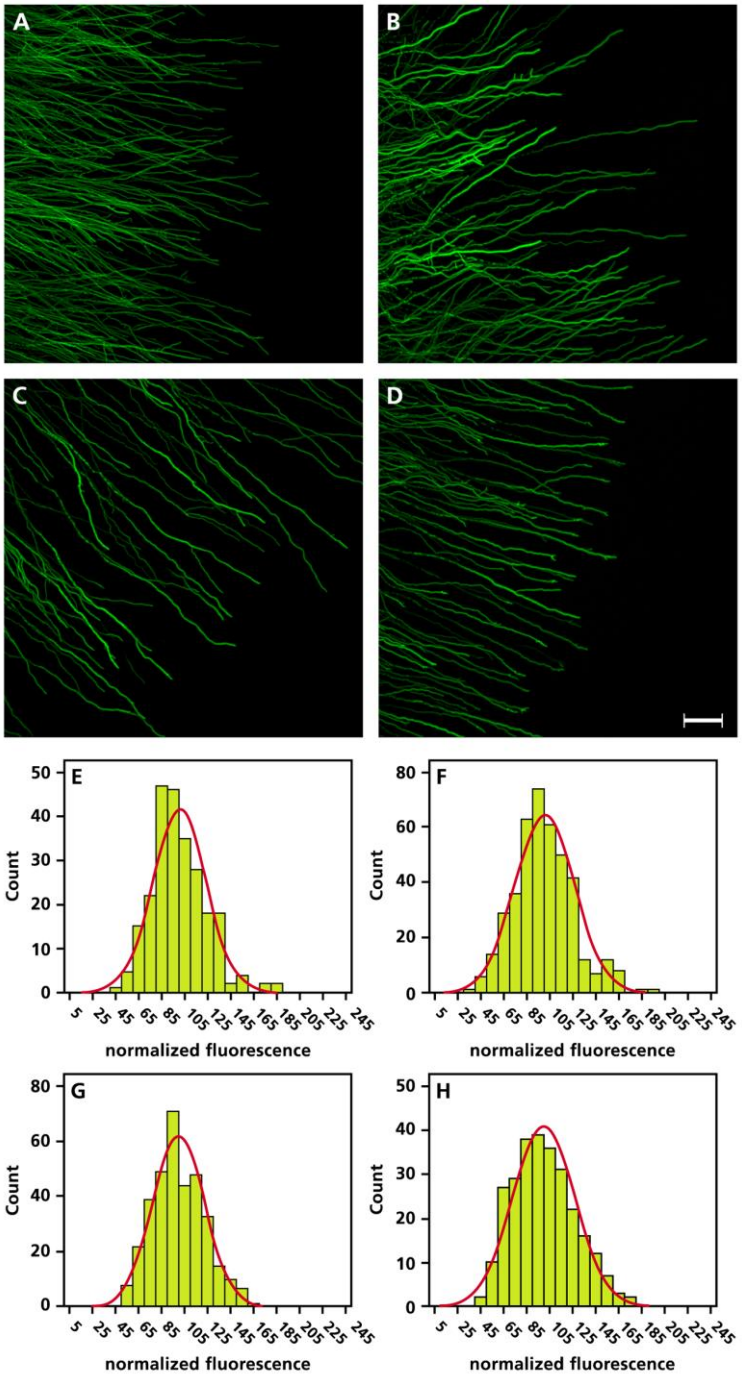

Figure 2. CLSM images (**A**-**D**) and the respective fluorescence intensity distribution (**E**-**H**) of hyphae in the exploration zone of colonies of strains AV11#3 (**A**, **B, E, F**) and AV11#4 (**C**, **D, G, H**). These strains that expresses *GFP* behind the *aguA* promoter were grown for 5 d on 25 mM xylose (**A**, **C, E, G**) or for 5 d on 50 mM glucose followed by 8 h on 25 mM xylose (**B**, **D, F, H**). Bar represents 40 µm.

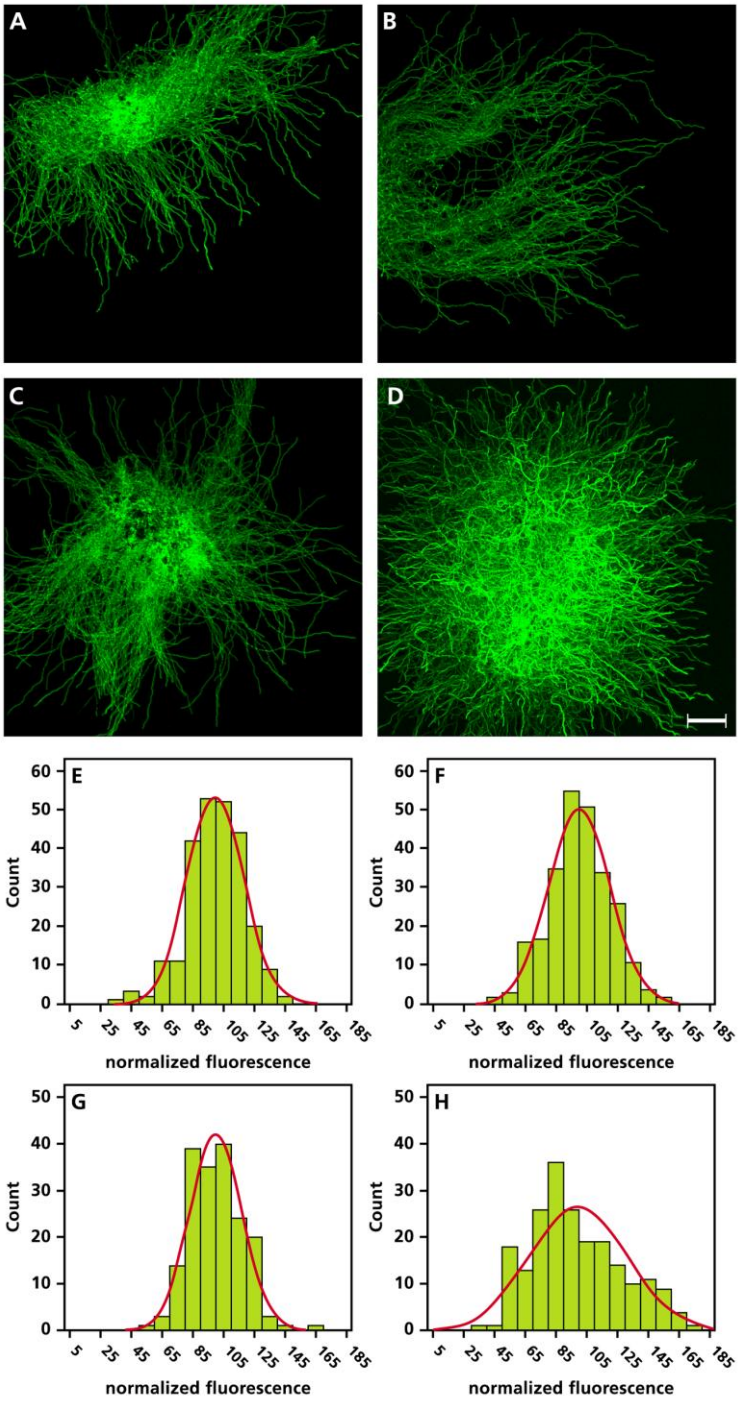

Figure 3. CLSM images (**A**-**D**) and the respective fluorescence intensity distribution (**E**-**H**) of hyphae at the periphery of 24-h-old pellets grown in liquid shaken medium. Strains AR19#1 (**A**, **E**) and AR9#2 (**B**, **F**) expressing *GFP* behind the *glaA* and *gpdA* promoter, respectively, were grown in 25 mM maltose containing medium. 25 mM xylose was used as carbon source for strains AV11#4 (**C**, **G**) and UU-A005.4 (**D**, **H**) that express *GFP* from the *aguA* and *faeA* promoter, respectively. Bar represents 40 µm.

Chapter 4

Translocation of cytosolic proteins from the vegetative mycelium into developing conidiophores of *Aspergillus niger*

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Abstract

The formation of asexual conidiospores in *Aspergillus* involves a complex differentiation programme including the formation of an aerial reproductive structure called the conidiophore. Cytoplasm is translocated from the substrate mycelium into the aerial reproductive structure to allow for its growth and development. At the moment, it is not clear how much of this apparent translocation of cytoplasm is due to movement of cytoplasm as such or to local synthesis of cellular components from molecules resulting from degradation of polymers in the substrate mycelium. Using the green fluorescent protein (GFP) as a reporter we here present evidence that cytosolic proteins can be translocated from the vegetative mycelium into the conidiophore. This was concluded from the fact that both the vegetative mycelium and the conidiophores were fluorescent when a cytosolic GFP was expressed from the *gpdA* or *glaA* promoter. In contrast, when a nuclear targeting signal was fused to the GFP only nuclei of the vegetative hyphae turned fluorescent. Quantification of fluorescence of cytosolic GFP indicates that the composition of the conidiospore depends, at least partially, on the expression profile of the vegetative mycelium that underlies the conidiophore.

Introduction

Vegetative growth of filamentous fungi starts with the germination of a spore. Germination leads to the formation of hyphae that grow by apical extension and that branch subapically. Subsequent hyphal fusion results in a network of interconnected cells known as the mycelium. Fungal mycelia can colonize distinct patches of substrate but can cover immense areas as well. For instance, genetic individuals of *Armillaria* have been identified that had colonized up to 1000 hectares of forest (Ferguson *et al.,* 2003; Smith *et al.,*, 1992). In most natural habitats (e.g. forest soil) the supply of nutrients is not uniform. Filamentous fungi circumvent this spatial heterogeneity by translocation of nutrients through the mycelium (Jennings, 1987). This is facilitated by porous septa that divide hyphae in compartments. In fact, the cytoplasm within a fungal mycelium is a continuous system.

After a sufficient amount of vegetative mycelium has been established the fungal colony initiates the formation of aerial structures that may further differentiate into asexual or sexual reproductive structures. Growth of these aerial hyphae depends on the translocation of

nutrients and water from the vegetative mycelium (Jennings, 1984; Wösten and Wessels, 2006). The formation of the asexual spore-bearing conidiophore of *Aspergillus* species begins with the growth 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 begins to swell forming the conidiophore vesicle. The cytoplasm of the stalk, vesicle and foot cell is in contact with that in the vegetative mycelium. Depending on the *Aspergillus* species, phialides develop directly from the vescicle or from metulae (Mims *et al.*, 1988). The phialides give rise to chains of uninucleate spores called conidia that are dispersed by wind and that can give rise to a new mycelium.

It has been estimated that in *A. nidulans* about 1200 genes are up-regulated during asexual spore formation (Timberlake, 1980). It is still unclear how different the expression profiles are of the substrate and aerial hyphae and to which extent the vegetative substrate mycelium contributes to the formation and the composition of conidiospores. We here present evidence that cytosolic proteins from the substrate mycelium can be translocated into the aerial reproductive structure and that the composition of the conidiospore at least partly depends on the expression profile of the underlying mycelium.

Materials and methods

Strains and plasmids

Aspergillus niger strains AB4.1 (*pyrG cspA1*) (van Hartingsveldt *et al.*, 1987) and NW249 (△*argB pyrA6 nicA1 leuA1 cspA1*) (P.J.I. van de Vondervoort and Y. Muller, unpublished) as well as their derivatives (Table 1) were used in this study. AB4.1 and NW249 are derived from strain N402 (△*cspA1*) (Bos *et al.*, 1988).

Growth conditions

A. niger was grown at 30 °C in minimal medium (de Vries *et al.*, 2004) containing 15 g l^{-1} agar and 25 mM xylose or maltose as a carbon source. UU0021.02 and UU0022.02 were supplemented with nicotine amide (2 μg/ml), leucine (200 μg/ml) and arginine (200 μg/ml). Cultures were grown as sandwiched colonies in a 0.2 mm thin layer of 1.25% agarose (D-1 agarose, type low EEO, Hispanagar, Spain) in between two perforated polycarbonate membranes (diameter 76 mm, pore size 0.1 μm; Osmonics, GE Water Technologies, Trevose,

PA, USA) placed on top of solidified (1.5% agar) minimal medium (Wösten *et al.*, 1991). Alternatively, cultures were grown between an object glass and a cover slip (24x50 mm) in 300 µl agar medium. Colonies grown between polycarbonate membranes were inoculated from mycelial plugs, while cultures grown between an object glass and a cover slip were grown from spores.

Table 1. Strains used in this study.

Strain	Transforming construct	Parental strain	Description
AR9#2	pAN52-10S65TGFPn/s **(a)**	AB4.1	Plasmid containing *sGFP* under regulation of the *glaA* promoter of *A. niger* (Siedenberg *et al.*, 1999).
AR19#1	P*GPD*GFP	AB4.1	As **(a)** but with the *gpdA* promoter of *A. nidulans* (Lagopodi *et al.*, 2002).
UU21.02	P*mtdA*_sGFP	NW249	As **(a)** but with the *mtdA* promoter of *A. niger* (Van Kuyk and de Vries, unpublished).
MA25#2	pMA25 **(b)**	AB4.1	Derivative of pAH2BG (Maruyama *et al.*, 2001) containing a fusion of histon H2B to EGFP under the regulation of the *glaA* promoter of *A. niger* (This work).
MA26#1	pMA26	AB4.1	As **(b)** but with the *gpdA* promoter of *A. nidulans*. (This work).
UU22.02	P*mtdA*_H2B::EGFP	NW249	As **(b)** but with the *mtdA* promoter of *A. niger*. (Van Kuyk and de Vries, unpublished).

Construction of vectors with nuclear targeted EGFP

To construct vector pMA25 (P*glaA*_H2B::EGFP) and pMA26 (P*gpdA*_H2B::EGFP) fusion PCRs were performed. For the construction of pMA25, the first 800 bp of the *A. niger glaA* promoter was amplified with primers PglaP2 (5'CggggATCCgAACTCCAA 3') and PglaP3 (5'CggCAgCTTTgggAggCATTgCTgAggTgTAATgATgC 3') using pAN52-7 (Dr. P. Punt, unpublished vector) as template DNA. The H2B::eGFP sequence was amplified from pAH2BG (Maruyama *et al.*, 2001) by PCR using primers H2BP2 (5'ATgCCTCCCAAAGCTgCCg 3') and CPY-GFPP2 (5' CgggATCCTTACTTgTACAgCTCgTCCAT 3'). The resulting PCR products were used as template for a fusion PCR with primers PglaP2 and CPY-GFP-P2. This resulted in a 2.1 kb product of the *glaA* promoter and the H2B::EGFP sequence with *Bam*H1 linkers at both ends. The amplified product was cloned in the *Bam*HI site of pAN52-7 resulting in pMA25.

To obtain pMA26 a PCR product of the *gpdA* promoter was generated using primers NW1For (5' AAgCTggCAgTCgACCCAT 3') and H2BP1revNew (5' CggCAgCTTTgggAggCATggTgATgTCTg-CTCAAg 3') with pAN56-1 (Punt *et al.*, 1990) as a template. The H2B::EGFP fragment was the same fragment as used for the construction of pMA25. The fusion PCR was performed with primers NW1For and CPY-GFP-P2 using the *gpdA* promoter and the H2B::EGFP fragment as templates. The 1.7 kb product of the fusion-PCR with a *Sal*1 and a *Bam*H1 linker at the 5' and 3' end, respectively, was cloned in pAN52-1Not (Dr. P. Punt, unpublished vector) using the *Sal*I/*Bam*HI restriction sites. Constructs pMA25 and pMA26 were checked by sequence analysis.

Microscopy

GFP fluorescence at the colony level was monitored using a Leica MZFLIII binocular equipped with a HBO 100 W mercury lamp and a standard FITC filter. Images were captured with a Photometrics Cool SNAP camera (1392 x 1024 pixels). Confocal laser scanning microscopy was performed using an inverted Zeiss LSM5 system equipped with a PLAN-Neofluar 20x /0.50 objective lens. GFP was excited with the 488 nm laser line and fluorescence was detected at 505-530 nm bandpass. Bright field images were made using the transmission channel. Laser intensity was kept to a minimum to reduce photobleaching and phototoxic effects. Images were captured as *z*-series of optical sections. The data sets were displayed as maximum intensity projections (1024 x 1024 pixels) using Zeiss software.

Results

***gpdA* and *glaA* are expressed in the substrate mycelium but not in aerial structures**

Expression of the glyceraldehyde-3-phosphate dehydrogenase gene *gpdA*, the glucoamylase gene *glaA*, and the mannitol dehydrogenase gene *mtdA* was studied in the substrate mycelium and in aerial structures of *A. niger* using GFP as a reporter. To this end, the *GFP* gene was either or not fused to the histon gene *H2B* that contains a nuclear localization signal (Maruyama *et al.*, 2001). The *glaA*, *gpdA* and *mtdA* promoters are active on maltose, while xylose is a repressing carbon source for the *glaA* promoter (Fowler *et al.*, 1990). Recombinant strains were grown in maltose containing solid medium between an object glass and a cover slip. After 1-2 days the colony had reached the boundary of the medium and conidiophores were formed. Nuclei of vegetative hyphae of strains MA25#2 and MA26#1 expressing the

H2B::EGFP fusion from the *glaA* and *gpdA* promoter, respectively, were highly fluorescent (Figure 1). In contrast, nuclei of the conidiophores and the conidiospores were only weakly fluorescent. The *mtdA* driven expression of the fusion in strain UU22.02 resulted in fluorescent nuclei in the conidia, whereas fluorescence in the substrate mycelium was negligible (Figure 1). From these results it is concluded that *mtdA* is specifically expressed in the conidia, whereas *glaA* and *gpdA* are expressed in the vegetative mycelium.

When *sGFP(S65T)* was expressed from either the *glaA* or *gpdA* promoter in the absence of a nuclear targeting signal (strains AR9#2 and AR19#1, respectively) both vegetative and aerial hyphae were fluorescent (Figure 2). In fact, the aerial structures were more fluorescent than the substrate hyphae. Fluorescence was only observed in conidia in case of *mtdA* driven expression. These results and the fact that *glaA* and *gpdA* are not expressed in the aerial structures indicate that cytosolic GFP streams from the vegetative mycelium into condiophores and spores.

Streaming of GFP into aerial structures was confirmed in the following experiment. Strain AR9#2 expressing *sGFP(S65T)* behind the *glaA* promoter was grown between porous polycarbonate membranes for 5 days on maltose containing medium. This inducing carbon source resulted in GFP fluorescence throughout the colony (data not shown). After transferring the colony to xylose-containing medium for 24 h, the upper polycarbonate (PC) membrane was removed allowing formation of conidiophores. These conidiophores were shown to be highly fluorescent. Since xylose is known to repress the *glaA* promoter, GFP fluorescence can only be explained by streaming of 24-h old reporter molecules from the vegetative mycelium into the aerial structure.

Streaming of GFP into spores formed in the centre and the periphery of the colony

Strain AR9#2 expressing *sGFP(S65T)* from the *glaA* promoter was grown as a sandwiched culture on solid medium with xylose or maltose as a carbon source. After 5 days, pores were punctured in the upper PC membrane allowing formation of conidiophores both in the centre and the periphery of the colony. Prior to making holes in the PC membrane, the xylose-grown colonies were transferred to maltose-containing medium. Spores formed in the centre of transferred colonies were two-fold less fluorescent than those formed at the periphery (Table 2). Differences were less pronounced when cultures had grown continuously on maltose. These results and those described in **Chapter 2** show that the GFP fluorescence of spores depends on the expression of the protein in the underlying mycelium.

Table 2. The average fluorescence intensity of conidiospores of sandwiched colonies of strain AR9#2, expressing *sGFP(S65T)* behind the *glaA* promoter. Pores were punctured in the upper PC membrane after growing the colony as indicated. Spores were allowed to form for 2 days. The spot indicates the location from which the spores were taken; from the center (cen) or the periphery (per). N is the sample size, SD is the standard deviation of the mean.

Growth Condition	spot	N	Mean	SD
5d 25mM xylose, 8h 25mM maltose	cen1	101	57	9,88
5d 25mM xylose, 8h 25mM maltose	per1	127	109	23,63
5d 25mM xylose, 8h 25mM maltose	cen2	49	51	10,36
5d 25mM xylose, 8h 25mM maltose	per2	218	98	19,18
5d 25mM maltose	cen1	82	73	14,90
5d 25mM maltose	per1	158	114	25,77
5d 25mM maltose	cen2	181	83	21,68
5d 25mM maltose	per2	118	84	21,80

Discussion

The *mtdA* gene was shown to be expressed in conidiospores by using a cytosolic and a nuclear targeted GFP as a reporter. The mannitol dehydrogenase encoded by this gene is expected to convert the large amounts of mannitol (10-15 % of the spore dry weight) into mannose during germination of the conidiospore (Ruijter *et al.*, 2003). Mannitol protects the spore against high temperature and oxidative stress and to a lesser extent to freezing and lyophilisation (Ruijter *et al.*, 2003). During germination, mannitol is no longer needed and can serve as a carbon source by reducing it to mannose. The localized expression of *mtdA* in the conidiospores is only the second example of a gene that has been shown to be expressed in the conidiophore and/or conidiospores. Previously, the catalase gene *catA* was shown to be expressed in the metulae, phialides and conidiospores (Navarro & Aguirre, 1998). This gene is also involved in protection of spores against oxidative stress.

Substrate hyphae, conidiophores and conidiospores were all fluorescent when cytosolic GFP was expressed from the *gpdA* or the *glaA* promoter. In contrast, fluorescent nuclei were observed in substrate hyphae and not in the aerial structures when GFP with a nuclear localization signal was expressed from these promoters. From the latter it is concluded that

gpdA and *glaA* are only expressed in substrate hyphae. GFP with a nuclear localization signal is rapidly imported in the nucleus after it has been formed and will thus not stream into the aerial reproductive structures. In contrast, cytosolic GFP is effectively translocated to conidiophores and conidiospores after it has been formed in substrate hyphae and accumulates at even a higher concentration than in the vegetative hyphae. The fact that cytosolic GFP is translocated into aerial reproductive structures suggests that also other cytosolic proteins stream from the substrate mycelium into conidiophores and conidiospores. Future studies should reveal whether this is indeed the case. It would answer one of the main questions of the development of fungal reproductive aerial structures; how much of the apparent translocation of cytoplasm from a feeding substrate mycelium into the aerial structure is due to movement of cytoplasm as such or to local synthesis of cellular components from molecules that result from degradation of polymers in the substrate mycelium (Wösten and Wessels, 2006).

Spores produced at the periphery of induced colonies contained more GFP than spores formed in the centre when the reporter was expressed from the glaA promoter. Indeed, the glaA promoter is more active at the periphery than in the centre (Vinck *et al.*, 2005; **Chapter 2**). These results indicate that spore composition depends on a restricted part of the underlying substrate mycelium. The colony is thus predicted to form spores with a variable composition when nutrients are not evenly distributed in the substrate. So far, we were unable to show differences in germination of spores formed at the colony centre or at the periphery before or after freeze/thawing or freeze-drying. However, it cannot be excluded that there are differences in viability under particular conditions. Previously, it has been shown that the age of the culture as well as environmental conditions affect properties (e.g. viability and cytotoxicity) of fungal spores (Cliquet and Jackson, 1999; Cliquet and Jackson, 2005; Hallsworth and Magan, 1996; Murtoniemi *et al.*, 2003). Normally, spores are collected from the whole mycelium. This study indicates that variability in spore properties can be reduced by extracting spores from selected parts of the colony. Defined spore properties are of interest for biocontrol applications (Cliquet and Jackson, 1999; Cliquet and Jackson, 2005) but may also be of interest for starter cultures of fungal fermentations.

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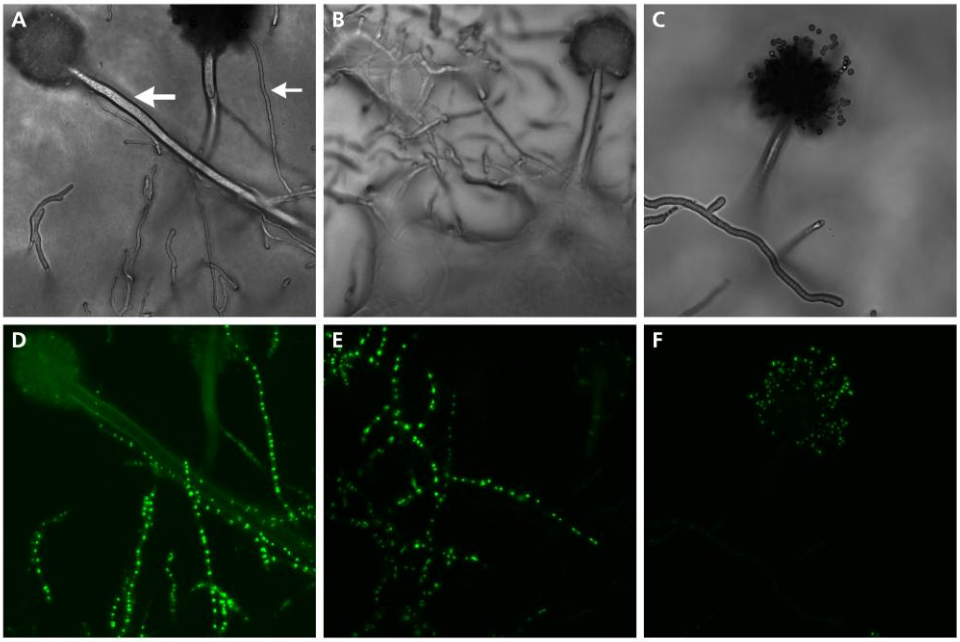

Figure 1. Expression of *H2B::EGFP* from the *gpdA* (**A**, **D**), *glaA* (**B, E**) and *mtdA* (**C, F**) promoter. Confocal microscopy images were taken using bright field (**A-C**) and 488 nm laser light (**D-F**). Large and small arrow point to a conidiophore and a vegetative hypha, respectively.

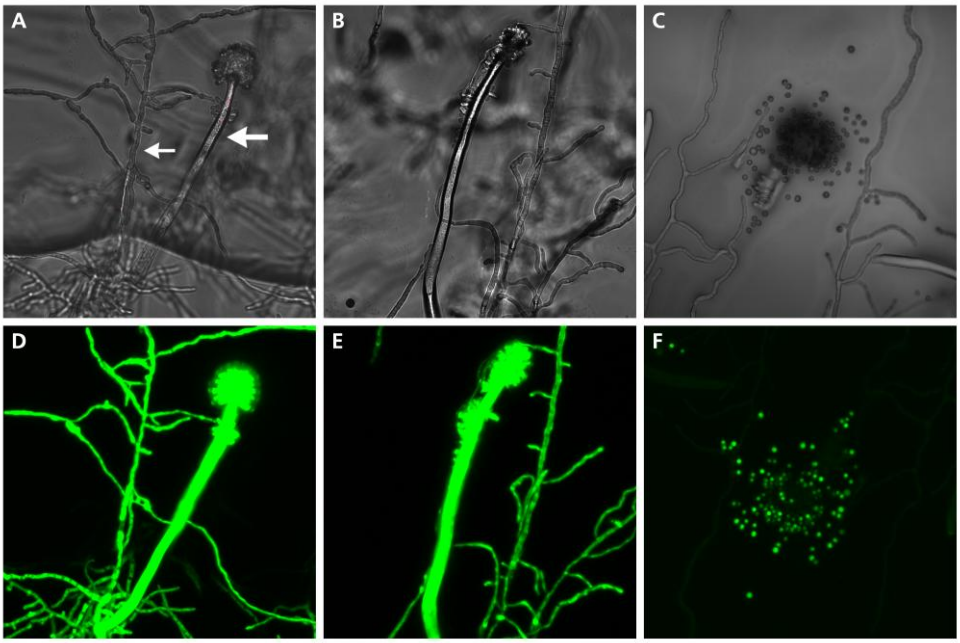

Figure 2. Expression of *sGFP(S65T)* from a *gpdA* (**A**, **D**), *glaA* (**B, E**) and a *mtdA* (**C, F**) promoter. Confocal microscopy images were taken using bright field (**A-C**) and 488 nm laser light (**D**-**F**). Large and small arrow point to a conidiophore and a vegetative hypha, respectively.

Chapter 5

Use of GFP and DsRed as reporters in the homobasidiomycete *Schizophyllum commune*

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Abstract

Expression of GFP and DsRed2 was studied in the homobasidiomycete *Schizophyllum commune*. Steady state mRNA levels of *CrGFP* and *sGFP* were similar when these reporter genes were expressed from the *SC3* promoter. Their level was considerably lower than that of endogenous *SC3*. However, accumulation of *CrGFP* mRNA was almost similar to that of endogenous *SC3* when the reporter gene was fused to the coding sequence of *SC3* corresponding to the N- or C-terminal part of the mature hydrophobin. Immuno-labelling showed that in both cases GFP was secreted. However, intra or extracellular fluorescence of the reporter was not observed. Fluorescence was also not observed when a *GFP* variant was expressed with a codon composition optimized for *S. commune*. A C-terminal fusion of CrGFP to the SC15 protein was also secreted, although part of the fusion protein was found intracellularly. From these data it is concluded that GFP can be used as a reporter in *S. commune* but that its application is restricted by limitations that occur at the transcriptional, translational and/or post-translational level. Moreover, use of GFP is limited by autofluorescence of *S. commune* hyphae. *DsRed2* was successfully expressed behind the *SC3* and *GPD* promoter in *S. commune*. The absence of red autofluorescence favors use of DsRed instead of GFP.

Introduction

Fluorescent proteins (e.g. the green fluorescent protein GFP and the red fluorescent protein DsRed) are valuable tools for the analysis of cellular processes (Lorang *et al.*, 2001). The development of GFP variants with altered spectral properties (Heim *et al.*, 1995) and preferential codon composition (Chiu *et al.*, 1996) has allowed for efficient expression of GFP in a broad range of fungal species. For instance, GFP has been used in the ascomycetes to tag organelles like the nucleus (Maruyama *et al.*, 2001) and the vacuole (Shoji *et al.*, 2006), to monitor promoter activity (Siedenberg *et al.*, 1999; **Chapter 2** and **3**) and to study protein secretion (Gordon *et al.*, 2000ab). DsRed and its derivatives that exhibit faster maturation and have reduced non-specific aggregation (Matz *et al.*, 1999; Bevis and Glick, 2002) also have been used in the ascomycetes to monitor gene expression and to label organelles like mitochondria and nuclei (Rodrigues *et al.*, 2001; Czymmek, 2002; Mikkelsen *et al.*, 2003; Toews *et al.* 2004). So far, DsRed has not been expressed in homobasidiomycetes, whereas expression of GFP has been limited to *Schizophyllum commune* (Lugones *et al.*, 1999),

Phanerochaete chrysosporium (Ma *et al.*, 2001), *Ganoderma lucidum* (Sun *et al.*, 2001), *Agaricus bisporus* and *Coprinus cinereus* (Burns *et al.*, 2005). In all these cases GFP was fused to promoter sequences and was not expressed as a fusion protein to monitor protein localization or to tag organelles. We here explored the use of the fluorescent proteins GFP and DsRed as reporters for gene expression and protein localization in *S. commune*. To this end, transcriptional and translational fusions of GFP variants and of DsRed2 were expressed in this homobasidiomycete.

Materials and Methods

Strains

The monokaryotic *S. commune* strains 4-39 (*MAT*A41*MAT*B41, CBS 341.81), 4-40 (*MAT*A43*MAT*B43, CBS 340.81), 72-3 and derivatives thereof were used (Table 1). 4-40 and 4-39 are co-isogenic but have different mating type loci. 72-3 is a derivative of 4-39, in which the *SC3* gene has been inactivated (van Wetter *et al.*, 2000; Wösten *et al.*, 1994b). *Escherichia coli* DH5α was used for cloning purposes.

Table 1. Recombinant *S. commune* strains used in this study.

Strain	parental strain	genotype	construct	description
AR1#1	4-39	P*sc3_DsRed2*	P3*Red2*MARTP **(a)**	*DsRed2* under control of *SC3* regulatory sequences and an intron directly downstream of stop codon.
AR2#1	4-39	P*sc3_DsRed2*	P3*Red2*Mui6diaTP **(b)**	As **(a)** and also an intron immediately upstream of start codon.
RR#1	4-39	P*gpd_DsRed2*	Pgpd*Red2*MARTP **(c)**	As **(a)** but under control of *GPD* promoter.
GDs2#6	4-39	P*gpd_DsRed2*	pSIDU1	As **(c)** and also an intron immediately upstream of start codon.
AV1, 2	4-39	P*sc3_CrGFP*	P3CrARTP	As **(a)** but with *CrGFP*.
RK4	4-39	P*sc3_sGFP*	pGFPi6	As **(a)** but with *sGFP(S65T)* (Lugones *et al.*, 1999)
AV27	72-3	P*sc3_ScGFP*	P3Scui6diaTP	As **(b)** but with *ScGFP*.
AV28#1	72-3	P*sc3_EGFP*	P3Eui6diaTP	As **(b)** but with *EGFP*.
AV22 #2, #4	72-3	*CrGFP*::*SC3*	P3ss3Crm3TP	N-terminal fusion of CrGFP to SC3
AV24 #13, #16, #17, #21	72-3	*SC3*::*CrGFP*	$SC3_{132}$::CrGFP	C-terminal fusion of CrGFP to SC3
FL1	4-39	*SC15*::*CrGFP*	pSC15gfp	C-terminal fusion of CrGFP to SC15

Growth conditions and media

E. coli was grown at 37 ºC in Luria-Bertani (LB) medium either or not supplemented with ampicillin (50 μg ml^{-1}) and agar (15 g l^{-1}). Liquid cultures were shaken at 200 rpm. *S. commune* was grown in the light at 25 or 30 ºC in minimal medium (MM) (Dons *et al.*, 1979) either or not solidified with agar (15 g l^{-1}). Liquid cultures were inoculated with a mycelial homogenate and shaken at 225 rpm. Cultures on agar plates were grown as a colony or as a sandwiched culture (Wösten *et al.*, 1991) from a plug inoculum.

For microscopy, *S. commune* was grown for 3 to 4 days at 30 ºC on microscope slides covered with a thin layer of solidified MM. A polycarbonate (PC) membrane (diameter 76 mm, pore size 0.1 μm; Osmonics, GE Water Technologies, Trevose, PA, USA) was placed on top of the inoculum to prevent formation of aerial hyphae that exhibit strong autofluorescence.

Construction of plasmids for transformation

Expression construct pE3SC and its derivatives

pE3SC is a derivative of plasmid pESC (Alves *et al.*, 2004), in which coding sequences can be placed in between the *SC3* regulatory sequences using unique *Nco*I and *Bam*HI sites. Construct pE3SC lacks an additional start codon in the *GPD* promoter that was generated 4 bp upstream of the coding sequence of the bleomycine resistance gene during the construction of pESC. The introduced ATG sequence was altered by site-directed mutagenesis using sense primer FpESc (5'gCCCATCACCAACATggCCAAgTTgACCAg 3') and anti-sense primer RpESc (5' CTggTCAACTTggCCATgTTggTgATgggC 3'). This resulted in a 3' end of the *GPD* promoter that conforms to the Kozak consensus sequence [C A c/a a/c A T G N G] (Kozak, 1992). To allow for expression of cDNA sequences an artificial intron was cloned in the *Bam*HI-site of pE3SC upstream of the *SC3* terminator as described (Lugones *et al.*, 1999). This resulted in plasmid pE3SCdi, which contains a regenerated *Bam*HI-site at the 5' end of the intron. A derivative of pE3SCdi with an additional intron directly downstream of the *SC3* promoter was constructed by replacing the 1022 bp *Hind*III/*Nco*I *SC3* promoter fragment of plasmid pE3SCdi by that of pSC3ciu, that encompasses the third intron of *SC6* directly downstream of the *SC3* promoter (Lugones *et al.*, 1999). This resulted in plasmid pE3SCui6dia. Like in pE3SC, coding sequences can be cloned in pE3SCdi and pE3SCui6dia in between the coding sequences of SC3 using *Nco*I and *Bam*H1 restriction sites.

DsRed2 expression constructs

The internal *Nco*I-site of *DsRed2* was altered by site-directed mutagenesis without affecting the amino acid composition of the encoded protein. For this, sense primer FDsRed2MUT (5' gCAgAAgAAgACgATgggCTgggAggCCTC 3') and anti-sense primer RDsRed2MUT (5' gAggCCTCCCAgCCCATCgTCTTCTTCTgC 3') were used with pDsRed2 (Clontech) as a template. This resulted in pDsRed2MUT, which was amplified using sense primer FDsRed2Nco (5' CCATggCCTCCTCCgAgAACg 3') and anti-sense primer RDsRed2Bam (5' ggATCCCTACAggAAC-AggTgg 3'). The resulting 678 bp PCR-fragment with a GC content of 63.7% and with *Nco*I and *Bam*HI linkers at the 5' and 3' ends, respectively, was cloned in pE3SCdi and pE3SCui6dia resulting in pP3*Red2M*ARTP and pP3*Red2M*ui6diaTP, respectively.

The *DsRed2* gene was placed under the control of the constitutive *GPD* promoter of S. *commune* by replacing the 1022 bp *Hind*III/*Nco*I *SC3* promoter fragment of pP3*Red2M*ARTP by a 650 bp *Hind*III/*Nco*I *GPD* promoter fragment from plasmid pGPHt (Schuren and Wessels, 1994). This resulted in plasmid pGPD*Red2M*ARTP. pSIDU1 (Kindly provided by Gerard Rouwendaal, Plant Research International, Wageningen, The Netherlands) resembles pGPD*Red2M*ARTP only that the *GPD* promoter fragment in this construct has been extended to contain the natural intron situated immediately after the start codon of this gene.

ScGFP, CrGFP and EGFP expression constructs

ScGFP, CrGFP and *EGFP* encode the GFPmut1 (P64L; S65T) variant (Cormack *et al.*, 1996) and have a valine insertion at position 1a (the Val is numbered 1a to maintain correspondence with the wild-type) to improve translational initiation (Kozak, 1989). The former mutation alleviates GFP's temperature sensitivity and the latter results in enhanced brightness and faster chromophore formation (Heim *et al.*, 1995). EGFP (Clontech Laboratories, Palo Alto, CA) and CrGFP (Entelechon GmbH, Regensburg, Germany) encode GFPmut1 variants with codon usage optimized for expression in human and *Chlamydomonas reinhardtii* cells, respectively. ScGFP has a Ala206Lys substitution to render the protein monomeric (Zacharias *et al.*, 2002) and has a codon usage optimized for *S. commune*. The CG-content of *ScGFP*, *EGFP*, *sGFP(S65T)* and *CrGFP* are 61.1%, 61.5%, 61.5% and 61.6%, respectively.

A 720 bp fragment of *ScGFP* with *Nco*I and *Bam*HI linkers at the 5' and 3' ends, respectively, was amplified using sense primer FscGFPnco (5' CCATggTCAgCAAgggCgAgg 3') and anti-sense primer RscGFPbam (5' ggATCCTTACTTgTAgAgCTCgTCC 3'). This fragment was cloned under

the control of the *SC3* regulatory sequences in plasmids pE3Scdi and pPE3ui6dia using the *Nco*I and *Bam*HI restriction sites. This resulted in constructs pP3ScdiaTP and pP3Scui6diaTP, respectively. Similarly, 720 bp fragments of *EGFP* and *CrGFP* with *Nco*I and *Bam*HI linkers at the 5' and 3' ends, respectively, were amplified using sense primer FGFPnco (5' CCATggTg-AgCAAgggCgAggAgC 3') and anti-sense primer BGFPbam (5' ggATCCTTACTTgTACAgCTCgTCC 3'). The *EGFP* PCR-fragment was cloned under the control of the *SC3* regulatory sequences in plasmid pE3SCui6dia, resulting in construct pP3Eui6diaTP. The *CrGFP* PCR-fragment was cloned under the control of the *SC3* regulatory sequences in plasmid pE3SCdi, resulting in construct pP3CrARTP.

To fuse CrGFP to the N-terminal part of mature SC3, the coding sequence of *CrGFP* was amplified using sense primer 2FGFPSmaI (5'ACCCgggCATggTgAgCAAgggCg 3') and anti-sense primer RGFPSmaI (5'CCCgggggCTTgTACAgCTCgTCCATgC 3'). The resulting 720 bp fragment with *Sma*I linkers at both ends was inserted in the *Sma*I-site of pgSC3. Plasmid pgSC3 is a derivative of pUC21 encompassing an *Nco*I/*Bam*H1 fragment representing the genomic *SC3* sequence between the start and stop codon and containing a *Sma*I-site 9 bp downstream of the SC3 signal sequence. Sequencing showed that the PCR-reaction resulted in the substitution of codon 105 in the CrGFP sequence from ggC to ggA both encoding glycine. The *Nco*I/*Bam*HI fusion fragment of the resulting plasmid (pGFPSC3) was placed under the control of the *SC3* regulatory sequences in pE3SC. This resulted in plasmid pP3ss3Crm3TP.

An *Xba*I-site was introduced in pgSC3 6 bp upstream of the stop codon of *SC3* to fuse the *CrGFP* gene to the 3' end of the coding sequence of *SC3.* To this end forward primer 2FSc3Xba (5'CggTTgCACCCCCTCTAgAATCCTCTgAggATC 3') and reverse primer 2RSc3Xba (5'gATCCTCAgAggATTCTAgAgggggTgCAACCg 3') were used with pgSC3 as a template for the site-directed mutagenesis. The *CrGFP* gene was amplified using sense primer 2FGFPXbaI (5'CCTCTAgAATggTgAgCAAgggCg 3') and anti-sense primer BGFPBam (5'ggATCCTTACTTgTA-CAgCTCgTCC 3'). The resulting 720 bp *Xba*I/*Bam*HI fragment was inserted in-frame with the *SC3* sequence after amino acid 132, using the unique *Xba*I and *Bam*HI-sites. The *Nco*I/*Bam*HI fragment of the resulting plasmid encompassing the fusion between *SC3* and *CrGFP* was placed under control of the *SC3* regulatory sequences by cloning it in plasmid pE3SC. This resulted in plasmid $SC3_{132}$::CrGFP.

A 357 bp *Mlu*I/*Kpn*I fragment of *SC15* was amplified by PCR using primers sc15fw1 (5'CATCgCCgAgATCCTCAAggTACgCgTTgC 3') and sc15rev1 (5'ATggTACCgATCCgCCgATCAACAACTC-CCgCCTTAgAgATCTAgCgCgg 3') to fuse the *CrGFP* gene to the 3' end of the *SC15* gene of *S.*

commune. This fragment that contains an introduced *Bgl*II site 6 nucleotides upstream of the stop codon was cloned in pGEM-T (Promega), resulting in plasmid pSC15KM. The *CrGFP* coding region was inserted in frame with the *SC15* sequence in the introduced *Bgl*II site using a *Bam*HI fragment from pCrGFP (Entelechon GmbH, Regensburg, Germany). Ligation did not regenerate the *Bam*HI and *Bgl*II sites (pSC15KMG). The *Mlu*I/*Kpn*I fragment of *SC15* contained in pSC15BS was replaced by the *Mlu*I/*Kpn*I fragment of pSC15KMG, resulting in pSC15BSG. PSC15BS is a derivative of pSP72 containing a downstream 1.3 kb *Bgl*II/*Sal*I *SC15* fragment. The *Bgl*II/*Sal*I fragment from pSC15GSPZ was replaced by the one of pSC15BSG. The former construct contains a 4.1 kb *Sal*I fragment encompassing the *SC15* gene (including 2500 and 800 bp up- and downstream sequences, respectively) and a phleomycin cassette for selection. For cloning purposes the upstream *Sal*I site has been eliminated. Replacement of the *Bgl*II/*Sal*I fragment resulted in plasmid pSC15gfp.

Transformation of *S. commune*

S. commune strains 4-39 and 72-3 were transformed as described (Schuren and Wessels, 1994) with the exception that they were protoplasted in 1 M $MgSO_4$ containing 1mg ml^{-1} Lysing enzymes from *Trichoderma harzianum* (Mushroom Research Unit, PPO, Horst, the Netherlands). DNA (5-10 μg) was added to 3 x 10^7 protoplasts in 100 μl 1 M sorbitol. Transformants of strains 4-39 were selected on MM agar plates containing phleomycin (25 μg ml^{-1}), while those of strain 72-3 were selected on plates containing 40 μg ml^{-1} phleomycin and 500 μg ml^{-1} caffeine. The latter allows selection of transformants on phleomycine despite the fact that 72-3 already contains a phleomycin resistance cassette.

RNA isolation and Northern blotting

RNA was extracted with the TRIzol® (Life Technologies) reagent according to the manufacture's protocol. RNA was separated using 1.5% TAE gels and transferred to Immobilon™-NY+ membrane (Millipore). The blots were hybridized with ^{32}P-labeled probes as described (de Vries *et al.*, 2002).

Protein extraction from medium of standing cultures and Western blotting

Proteins in the medium of 5-day-old standing cultures were precipitated with 10% TCA (v/v). After centrifugation for 30 min at 10.000 rpm, pellets were washed with 10% TCA and cold acetone. Samples were dried and taken up in SDS-sample buffer with or without prior TFA-

treatment (Wösten *et al.*, 1993). Proteins were separated in 12% SDS-PAA gels and stained with coomassie brilliant blue (Neuhof, 1988) or silver (Merril *et al.*, 1981). Alternatively, proteins were blotted to PVDF membranes. Immunodetection was done as described (Wösten *et al.*, 1991) using anti-SC3 serum (Wösten *et al.*, 1994a) or a serum against GFP (Roche; 1:1000 dilution).

Immunolocalization at the colony level

For localization of secretion of the GFP fusion proteins colonies were grown for 4 days at 30 ºC days on a PC-membrane overlying solidified MM. A polyvinylidenedifluoride (PVDF) membrane was placed underneath the PC-membrane supporting the colonies for 4 hours to trap the secreted proteins. Immunodetection of SC3 and GFP was performed as described above.

Microscopy

GFP and DsRed2 fluorescence was monitored with an Axioskop 2 plus microscope (Zeiss, Germany) equipped with a HBO 100 W mercury lamp and a Photometrics Cool SNAP camera (1392 x 1024 pixels) using standard FITC and TRITC filters, respectively.

Confocal laser scanning microscopy (CLSM) of the *DsRed2* expressing strains was performed with an inverted Zeiss LSM5 system using a PLAN-Neofluar 25x/0.8 Imm objective. DsRed2 was excited with the 543 nm laser line and fluorescence was detected with a longpass LP560 emission filter. The data sets were displayed as maximum intensity projections (1024 x 1024 pixels) using Zeiss software. GFP fluorescence was visualized with an inverted Leica TCS SPII system equipped with a PLAN APO 40x/0.85 corr objective lens. The GFP variants were excited with the 488 nm laser line and fluorescence was detected with a bandpass BP500-550 filter. The data sets were displayed as maximum intensity projections (1024 x 1024 pixels) using Leica LSM software.

Results

GFP and DsRed2 as reporters for gene expression

The use of the fluorescent proteins GFP and DsRed2 as reporters for gene expression and protein localization was explored in the homobasidiomycete *S. commune*. *GFPmut1* variants

(*EGFP*, *CrGFP* or *sGFP*) and the *DsRed2* coding sequence were cloned behind the *SC3* or the *GPD* promoter and one intron was inserted within the transcriptional unit (Table 1). *S. commune* transformants expressing either reporter construct exhibited green or red fluorescence throughout the cytoplasm (Figure 1, 2 and 3). Intensity was not increased when two instead of one intron was present in the expression construct (Figure 2). To further improve expression of GFP, codon usage of *GFPmut1* was adapted for *S. commune.* Surprisingly, transformants expressing the *ScGFP* gene did not display green fluorescence, despite the fact that the reporter was functional in *E. coli* (results not shown).

Total RNA of 5 and 6 days old colonies of strains transformed with either of the *GFP*- and *DsRed2*-expression constructs (in the presence of a single intron in the transcriptional unit) was isolated and subjected to Northern analysis. By using the *SC3* 5' untranslated region (UTR) as a probe reporter mRNA levels could be compared to that of the endogenous *SC3* transcripts. Since only the *SC3* transcript could be detected, it is concluded that steady state levels of *CrGFP* (Figure 4B), *sGFP* and *DsRed2* mRNA (data not shown) was much lower than that of the endogenous *SC3* transcripts. However, the reporter mRNA could be detected using a *GFP* (Figure 4A) or a *DsRed2* (data not shown) probe.

Expression of GFP as a fusion with a secreted protein

Constructs encoding CrGFP fused to the N- or C-terminal parts of the mature forms of the secreted proteins SC3 or SC15 were introduced into a Δ*SC3 S. commune* strain and the wild-type strain4-39, respectively. Northern analysis using a *SC15*-probe showed that the gene encoding the C-terminal fusion of GFP to SC15 was highly expressed, albeit at a lower level than the endogenous *SC15* gene (Figure 5). Fluorescence was detected intracellularly (Figure 6AB). Similar observations were made after introducing a construct encoding a SC15::GFP fusion with a spacer sequence [GSAGSAAGSGEF] (Waldo *et al.*, 1999) between both moieties (results not shown). To examine whether part of the SC15::GFP fusion was secreted into the culture medium, medium proteins of standing cultures of the wild-type, a Δ*SC15* strain and FL1 (*SC15*::*GFP*) were subjected to SDS-PAGE. After staining with Coomassie Brilliant Blue a SC15 band (15 kDa) was observed in the wild-type and FL1 strain (Figure 7A). This protein band was absent in the Δ*SC15* strain. The fusion protein with an expected molecular weight of 42 kDa was not detected in FL1. Western analysis using an SC15-antiserum resulted in a strong immunosignal at the position of SC15 in the wild-type and the

FL1 strain. In FL1 an additional less intense immunosignal was detected at the expected molecular weight of the fusion protein (Figure 7B).

Transformants expressing the gene encoding GFP fused to the N- or C-terminus of the mature SC3 protein did not show any fluorescence (~ 40 transformants screened in each case). However, the fusion was expressed in 5-day-old colonies of transformants as shown by hybridization of RNA with a 5' *SC3* UTR and a *GFP* probe (Figure 8). Hybridization with the 5' *SC3* UTR probe showed that the fusion gene was expressed at a lower level than that of *SC3* in the wild-type. To investigate whether the chimaeric protein was secreted, immuno-labelling was performed on PVDF membranes that had been positioned under colonies. Transformants expressing the N-terminal fusion reacted with both the SC3 and GFP anti-serum (Figure 9AB). Transformants expressing the C-terminal fusion showed no reaction with SC3 antisera but did react with the GFP antiserum (Figure 9CD). Strong SC3 signals were obtained with the wild-type and FL1 strains. The GFP antiserum only reacted with FL1 of these three control strains.

Discussion

Previously, *sGFP(S65T)* was expressed behind the *SC3* promoter of *S. commune.* Accumulation of *GFP* mRNA was not observed with an intron-less construct but was detectable when an intron was added to the coding sequence of the reporter (Lugones *et al.*, 1999). However, the fluorescence was weak despite the use of the very strong *SC3* promoter. Here, we expressed the *GFP* variants *sGFP*, *EGFP*, *CrGFP* and *ScGFP* in *S. commune* either or not fused to coding sequences of the secreted proteins SC3 and SC15. Production of GFP was hampered by transcription and/or by posttranscriptional processes. The *DsRed2* gene was expressed in *S. commune* as an alternative to GFP.

Expression analysis showed that steady state levels of *CrGFP* and *sGFP* were considerably lower than that of *SC3* when these GFP variants were expressed from the *SC3* promoter. This shows that production of the reporter is hampered at the level of transcription or mRNA stability. In this study introns were positioned at the 3' end or at the 3'and the 5' ends of the open reading frame. Possibly, these positions are not optimal for *GFP* mRNA accumulation. Future experiments are planned to insert introns within the coding sequence.

A fusion of GFP to the N-terminal part of mature SC3 was secreted into the medium as indicated by a positive immuno-response for both partners of the fusion. The reporter was also secreted when it was placed at the C-terminus of the SC3 hydrophobin. However, fluorescence was not detected intracellularly and not in septa or the cell walls. In contrast, secreted GFP fusion proteins give rise to fluorescent hyphal tips and to fluorescent cell walls and septa in the ascomycetes *A. niger* (Gla::sGFP) (Gordon *et al.*, 2000ab) and *A. oryzea* (RntA::EGFP) (Masai *et al.*, 2003). These data suggest that GFP fused to the SC3 hydrophobin does not fold correctly. Hyphae expressing a SC15::CrGFP fusion did fluoresce. Fluorescence was detected intracellularly contrasting the pattern of septa, cell wall and hyphal tip fluorescence in the aspergilli (Gordon *et al.*, 2000ab; Masai *et al.*, 2003). Addition of a spacer sequence in between the reporter and the SC15 protein gave similar results. Possibly, the SC15 protein is not correctly folded due to the presence of GFP to the hydrophobic C-terminal part. This may retain the protein in the secretion pathway and may ultimately lead to the translocation of the fusion to the cytoplasm, where it will be degraded as part of the ER quality control (Brandizzi *et al.*, 2003). We can thus conclude that cytoplasmic versions of GFP can be used to study gene expression but that the reporter cannot yet be used to study secretion processes by targeting it into the secretion pathway.

Application of GFP in *S. commune* is not only restricted by limitations at the transcriptional and (post)translational level, it is also hampered by autofluorescence of the hyphae. In contrast, red autofluorescence is very low thus favouring use of DsRed as a reporter. The *DsRed2* variant was successfully expressed in *S. commune* using the homologous *GPD* or *SC3* promoter. The DsRed derivative dTomato (Shaner *et al.*, 2004) is now used to study temporal and spatial expression of genes in fruiting bodies of *S. commune* (R.A. Ohm and L.G. Lugones, unpublished results). This reporter has the advantage that it folds about 10 times faster than DsRed (1 and 10 h respectively at 37 °C).

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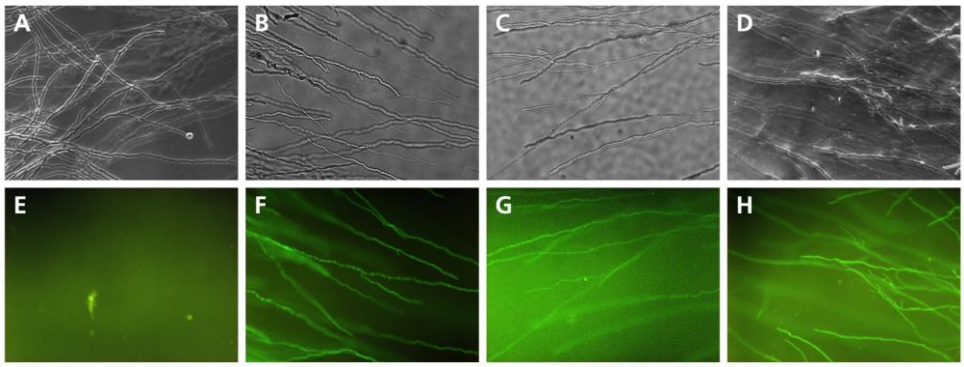

Figure 1. Brightfield (**A**-**D**) and fluorescence (**E**-**H**) microscopy of *S. commune* strains RK4 (**B**, **F**), AV2 (**C**, **G**) and AV28#1 (**D**, **H**), wild-type 4-40 (**A**, **E**) serving as a control. RK4, AV2 and AV28#1 express *sGFP*, *CrGFP* and *EGFP*, respectively, under the control of the *SC3* regulatory sequences.

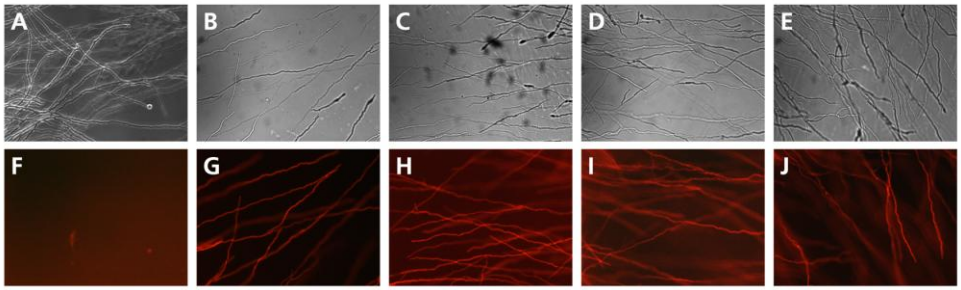

Figure 2. Brightfield (**A**-**E**) and fluorescence (**F**-**J**) microscopy of *S. commune* strains AR1#1 (**B**, **G**), AR2#1 (**C**, **H**), RR#1 (**D**, **I**) and GDs2#6 (**E**, **J**), wild-type 4-40 (**A**, **F**) serving as a control. AR1#1 and AR2#1 express *DsRed2* under the control of the *SC3* regulatory sequences in the presence of one or two introns within the transcriptional unit, respectively. Similarly, RR#1 and GDs2#6 express *DsRed2* under the control of the *GPD* promoter.

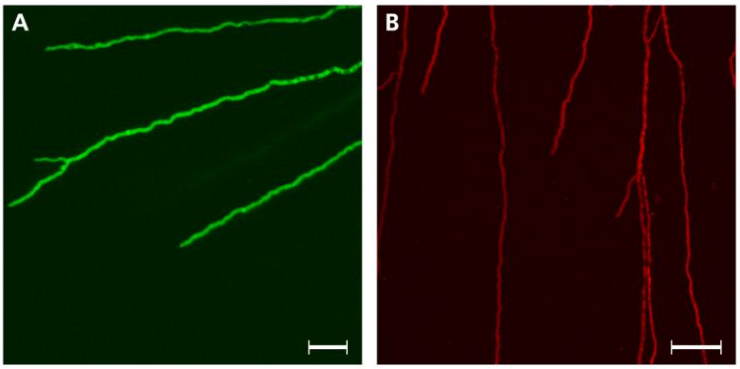

Figure 3. Confocal laser scanning microscopy of *S. commune* strains AR2#1 (**A**) and RK4 (**B**) expressing *DsRed2* and *sGFP(S65T)*, respectively, under the control of the *SC3* regulatory sequences. Bar represents 25 µm (**A**) and 40 µm (**B**).

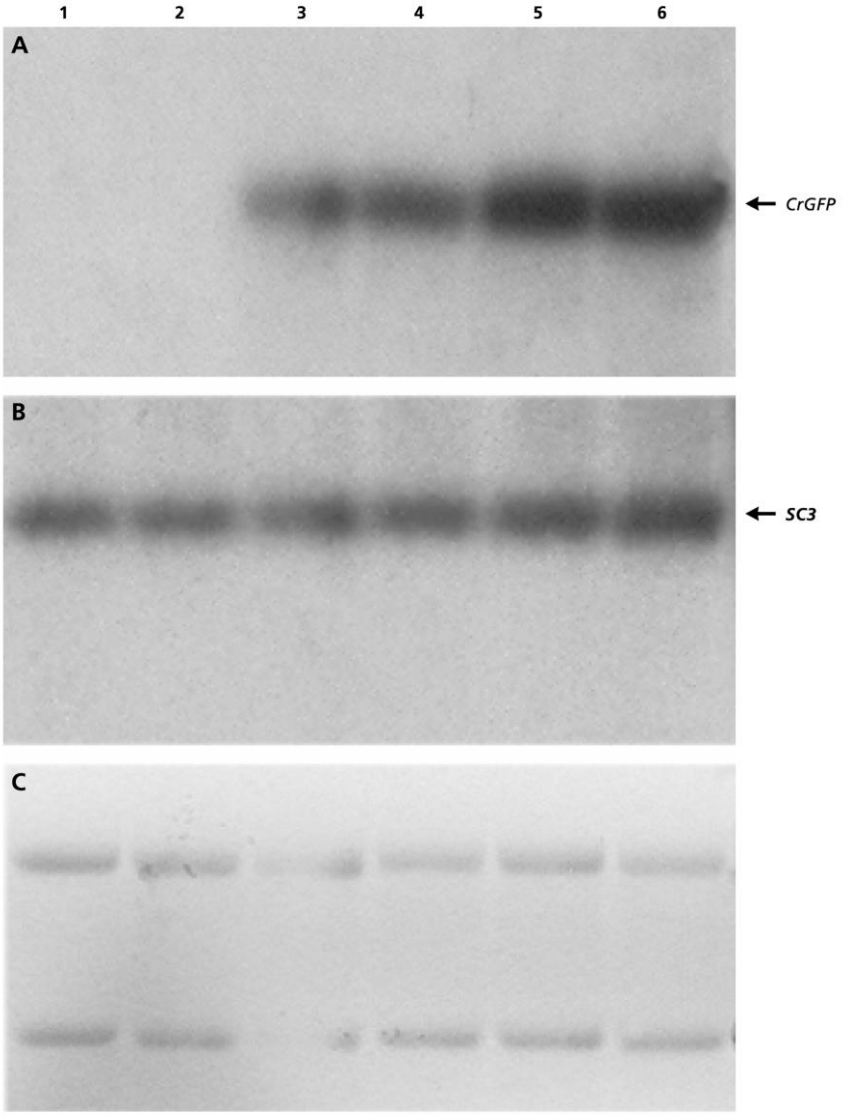

Figure 4. Northern blot analysis of total RNA isolated from 5 (lane 1, 3, 5) and 6-day-old colonies (lane 2, 4, 6) of strains 4-39 (lane 1, 2), AV1 (lane 3, 4) and AV2 (lane 5, 6) using a probe against *GFP* (**A**) or the 5' untranslated region of *SC3* (**B**). Methylene Blue staining served as an RNA loading control (**C**). AV1 and AV2 express *CrGFP* under the control of the *SC3* regulatory sequences.

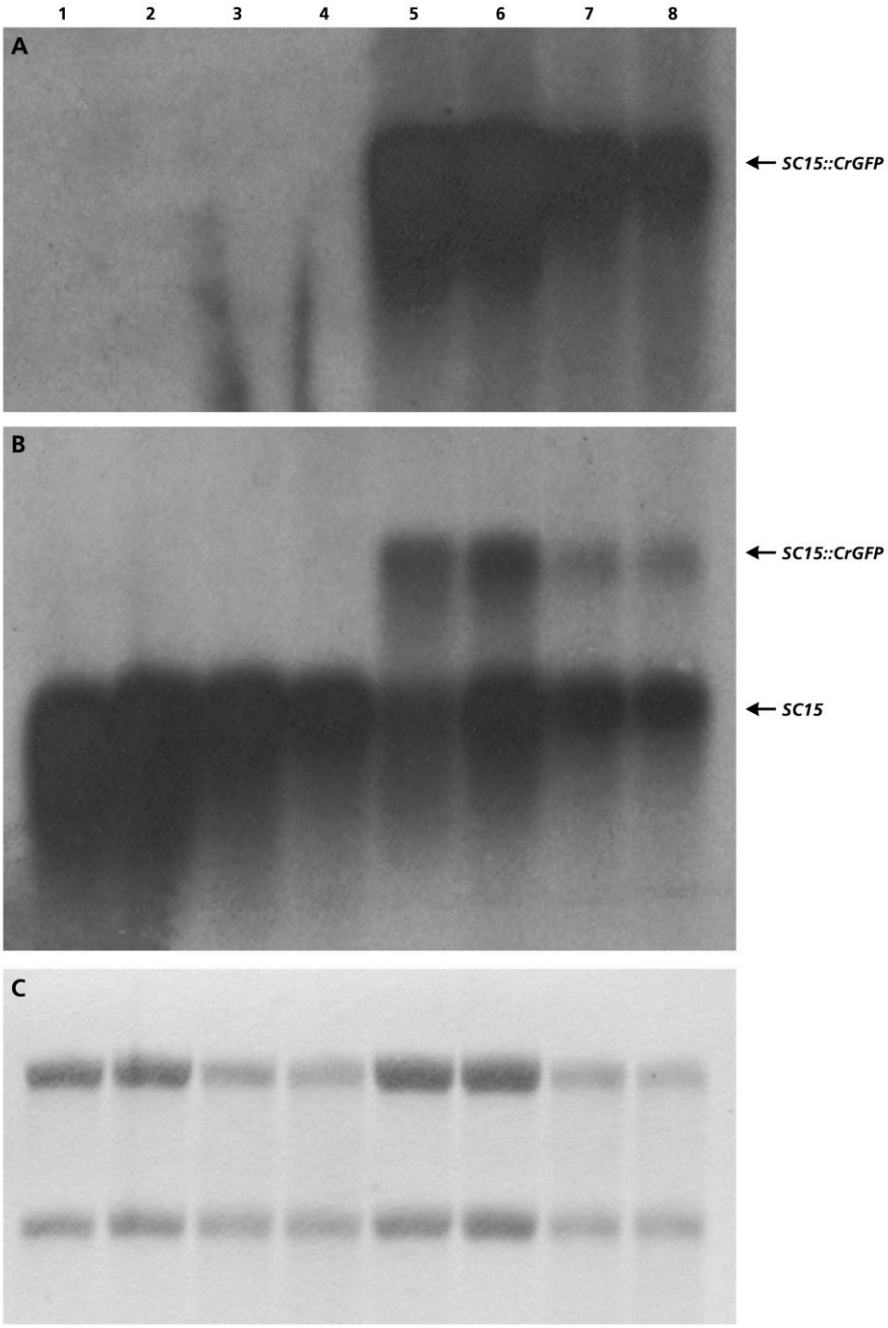

Figure 5. Northern blot analysis of total RNA isolated from 3 to 6-day-old colonies of strain 4-40 (lanes 1- 4) and of the FL1 strain expressing a gene encoding a C-terminal fusion of CrGFP to SC15 (lane 5-8). RNA was hybridized with *GFP* (**A**) or *SC15* (**B**) cDNA. Methylene Blue staining served as an RNA loading control (**C**).

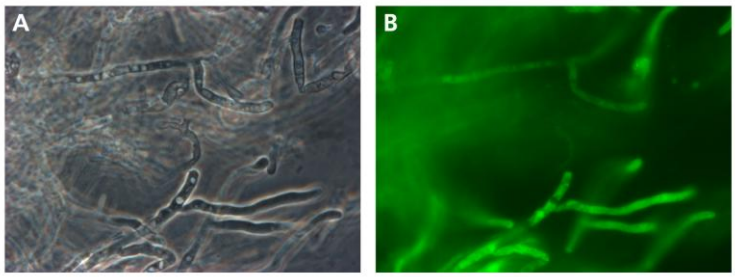

Figure 6. Brightfield (**A**) and fluorescence (**B**) microscopy of *S. commune* strain FL1, expressing a gene encoding a C-terminal fusion of CrGFP to SC15.

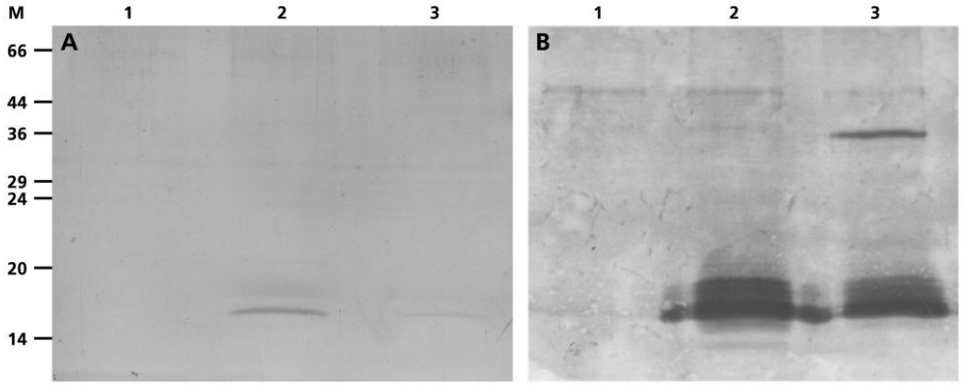

Figure 7. SDS-PAGE (**A**) and Western (**B**) analysis of proteins isolated from the medium of standing cultures of the Δ*SC15* strain (lane 1), the wild-type strain (lane 2) and strain FL1 expressing a *SC15*::*GFP* construct (lane 3). Proteins were stained with Coomassie Brilliant Blue (**A**) or labelled with SC15 antibodies (**B**). SC15 and the SC15::GFP fusion run at 17 and 44 kDa, respectively.

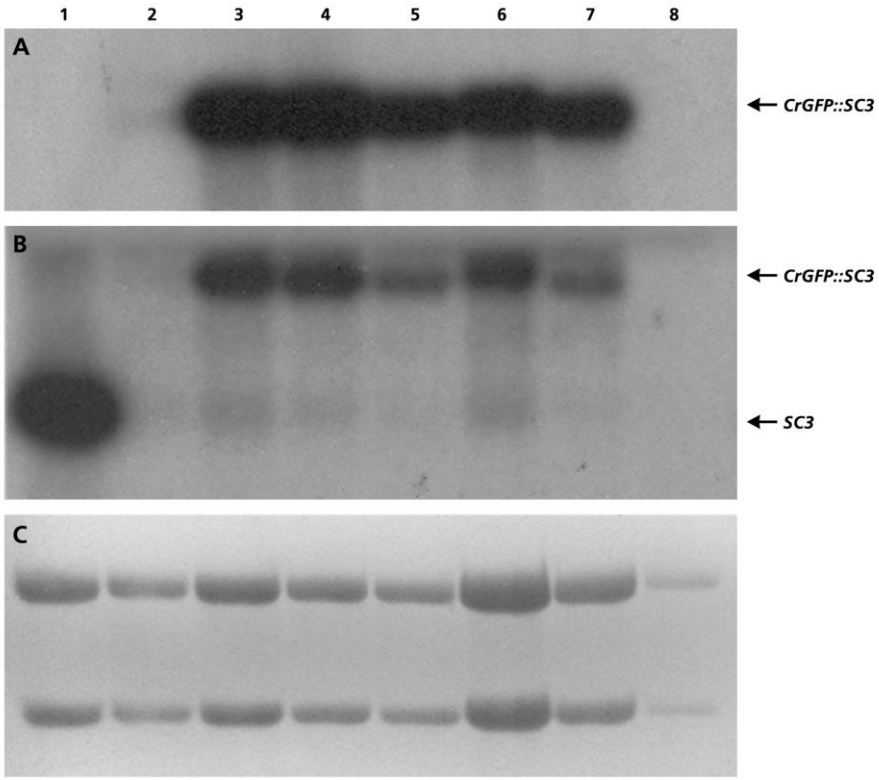

Figure 8. Northern blot analysis of total RNA isolated from 5-day-old colonies of strains 4-39 (lane 1), AV22#1 (lane 2), AV22#2 (lane 3), AV22#4 (lane 4), AV22#5 (lane 5), AV22#9 (lane 6), AV22#14 (lane 7) and 72-3 (lane 8) using a probe against *GFP* (**A**) and the 5' untranslated region of *SC3* (**B**). Methylene Blue staining served as an RNA loading control (**C**). The AV22 transformants express a gene encoding the N-terminal fusion of CrGFP to SC3.

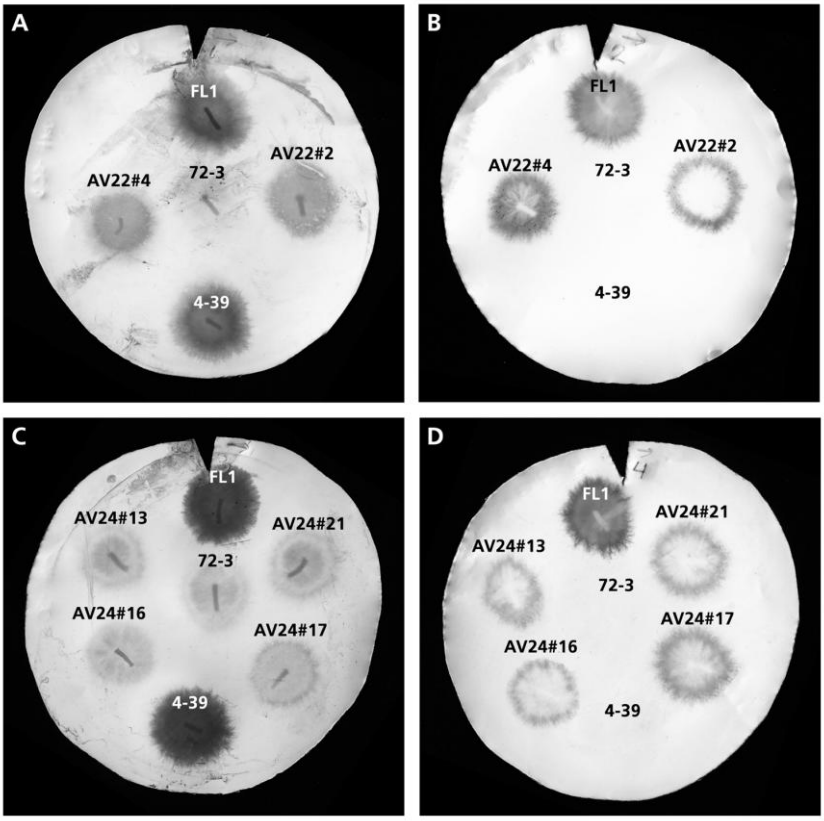

Figure 9. Immunodetection of SC3 (**A, C**) and GFP (**B, D**) on PVDF membranes that had been positioned under colonies of strains of *S. commune* expressing a gene encoding a fusion of GFP to the N-terminal (**A, B**) and C-terminal part (**C, D**) of SC3, respectively. Strains 4-39, 72-3 and FL1 served as controls.

Chapter 6

General discussion

Filamentous fungi (e.g. *Aspergillus niger* and *Schizophyllum commune*) colonize dead and living substrates by means of hyphae that grow at their tips. These hyphae branch subapically and subsequent hyphal fusion results in a network of interconnected cells known as a mycelium. In many fungi, the hyphae are divided into individual compartments by septa. These septa are porous (≥ 100 μm), which enables filamentous fungi to translocate water and nutrients from those parts of the colony that have a surplus of these resources to those parts that have a deficit (Jennings, 1984; 1987; Lindahl & Olsson, 2004). As a result, filamentous fungi can efficiently colonize habitats with a heterogeneous distribution of nutrients, such as forest soil.

After a sufficient amount of vegetative mycelium has been established, the fungal colony initiates the formation of aerial hyphae. Growth of these hyphae depends on the translocation of water and nutrients from the vegetative mycelium (Jennings, 1984; Wösten & Wessels, 2006). These aerial hyphae may further differentiate into (a)sexual reproductive structures such as conidiophores in the case of *A. niger* and fruiting bodies in the case of *S. commune*. The spores that are formed by these structures are dispersed and under the right environmental conditions may give rise to a new colonizing mycelium.

Filamentous fungi secrete enzymes into the environment to enable growth of the vegetative and the aerial mycelium. These enzymes degrade organic polymers in the substrate into small molecules that can be taken up to serve as nutrients. In fact, filamentous fungi secrete a wide variety and large amounts of these enzymes. *A. niger* is an example of a fungus with an enormous secretion capacity. Some strains produce more than 20 grams per liter of the enzyme glucoamylase (Finkelstein *et al*., 1989). The high secretion capacity in combination with the GRAS (generally regarded as safe) status and the established fermentation technology and molecular biology makes *A. niger* an attractive cell factory for the large-scale production of homologous and heterologous proteins. *A. niger* is one of the main fungal production platforms used by the industry and its secreted proteins are used in the production of a wide variety of products (e.g. foods, feeds, drinks, paper, and clothing) or as an active compound thereof (e.g. detergents, pharmaceuticals) (Punt *et al.*, 2002). It should be noted that a variety of proteins can not be produced with the fungi that are used by the industry. Basidiomycetes seem to be a good alternative as a cell factory. For instance, a recombinant strain of *Pycnoporus cinnabarinus* secreted 20 times more laccase than recombinant *Aspergillus* strains (Alves *et al.*, 2004).

Despite the long history of exploitation of filamentous fungi as a cell factory, knowledge of fungal secretion at the molecular and cellular level is still limited. Most studies are performed on whole cultures and thus do not acknowledge the fact that secretion may be different between different parts of the colony or even between hyphae within the same zone. Yet, evidence trickles through that a fungal colony is highly differentiated and is more than just a mass of identical hyphae (Wösten *et al.*, 1991; Moukha *et al.*, 1993; Levin, 2007). For example, glucoamylase (Gla) is only secreted at the periphery of the colony and not by its central secretion zone, a phenomenon called zonal differentiation. In addition, immuno-labelling indicated that only a subset of hyphae within the outer zone of the colony secrete Gla (Wösten *et al.*, 1991), suggesting the existence of hyphal differentiation within zones of the fungal colony. The phenomena of zonal and hyphal differentiation suggest that protein production can be improved by increasing the number of hyphae in the mycelium that are engaged in secretion.

The aim of this PhD Thesis was to study zonal and hyphal differentiation in the fungal mycelium with respect to expression of genes encoding secreted proteins. Using the green fluorescent protein GFP as a reporter it was shown that the glucoamylase gene *glaA* and the α-glucoronidase gene *aguA* are not uniformly expressed in the mycelium of *A. niger*. Moreover, these genes as well as the *faeA* gene were shown to be subject to hyphal differentiation within the periphery of the *A. niger* mycelium. It was also demonstrated that proteins, at least GFP, can stream through the mycelium. Intercellular protein streaming may alleviate hyphal differentiation. It would be interesting to see whether hyphal differentiation and protein streaming also occur in basidiomycetes that are characterized by a complex hyphal septum. To this end, a reporter system was developed for the model basidiomycete *S. commune.*

Zonal and Hyphal differentiation

Differentiation within the colony was studied using GFP as a reporter. Its encoding gene was expressed in *A. niger* from the promoter of the glucoamylase gene *glaA*, the α-glucuronidase gene *aguA*, and the feruoyl esterase gene *faeA*. As a control, *GFP* was fused to the promoter of *gpdA*. This gene, which is part of the central metabolism, is constitutively expressed. Maltose and xylose were used as the inducing carbon sources. These sugars activate the transcriptional activators AmyR (Petersen *et al.*, 1999) and XlnR (van Peij *et al.*, 1998), respectively. The AmyR-regulated gene set includes the *glaA* gene, whereas the gene set regulated by XlnR includes *aguA* and *faeA*. To facilitate microscopic studies and to enable

transfer of the mycelium to an inducing medium, colonies were grown as sandwiched cultures (Wösten *et al.*, 1991). To this end, mycelium is grown in a thin layer of agarose in between two perforated polycarbonate membranes. Alternatively, mycelium was grown in liquid shaken medium.

Zonal differentiation

GFP fluorescence resulting from expression from the *glaA* promoter was mainly observed at the periphery of 5-day-old colonies of *A. niger* that had been transferred from a repressing to an inducing medium for 8 h (**Chapter 2**). In contrast, *gpdA*-driven expression resulted in fluorescence throughout the colony. The zonal expression of *glaA* as monitored with GFP agrees with the accumulation of *glaA* mRNA and the abundant secretion of glucoamylase at the periphery of 5-day-old sandwiched colonies (Wösten *et al.*, 1991; Wösten, 1994; **Chapter 3**). In contrast, *aguA* expression was shown to be more evenly distributed in 5-day-old colonies after 8 h of induction. The spatial expression of *glaA* and *aguA* correlates with the expression of the genes encoding their activators; *amyR* and *xlnR*, respectively (Levin, 2007).

Interestingly, *aguA* expression was somewhat lower in the zone just behind the periphery. This position correlates with the zone that starts to sporulate when the upper polycarbonate membrane is removed from sandwiched cultures (Levin, 2007). This zone is also characterized by its inability to secrete. In contrast, its neighbouring zones secrete high amounts of protein in the medium when cultures are transferred to fresh medium for 24 h. Taken together; I propose that the inability of the sporulating zones to secrete is partly due to repression of genes encoding secreting proteins. This repression may be directed by a pathway leading to sporulation.

Despite the difference in the *glaA* and *aguA* expression patterns, both glucoamylase and α-glucuronidase were primarily secreted at the periphery of a 5-day-old colony (**Chapter 3**). This would agree with the finding that only growing hyphae secrete proteins. These growing hyphae are mainly found at the outer zone of the mycelium (Wösten *et al.*, 1991). However, recently it was shown that central zones of 7-day-old *A. niger* colonies that had been transferred for 24 h to a fresh medium secrete proteins in the absence of growth (Levin, 2007). At the moment it is not clear how proteins would be able to pass the highly rigidified cell wall of non-growing hyphae and why this mechanism develops after 24 h of transfer and not after 4-8 h as was done in this study and in the study of Wösten *et al.* (1991).

Hyphal differentiation

Fluorescence of hyphae at the periphery of induced sandwiched colonies of *A. niger* that expressed *GFP* from the *glaA* or *aguA* promoter was studied using confocal microscopy (Vinck *et al.*, 2005; **Chapter 2 and 3)**. Although all hyphae exhibited green fluorescence large differences in the intensity of the signal between hyphae were observed. Statistical analysis showed that the distribution of fluorescence intensities deviated from normality after inducing *glaA* or *aguA*. Using the bootstrap resampling technique it was inferred that the fluorescence distributions are composed of a weighted mixture of two normal components. Hence, it is concluded that two types of hyphae exist at the periphery of 5-day-old sandwiched colonies; namely, those hyphae that express *glaA* or *aguA* at a high level and those that express these genes at a low level. This is a remarkable finding considering the fact that the high and low expressers experience identical environmental conditions. So far, it is not known whether the hyphae that highly express *glaA* on a maltose inducing medium also express *aguA* highly on a medium with xylose as the carbon source. In other words, are certain hyphae specialized in secreting, while other hyphae fulfil other functions like uptake of nutrients?

When *GFP* was expressed from the *glaA* promoter, 73% of the peripheral hyphae belonged to the high expressing type after 8 hrs of induction. However, intercellular streaming of the GFP affected the quantitative analysis. This was shown by using a photo-activatable variant of GFP (PA-GFP; Patterson and Lippincott-Schwartz, 2002) (Vinck *et al.*, 2005; **Chapter 2**). It was demonstrated that PA-GFP activated in subapical parts of hyphae streamed to adjacent compartments and into branches within seconds, thus illustrating that cytoplasmic streaming interferes with localization studies at the hyphal level, and possible also at the colony level, by leveling out differences in GFP fluorescence. Indeed, when the effect of cytoplasmic streaming was minimized by targeting GFP to the endoplasmic reticulum (ER) (Vinck *et al*., **Chapter 2**) the number of hyphae expressing *glaA* at a low level increased to 50%.

Hyphal differentiation was also studied in liquid shaken cultures. This growth condition is more similar to submerged fermentation as used by the industry than growth of colonies on a solid agar medium. Loose pellets had formed after 24 h of growth in inducing medium. The fluorescence intensity between hyphae at the outer zone of these pellets was quite variable. However, statistical analysis did not confirm the existence of hyphal differentiation in the case of *glaA* and *aguA*. It thus seems that differential expression of *glaA* and *aguA* depends on the growth condition. Indeed, *glaA* is differentially expressed in 1-day-old sandwiched-grown

germlings but differential expression could not be unequivocally shown in case germlings were grown in the absence of a covering membrane (Vinck *et al.*, 2005; **Chapter 2**). Of interest, hyphal differentiation in liquid cultures was observed in the case of *faeA*. Yet, *faeA*-driven expression of GFP could not be monitored in sandwiched colonies of *A. niger*, despite the fact that *faeA* is relatively highly expressed as concluded from micro-array analysis (Levin, 2007). Possibly, translation is regulated by the 5' non-translated part of the mRNA that originates from *faeA.*

Future studies will focus on the mechanisms underlying hyphal differentiation. Possibly, inducers are inefficiently, if at all, being taken up by the low expressers. These hyphae could for instance lack a sugar transporter. Alternatively, differential expression is due to the absence or presence of an activator or repressor. The fact that germlings already display differential expression suggests that spores are already predestined with respect to differential expression and that this trait is passed on to part of the hyphae in the resulting mycelium. Future research should also establish whether other genes are differentially expressed and how many hyphal types exist within the substrate mycelium. Moreover, it should be established whether this differentiation operates during fungal pathogenesis or during protein production in industrial fermentations. It would also be interesting to study hyphal differentiation in hyper-branching mutants (Bocking *et al.*, 1999). It may well be that this type of differentiation explains why these branching mutants do not secrete more proteins than wild-type strains.

Streaming of proteins through the mycelium

The pores in septa of fungal cells (≥100 nm) allow streaming of water and nutrients and even organelles. In **Chapter 2** it was shown that PA-GFP activated in subapical parts of hyphae streamed to adjacent compartments within seconds. In **Chapter 4** long distance streaming of GFP was studied; i.e. streaming from the vegetative mycelium to the condiophores.

The genes *gpdA* and *glaA* of *A. niger* are expressed in the substrate mycelium but not in the conidiophores. This was shown by Northern analysis and by using GFP that had been fused to a nuclear localization signal. Nuclei of the vegetative mycelium were highly fluorescent whereas those in the conidiophores were not. However, both the substrate mycelium and the aerial structures became highly fluorescent when the green fluorescent protein (GFP) was expressed in the absence of a nuclear localization signal. From these data it is concluded that the cytosolic version but not the nuclear version of the reporter streams into the aerial

structure. Do endogenous cytosolic proteins of *A. niger* also stream? Glyceraldehyde-3-phosphate dehydrogenase (GPD) apparently does not. Its activity was only detected in the substrate mycelium (ZC Wu and A. Vinck, unpublished), correlating with the expression of the encoding gene. Of interest, GPD of the yeast *Saccharomyces cerevisae* is part of a multi-protein complex (Gavin *et al.*, 2006; Avilan *et al.*, 1997; Svedruzic and Spivey, 2006) that interacts with other protein complexes being anchored to membranes (Gavin *et al.*, 2006). This observation may well explain why GPD does not stream in *A. niger* and this may also hold for other proteins. Gavin *et al.* (2006) showed that 88% of 1993 successfully purified tagged proteins had at least one partner and that 2760 proteins (60% of the estimated proteome for exponentially growing yeast) are contained in 491 protein complexes that may be directly or indirectly anchored to a membrane.

To summarize, cytosolic proteins may be non-interacting or part of a small or large complex that either or not interacts with other complexes. It may thus be that proteins that are part of a (certain) complex do not stream, while non-interacting proteins are free to flow. It would be of major interest to establish which of these proteins and complexes stream through the cytoplasm. The results will complement transcriptome analysis to understand local metabolic and developmental processes in fungal multicellular structures.

Fluorescent reporters for *Schizophyllum commune*

A fluorescent reporter system was developed to facilitate molecular and cellular analysis of *S. commune,* and possibly other (homo)basidiomycetes as well (**Chapter 5)**. Previously, *sGFP(S65T)* was expressed in *S. commune.* Accumulation of *GFP* mRNA was not observed with an intron-less construct but was detectable when an intron was added to the coding sequence of the reporter (Lugones *et al.*, 1999). However, fluorescence was weak despite the use of the very strong *SC3* promoter. In **Chapter 5** expression of GFP variants was studied to find targets to improve the fluorescence of the reporter.

Expression analysis showed that steady state levels of *sGFP* and *CrGFP* were considerably lower than that of *SC3* when these *GFP* variants were expressed from the *SC3* promoter. This was also observed when two instead of one intron were added to the expression construct. Possibly, the position(s) of the intron(s) in the expression constructs is not yet optimal. Future experiments are planned to insert introns within the coding sequence instead of being positioned at its 3' and/or 5'ends.

GFP fused to the N- or C-terminal part of mature SC3 was secreted into the medium as indicated by immuno-localization. However, fluorescence was not detected, neither in the medium nor associated with the mycelium. These data suggest that GFP fused to the SC3 hydrophobin does not fold correctly. Misfolding also appears to occur when SC15 was fused to GFP. Hyphae expressing a *SC15::CrGFP* fusion did fluoresce. However, fluorescence was not observed in the ER or in the septa and the cell wall, as expected, but was found in the cytosol. This location may be explained by translocation of the misfolded fusion (i.e. the SC15 partner) to the cytoplasm. We can thus conclude that GFP is not an optimal reporter to study gene expression or secretion of proteins. The green autofluorescence of hyphae further hampers use of GFP.

Red autofluorescence is very low in *S. commune* thus favouring use of DsRed (or its derivatives) as a reporter. The *DsRed2* variant was successfully expressed in *S. commune* using the homologous *GPD* or *SC3* promoter. This result stimulated us to further explore the use of this reporter and its derivatives. The DsRed derivative dTomato (Shaner *et al.*, 2004) is now used to study temporal and spatial expression of genes in fruiting bodies of *S. commune* (R.A. Ohm and L.G. Lugones, unpublished results). This reporter has the advantage that it folds about 10 times faster than DsRed (1 and 10 h respectively at 37 °C). In the future dTomato will be used to study zonal and hyphal differentiation in *S. commune* as well as cytoplasmic streaming of proteins in this basidiomycete.

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Samenvatting in het Nederlands

Filamenteuze schimmels koloniseren dode en levende substraten middels hyfen (schimmeldraden) die groeien aan hun top. Deze eigenschap van topgroei maakt dat hyfen zich door het substraat kunnen boren. Voorts vertakken de schimmeldraden zich en versmelten ze met elkaar, waardoor een netwerk van aaneengesloten cellen ontstaat wat een mycelium of een kolonie wordt genoemd. In veel schimmels zijn de hyfen onderverdeeld in compartimenten door middel van tussenwanden, septa genaamd. Deze septa zijn poreus en maken transport van moleculen door het mycelium mogelijk. Op deze manier worden water en voedingsstoffen vervoerd vanaf delen van de schimmelkolonie die een overvloed aan deze stoffen hebben naar delen die hieraan een tekort hebben. Hierdoor kunnen schimmels heel efficient habitats (leefomgeving) koloniseren met een ongelijkmatige verdeling van voedingsstoffen, zoals bijvoorbeeld bosgrond.

Als er genoeg substraatmycelium gevormd is, begint de schimmelkolonie met de vorming van luchthyfen. De groei van deze luchthyfen is volledig afhankelijk van transport van water en voedingsstoffen uit het substraatmycelium. De luchthyfen kunnen zich weer verder ontwikkelen tot (a)-sexuele voortplantingsstructuren, zoals bijvoorbeeld de conidiophoren op bedorven voedsel of de paddenstoelen in het bos. De sporen die gevormd worden door deze structuren worden verspreid door bijvoorbeeld water, wind of insekten en kunnen onder de juiste condities uitgroeien tot een nieuwe schimmelkolonie.

Filamenteuze schimmels scheiden eiwitten in hun milieu uit die de groei van het substraat- en luchtmycelium mogelijk maken. Deze enzymen breken de grote organische verbindingen in het substraat af tot kleine moleculen, die door de schimmel opgenomen worden om te dienen als voedsel. Schimmels scheiden een grote verscheidenheid en buitengewoon grote hoeveelheden van deze enzymen uit. *Aspergillus niger* is een voorbeeld van een schimmel met een reusachtige uitscheidingscapaciteit. Sommige *A. niger* stammen produceren wel 20 gram per liter van één bepaald enzym, glucoamylase genaamd. Dit enzym breekt zetmeel af tot glucose (druivensuiker).

Filamenteuze schimmels worden tegenwoordig grootschalig ingezet als fabriek voor de productie van voor de mens relevante eiwitten. Deze eiwitten worden bijvoorbeeld gebruikt als medicijn of in de voedingsmiddellenindustrie. *A. niger* is een aantrekkelijke fabriek vanwege zijn hoge secretiecapaciteit, zijn voedselveiligheid, en het feit dat er al veel ervaring is met de genetische modificatie en het grootschalig groeien van deze schimmel. *A. niger* kan zelfs gebruikt worden om grote hoeveelheden van niet-eigen eiwitten te produceren. Er dient opgemerkt te worden dat veel enzymen niet door de nu industrieel gebruikte schimmels vervaardigd kunnen worden. De basidiomyceten lijken in dit opzicht een goed alternatief. Zo

kan de basidiomyceet *Pycnoporus cinnabarinus* 20 maal meer laccase maken dan *Aspergillus* stammen.

Het gebruik van filamenteuze schimmels als fabriek kent een lange geschiedenis. Nochthans, is de kennis van het mechanisme van eiwituitscheiding op moleculair en cellulair niveau beperkt. Er is bewijs dat een mycelium sterk gedifferentieerd is. Het mycelium is dus niet een samenraapsel van identieke hyfen, maar blijkt te bestaan uit hyfen die een sterke taakverdeling hebben. Zo wordt bijvoorbeeld het enzym glucoamylase alleen uitgescheiden aan de buitenkant van een *A. niger* mycelium. Dit verschijnsel wordt zonale differentiatie genoemd. Voorts is er middels antilichamen aangetoond dat slechts een beperkt deel van de hyfen in de buitenste zone het glucoamylase uitscheiden. Dit laatste spreekt voor het bestaan van hyfendifferentiatie binnen de verschillende zones van een schimmelkolonie. De verschijnselen van zonale en hyfen-differentiatie suggereren dat eiwitproductie verhoogd kan worden door het aantal hyfen die betrokken zijn bij secretie in een kolonie te verhogen. Het doel van dit proefschrift was dan ook om zonale en hyfen-differentiatie in een schimmelmycelium te onderzoeken met betrekking tot expressie van genen die coderen voor uitgescheiden eiwitten.

Zonale en hyfendifferentiatie

Differentiatie binnen het schimmelmycelium werd onderzocht met behulp van het groene fluorescerende eiwit GFP. Het *GFP* gen werd tot expressie gebracht in *A. niger* onder de regulatie van de *glaA*, de *aguA*, of de *faeA* promoter. Deze genen coderen allen voor uitgescheiden eiwitten, waaronder glucoamylase. Ter controle werd GFP expressie bestudeerd wanneer het onder de regulatie van de *gpdA* promoter was geplaatst. Het *gpdA* gen is onderdeel van het centrale metabolisme en komt altijd tot expressie. De suikers maltose en xylose werden gebruikt als koolstofbron voor de schimmel. Deze suikers activeren respectievelijk de activatie-eiwitten AmyR en XlnR. De AmyR-gereguleerde genengroep bevat het *glaA* gen, terwijl *aguA* en *faeA* door XlnR worden gereguleerd.

Indien een vijf dagen oud mycelium acht uur werd overplaatst op een vast medium met maltose als koolstofbron, dan bleek GFP-fluorescentie als gevolg van expressie vanaf de *glaA* promoter voornamelijk aan de rand van het mycelium plaats te vinden. De expressie van *aguA* was meer egaal door de kolonie aanwezig na acht uur inductie. Ondanks het verschil in hun expressiepatroon werden de gecodeerde eiwitten voornamelijk aan de rand van de kolonie

uitgescheiden. Hier bevinden zich groeiende hyfen en deze kunnen eiwitten efficiënt uitscheiden.

Expressie van GFP onder de regulatie van de *glaA* danwel de *aguA* promoter werd in meer detail onderzocht met behulp van confocale microscopie. Dit werd gedaan aan de hyfen die zich aan de buitenkant van de kolonie bevinden. Hoewel alle hyfen groen fluoresceerden was duidelijk dat de signaalsterkte van hyfe tot hyfe sterk variëerde. Statistische analyse liet zien dat de distributie van de signaalintensiteiten afweken van normaliteit na inductie van de *glaA* en *aguA* genen. De waargenomen distributies konden verklaard worden door ze te beschouwen als een mengsel van twee normaal verdeelde componenten. Er werd bijgevolg geconcludeerd dat er twee soorten hyfen voorkomen aan de buitenkant van vijf dagen oude kolonies, namelijk hyfen die de *glaA* en *aguA* genen hoog tot expressie brengen en hyfen die deze genen laag tot expressie brengen. Dit is opmerkelijk, want alle hyfen in deze zone van het mycelium staan aan dezelfde omstandigheden bloot. Vooralsnog is het onduidelijk of bepaalde hyfen gespecialiseerd zijn in de uitscheiding van eiwitten en of de overige hyfen andere functies vervullen, zoals bijvoorbeeld de opname van voedingsstoffen.

73% van de hyfen die GFP tot expressie brachten vanaf de *glaA* promoter behoorde tot de hoog-expresserende hyfen. Echter, intercellulaire stroming van GFP beinvloedde deze kwantitatieve analyse. Dit werd aangetoond met behulp van een lichtactiveerbare GFP-variant (PA-GFP). Deze variant werd achter de hyfentop geactiveerd waarna deze naar naast gelegen compartimenten stroomde. Cytoplasmatische stroming van GFP lijkt verschillen in fluorescentie tussen hyfen dus te nivelleren. Inderdaad, als het effect van cytoplasmatische stroming werd geminimaliseerd door het GFP te verankeren in het endoplasmatisch reticulum daalde het aantal hyfen dat *glaA* hoog tot expressie bracht tot 50%.

Hyfendifferentiatie werd ook onderzocht in schuddende vloeistofcultures. Deze groeiomstandigheid is meer in overeenstemming met de manier waarop men in de industrie schimmels groeit. Na 24 uur groei hadden zich losse pellets (een soort van bollen) gevormd. Ook hier was de diversiteit in signaalsterkte van GFP omvangrijk. Hyfendifferentiatie onder deze groeiconditie werd aangetoond voor *faeA.* Nochthans kon met behulp van statistische analyse geen bewijs voor hyfendifferentiatie van de *glaA* en *aguA* genen gevonden worden. Uit deze en andere resultaten blijkt dat hyfe differentiatie van deze genen afhankelijk is van de groeimethode.

Samenvattend kan gesteld worden dat een nieuw fenomeen is ontdekt bij schimmels. We weten echter nog niet hoe hyfendifferentiatie plaatsvindt. Dit zal in de nabije toekomst worden onderzocht.

Stroming van eiwitten door het mycelium

De openingen in de septa van hyfen (>100 nm) maken stroming van water, voedingsstoffen en zelfs hele organellen mogelijk. Het langeafstandstransport van GFP in *A. niger* werd bestudeerd vanuit het substraatmycelium naar de luchtstructuren en in het bijzonder naar de conidiophoor. De *A. niger* genen *gpdA* en *glaA* bleken tot expressie te komen in het substraatmycelium, maar niet in de luchtstructuren. Dit werd onder andere aangetoond middels een fusie van GFP aan een kernlokalisatiesignaal. De kernen van het substraatmycelium lichtten sterk fluorescent op, terwijl die in de conidiophoren dit niet deden. Echter, zowel het substraatmycelium als de luchtstructuren werden sterk fluorescent wanneer GFP tot expressie werd gebracht zonder het kernlokalisatiesignaal. Hieruit werd geconcludeerd dat alleen de cytosolische versie van het GFP naar de luchtstructuren kan stromen. Stromen de eigen cytosolische eiwitten van *A. niger* dan ook? Het eiwit glyceraldehyde-3-fosfaat-dehydrogenase (GPD) lijkt dit niet te doen, want zijn activiteit werd alleen waargenomen in het substraatmycelium. Toekomstig onderzoek zal moeten uitwijzen welke eiwitten van *A.niger* stromen en wat de invloed van deze stroming is op de differentiatie van hyfen.

Fluorescente reporters voor *Schizophyllum commune*

Basidiomyceten zoals *Schizophyllum commune* hebben veel complexere septa dan ascomyceten zoals *A. niger*. Vraag is danook of hyfendifferentiatie optreedt in de basidiomyceten. Dit kon niet worden onderzocht omdat er geen fluorescent reportersysteem was ontwikkeld. In het verleden is het synthetische GFP gen *sGFP(S65T)* tot expressie gebracht in *S. commune.* Accumulatie van het *GFP* mRNA werd alleen waargenomen als er een intron (niet-coderend onderdeel van het mRNA) in de transcriptionele eenheid was geplaatst. Nochthans was de fluorescentie heel zwak hoewel het gen onder regulatie van de sterke *SC3* promoter was gezet. Fluorescentie kon niet worden verhoogd door gebruik te maken van andere GFP-varianten. De expressieniveaus van de GFP-varianten bleef laag en het deed er niet toe of er één danwel twee intronen werden toegevoegd aan het gen. Wellicht is de positie van de ingevoegde intronen nog niet optimaal.

Fusie van GFP aan zowel de N-terminus als aan de C-terminus van het SC3 eiwit leidde tot uitscheiding van het fusie-eiwit in het groeimedium. Toch werd er geen fluorescentie waargenomen in het mycelium of in het medium. Het lijkt erop dat het fusieeiwit van GFP en SC3 niet goed vouwt, waardoor fluorescentie uitblijft. Deze misvouwing schijnt ook op te treden bij fusie van GFP aan het SC15-eiwit. De aanmaak van deze fusie in *S. commune*

leidde tot fluorescentie in het cytoplasma, in plaats van in het ER, de septa en de celwanden, zoals dat in *Aspergillus* stammen is geconstateerd. Samengevat kan gesteld worden dat GFP niet de optimale reporter is om genexpressie en eiwitlokalisatie in *S. commune* te bestuderen. Verder is ook de groene autofluorescentie van de *S. commune* hyfen een probleem. De rode autofluorescentie is heel laag en dit maakt het gebruik van het rood fluorescerende reportereiwit DsRed (of varianten hiervan) aantrekkelijk. Het *DsRed2* gen werd met succes tot expressie gebracht in *S. commune*. Een DsRed-variant (dTomato) wordt nu gebruikt om genexpressie te bestuderen in paddestoelen van *S. commune*. In de toekomst zal dit reportereiwit gebruikt gaan worden om zonale en hyfendifferentiatie te bestuderen in deze fascinerende schimmel.

Curriculum Vitae

Arman Vinck is geboren in Willemstad te Curaçao op 25 september 1975. De middelbare school werd doorlopen aan het Emmelwerda College te Emmeloord en deze werd in 1994 afgesloten met het vwo-diploma. In september van hetzelfde jaar werd begonnen met de studie biologie aan de Rijksuniversiteit Groningen. In het kader van deze opleiding werd een stage doorlopen bij de vakgroep Microbiële Fysiologie onder begeleiding van Dr. H.A.B. Wösten. Tussentijds werd de studie biologie gevolgd aan King's College London en aan de Vrije Universiteit Amsterdam. Aan de Rijksuniversiteit Groningen werd in februari 2001 het doctoraaldiploma biologie behaald. Sinds september 2001 is Arman werkzaam geweest als assistent in opleiding (aio) bij de vakgroep Moleculaire Microbiologie aan de Universiteit Utrecht. Tijdens deze periode werd het in dit proefschrift beschreven onderzoek verricht onder begeleiding van Prof. Dr. H.A.B. Wösten. Sinds oktober 2007 is Arman bij dezelfde vakgroep werkzaam als post-doc op een STW-project dat ten doel heeft de mechanismen van hyfendifferentiatie in *Aspergillus niger* te ontrafelen.