The role of autophagy during carbon starvation in Aspergillus niger

Burggraaf, M.A.

Chapter 1

Post-genomic approaches to dissect carbon starvation responses in *Aspergilli*

Jolanda M. van Munster[1*], Anne-Marie Burggraaf[2*], István Pócsi[3], Melinda Szilágyi[4], Tamás Emri[3] and Arthur F.J. Ram[2]

[1] School of Life Sciences, University of Nottingham, University Park, Nottingham, NG7 2RD, United Kingdom

[2] Leiden University, Institute of Biology Leiden, Molecular Microbiology and Biotechnology, Sylviusweg 72, 2333 BE Leiden, The Netherlands

[3] University of Debrecen, Faculty of Sciences and Technology, Department of Biotechnology and Microbiology, Egyetem tér 1, H-4032 Debrecen, Hungary

[4] University of Debrecen, Faculty of Medicine, Department of Human Genetics, Egyetem tér 1, H-4032 Debrecen, Hungary

*Equal contribution

Aspergillus and Penicillium in the post-genomic era (2016). Caister Academic Press pp. 89–112

Abstract

Most filamentous fungi have a saprophytic lifestyle and proliferate on organic materials from plants. They contain a large arsenal of enzymes that are expressed and secreted in response to available carbon sources. Regulatory networks in which carbon-specific transcription factors and wide-domain transcription factors play essential roles tightly control the expression of these enzymes. While a suitable carbon source is present, the entire metabolism of the fungus is directed to grow as fast as possible via hyphal tip extension. At a certain point however, the available exogenous carbon source will be limited, resulting in a carbon starvation response. Filamentous fungi react to carbon starvation with some very specific responses including the induction of glycosyl hydrolases involved in fungal cell wall degradation (autolysis) and the onset of asexual spore formation. Recent advances in genome-wide transcriptomic and proteomic approaches have revealed new insights into molecular mechanisms involved in these fungal-specific carbon starvation responses. Together with the genetic accessibility of filamentous fungi, the omics technologies have made a large contribution in broadening our understanding of the carbon starvation response in recent years. In this review, we have focussed on summarizing and integrating the most important cellular responses of filamentous fungi towards carbon starvation. As most studies to date have been carried out with *Aspergillus nidulans* and *Aspergillus niger,* the focus will be further on these two species and to compare their responses to carbon starvation.

Introduction

The morphology of filamentous fungi is characterized by the presence of hyphae. Filamentous growth starts with the germination of a spore and the subsequent formation of a germling. Via branching or hyphal fusion events, a network of hyphae is formed which is referred to as a hyphal mycelium. Such a hyphal mycelium can be considered as a multicellular structure. In most filamentous fungi, except the zygomycete fungi, hyphae are divided into individual cells via the formation of internal walls named septa. Each septum contains a septal pore, which is large enough to allow transport of organelles and nutrients from one hyphal compartment to the other. Filamentous fungi can control the flow of cytosol or organelles between compartments by the controlled closure of the septal pore via Woronin bodies. Woronin bodies are peroxisome-derived, spherical organelles that are located near the septal pore. By plugging the septal pores with Woronin bodies, a multicellular structure with individual cells is formed. The opening and closing of septal pores are highly dynamic and well-controlled events (Bleichrodt *et al.*, 2015).

A fungal mycelium is typically formed on solid medium such as agar plates. To analyse growth on agar plates, spores are often inoculated in the centre of the plate from where they will gradually cover the plate through radial extension. During growth, the fungus will utilize the available nutrients until they become limiting (for most of the media this will be the carbon source). At that point, the mycelium starts a developmental program leading to sexual or asexual reproduction. Since nutrient limitation first occurs in the centre of the colony, differentiation will start here also. Whether the sexual or asexual developmental pathway is chosen depends on environmental factors such as light (Bayram and Braus, 2012). Some fungi, such as *Aspergillus niger*, are only known to proliferate asexually. The ability to reproduce sexually has either been lost through evolution or remains undiscovered.

Besides the growth on solid medium, fungi are also cultured in liquid medium, referred to as submerged cultivation. To start a submerged cultivation, spores can be used as an inoculum. Depending on the spore density and ambient pH (and possibly other factors), fungal hyphae develop as a dispersed fungal mycelium (individual hyphal filamentous cells) or as pellets (dense aggregates of filamentous cells). The tendency of a culture to form pellets is mainly caused by aggregation of the spores during the initial stages of spore swelling. Low pH values reduce the aggregation of swollen spores and are therefore used to prevent pellet formation. The macromorphology of the submerged culture (dispersed mycelia or pellets) can have important consequences on the productivity and heterogeneity of the culture and is therefore an important parameter. As on solid medium, nutrients can become limiting during batch growth and filamentous fungi respond to starvation with the activation of asexual reproduction program leading to the formation of conidiospores

(Nitsche *et al.*, 2012; Sarkar *et al.*, 2012). The advantages of studying starvation responses in filamentous fungi using bioreactors are reviewed in the next paragraph.

Controlled cultivation of filamentous fungi to study starvation responses

Responses to carbon starvation have been most elaborately studied in submerged cultures. Batch cultivation in controlled bioreactors is preferred as growth can be monitored on-line and is more reproducible (Nitsche *et al.*, 2012). Batch cultures in shake flasks have also been successfully used to perform C-starvation responses (Szilágyi *et al.*, 2013; de Sousa Lima *et al.*, 2014; van Munster *et al.*, 2014; Xiong *et al.*, 2014). However, more detailed measurements regarding the physiology of the fungus cannot be measured in shake flask cultures. On-line analysis of O_2 consumption and CO_2 production in a bioreactor is a convenient method to monitor carbon limitation as the parameters will dramatically change upon starvation (Nitsche *et al.*, 2012). Yet another advantage of bioreactor cultivation is that when sufficient large vessels are used (e.g. 5L), multiple samples for analysis can be withdrawn without affecting the total volume of the culture to such an extent that it changes the physiology of the culture. Substrate-limited growth has also been studied in chemostat cultures. There is an important difference between carbon starvation in batch growth and C-limited chemostat cultures, as additional carbon source is continuously added during chemostat cultivations. However, at low dilution rates (resulting in low growth rates), starvation-like responses such as sporulation have been observed (Bushell and Bull, 1999; Jørgensen, personal communication). An extreme form of chemostat cultivation which leads to a very low growth rate is retentostat cultivation. In a retentostat system, the biomass is retained in the bioreactor while keeping the dilution rate of the medium constant, leading to a severe reduction of the energy source per biomass unit. Cultivation of *A. niger* in retentostats also results in the onset of conidiation (Jørgensen *et al.*, 2010) indicating that limited metabolic fluxes might be sufficient to activate the sporulation program. Based on the phenotype of conidial mutants both on solid plates and in submerged conditions, it is likely that the same genetic regulatory program is involved under both conditions (Nitsche and Jørgensen, personal communication). Although there are obvious benefits, maintaining the environmental parameters (*e.g.* pH, oxygen levels and medium composition) at a constant/controlled level may alter the response of the fungus. In nature, fungi are continuously modifying their environment by secreting enzymes and metabolites during carbon starvation and these changes (*e.g.* alkalizing pH, liberation of nutrients from the hydrolyzing cell wall) can alter their behaviour. When controlling conditions in the bioreactor, we can gain precise data on chosen stress responses, but may lose information on the complexity of the whole stress response.

Carbon starvation responses

Carbon starvation responses in filamentous fungi have been studied both on surface cultures (mostly agar plates) and in submerged conditions. Several transcriptomic and

proteomic studies have been performed in the last few years to describe the carbon starvation responses and are discussed in the next two paragraphs.

Carbon starvation responses on surface cultures

The Wösten group developed an elegant ring system to analyse differential gene expression in a fungal colony and defined five zones, representing differently aged fungal mycelium. The differential gene expression between the different zones as well as the different extracellular proteomes demonstrates the multicellular nature of a fungal colony (Levin *et al.*, 2007; Krijgsheld *et al.*, 2012). Comparison of mRNA profiles between the central zone of an *A. niger* colony and the most outer zone revealed major differences in expression of genes from various functional classes (Levin *et al.*, 2007). Only the broad functional categories 01 (metabolism) including 01.01.10 (amino acid degradation), 01.05.01 and 01.05.07 (C-compound and carbohydrate utilization and transport) and 01.06.01 (lipid, fatty acid and isoprenoid biosynthesis), 06 (protein fate) including 06.07.03 (modifications by (de)-phosphorylation), 06.07.07 (modifications by (de)-ubiquitination) and 06.13.04 (lysosomal and vacuolar degradation), were consistently enriched in the central zones independent of the carbon source used (Levin *et al.*, 2007). It should be noted that in the experiments by Levin et al., the *A. niger* colony was covered with a perforated polycarbonate membrane to prevent sporulation in the centre of the colony. Therefore genes related to conidiation were not induced in these experiments.

mRNA profiling to dissect colony development has also been studied in *Neurospora crassa* (Kasuga and Glass, 2008) and despite the relatively large evolutionary distance between *A. niger* and *N. crassa* a comparative study between the zonal transcriptomes between the two filamentous fungi has been performed. Even though such a global comparison has its limitations, as it is highly dependent on the annotation and the classification in GO-terms or functional categories, the cross-species examination indicated conserved patterns of gene expression during colony development. These conserved patterns were mostly found in the outer parts of the colony and included genes involved in metabolism of lipids and fatty acids, which were predicted to be involved in membrane biosynthesis, and genes involved in biogenesis of cellular components which included genes involved in actin cytoskeleton organisation (Kasuga and Glass, 2008).

Carbon starvation responses under submerged conditions

Several genome-wide transcriptomic or proteomic studies have been reported in the last few years to study the response of filamentous fungi towards carbon starvation under submerged growth conditions. Initial genome-wide transcriptomic studies were performed with *A. niger* for which a genome sequence and microarrays were available (Pel *et al.*, 2007; Nitsche *et al.*, 2012; van Munster *et al.*, 2015b). Since then, other reports have been published, including carbon starvation responses in the medically relevant fungus

Paracoccidioides spp (de Sousa Lima *et al.*, 2014) and a transcriptome study including qRT-PCR for 99 selected genes in *Aspergillus nidulans* to analyse the response to carbon starvation (Szilágyi *et al.*, 2013). Because of the different experimental designs in each of the studies, a detailed comparison between the studies is challenging. Whereas in *A. niger* three different time points were analysed (16h, 60h and 140h after carbon starvation), studies in *Paracoccidioides* and *A. nidulans* included a single time point (6h and 4h after starvation, respectively). Starvation leads to important morphological changes, including vacuolization, the formation of empty compartments, hyphal fragmentation, the formation of thin hyphae and conidiation. These morphological changes are accompanied by massive changes in gene expression patterns. About 50% of the genes (7,292 out of 13,989) are differentially expressed during starvation compared to the exponential growth phase in *A. niger* (Nitsche *et al.*, 2012). Analysis of the differentially expressed genes during carbon starvation in submerged conditions identified several commonly induced functional categories or GO terms. As expected, many of these terms were related to dealing with the carbon limitation and the use of endogenous or reserve carbon sources. Obviously, these endogenous carbon sources include extracellular sources such as the fungal cell wall, but also intracellular sources. Therefore in all three genome-wide studies performed (Nitsche *et al.*, 2012; Szilágyi *et al.*, 2013; de Sousa Lima *et al.*, 2014) terms related to amino acid metabolism, fatty acid oxidation, C-compound and carbohydrate metabolism were upregulated in response to starvation. Terms related to cell biological processes such as cell cycle arrest *(A. niger*), protein synthesis and secretion (*A. nidulans*), autophagy (*A. niger* and *A. nidulans*), vacuolar transports (*A. niger* and *A. nidulans*) were enriched in the upregulated genes in response to carbon starvation. The energy and building blocks obtained from recycling its own mycelium, allow the fungus to respond to the starvation condition whereby the energy is used for maintenance and to fuel conidiation. In the next parts, we will further focus on the morphological responses observed in carbon starved cultures of filamentous fungi.

Morphological responses during C-limitation

Carbon starvation induces hyphal fragmentation in submerged *A. nidulans* cultures (Emri *et al.*, 2004, 2005; Pócsi *et al.*, 2009; Szilágyi *et al.*, 2010a, 2013). During this process, hyphal fragments are liberated from the surface of pellets and as a consequence the pellet size is decreased until it is completely disintegrated (Emri *et al.*, 2004). The disintegration of hyphae is followed by the complete degradation of the released hyphal branches until only small numbers of "yeast like cells" (hyphal fragments of one to two cells) are present in the fermentation broth (Emri *et al.*, 2004). These short fragments can grow out and form hyphae after glucose supplementation (Emri *et al.*, 2004). Carbon starvation in *A. niger* is also accompanied by several morphological changes which include: the formation of empty compartments (starting to emerge after 16 h of glucose depletion), the outgrowth of thin hyphae or thin hyphae within empty hyphae (16 h after glucose depletion), and conidiation

(60 h after glucose depletion) (Nitsche *et al.*, 2012). These morphological changes are similar to the morphological changes upon starvation which have been described before (White *et al.*, 2002). The formation of thin hyphae in response to carbon starvation was reported also for *Aspergillus oryzae* (Pollack *et al.*, 2008). An important difference in comparison to morphological changes upon starvation in *A. nidulans* (Emri *et al.*, 2004) is that in *A. niger* no mycelial fragmentation was observed. Additional unpublished studies have shown that the lack of fragmentation in *A. niger* is probably due to the low pH value (pH3.0) which is maintained in the bioreactor during the experiments. Fragmentation of the mycelium upon starvation in *A. niger* is observed when the pH is not controlled and reaches values around 6.0 during starvation (Nitsche, unpublished results). Likely, the low pH (3.0) conditions lower the activity of cell wall hydrolytic enzymes that are involved in hyphal fragmentation. In *A. nidulans*, fragmentation and formation of empty hyphae is also accompanied by the production of cell wall glycosyl hydrolases (Emri *et al.*, 2004, 2005; Pócsi *et al.*, 2009; Szilágyi *et al.*, 2010a, 2013). *A. nidulans* mutants with defects in cell wall glycosyl hydrolase production show reduced hyphal fragmentation but these mutations do not inhibit vacuolization and formation of empty hyphae (Emri *et al.*, 2005; Pócsi *et al.*, 2009; Szilágyi *et al.*, 2010a). It clearly demonstrates that hyphal fragmentation is an enzymatic process and not the passive consequence of mechanic forces occurring in shaken cultures.

In addition to the formation of empty compartments and hyphal fragmentation, some hyphal cells remain viable and these surviving cells differentiate and can produce conidiospores (Schrickx *et al.*, 1993; Emri *et al.*, 2004; Jørgensen *et al.*, 2010; Nitsche *et al.*, 2012; Szilágyi *et al.*, 2013). BrlA encodes a transcription factor which is essential for asexual reproduction in Aspergilli both in surface cultures as well as in submerged cultures (Adams *et al.*, 1998; Twumasi-Boateng *et al.*, 2009; van Munster *et al.*, 2015b). The regulation of conidiation in submerged conditions as well as the regulation of conidiation at surface cultures is dependent on BrlA and therefore BrlA is likely to be a key regulator for conidiation under both conditions. Asexual development (conidiogenesis) is supposed to be initiated by internal signals and/or several stress conditions including carbon starvation (Adams *et al.*, 1998; van Munster *et al.*, 2013). The stress-dependent activation of conidiogenesis is mandatory during submerged cultivation but thought to be secondary in surface cultures (Skromne *et al.*, 1995; Adams *et al.*, 1998; Krijgsheld *et al.*, 2013). In surface cultures, asexual development generally starts well before carbon depletion and continues during starvation (Adams *et al.*, 1998). Autolytic processes (see below) induced by carbon starvation are supposed to support asexual development with nutrients during this period (Emri *et al.*, 2008; Pócsi *et al.*, 2009).

In addition to asexual development (formation of conidiophores and conidia), developmental processes also involve sexual development (formation of cleistothecia and ascospores) in aspergilli with a sexual cycle such as *A. nidulans* (Adams *et al.*, 1998; Etxebeste

et al., 2010; Dyer and O'Gorman, 2012; Park and Yu, 2012; Krijgsheld *et al.*, 2013). Carbon starvation induces sexual development in several pezizomycete fungi (Dyer *et al.*, 1992; Dyer and O'Gorman, 2012). *A. nidulans* is among the exceptions where sufficient nutrition is needed for the initiation of cleistothecia formation (Han *et al.*, 2003; Dyer and O'Gorman, 2012). However, further research is needed to elucidate whether carbon starvation can block sexual development if it has already started. If not, autolytic processes could support sexual development with nutrients as was suggested in the case of asexual development.

Recycling responses to deal with carbon starvation

Under carbon starvation, fungi have two options to cope with this type of environmental stress: i) to recycle the biopolymers of old hyphal parts to provide energy for surviving cells (autolysis) or ii) to use other, non-easily degradable substrates as carbon source (*e.g.* plant materials). The recycling of cell contents is coupled tightly to the (controlled) death of old cells and proliferation of other cells, and includes the utilization of inner cell material by autophagy as well as the degradation of cell wall constituents by extracellular hydrolytic enzymes. In the next few paragraphs we will discuss in more detail the role of autophagy during starvation and the roles of glycosyl hydrolases (GH) during cell wall recycling and cell wall active enzymes involved in conidiation. We will also discuss the strategies employed by fungi to scout for non-easily degradable substrates that could be used as alternative C-sources.

Recycling responses: The role of autophagy in the carbon starvation response

To survive carbon starvation conditions and support the formation of reproductive structures and substrate exploring hyphae, endogenous recycling of nutrients has been proposed to be an important strategy (White *et al.*, 2002; Shoji *et al.*, 2006; Richie *et al.*, 2007; Shoji and Craven, 2011). As such, the autophagic pathway is one of the most dominantly induced processes during carbon limitation in filamentous fungi (Nitsche *et al.*, 2012; Szilágyi *et al.*, 2013; Krohn *et al.*, 2014; Ellström *et al.*, 2015). Autophagy is a non-selective catabolic pathway which targets cytoplasmic contents to the vacuole for degradation and recycling of the building blocks of the cell. It includes the sequestering of cytosolic components and organelles in double-membrane vesicles which subsequently fuse with the vacuole, releasing their contents.

The autophagy machinery is negatively regulated by the kinase activities of the target of rapamycin (Tor) and the cAMP-dependent protein kinase (PKA), which independently act through control of the interaction between the autophagy-related proteins Atg1 and Atg13 (Kamada *et al.*, 2000; Stephan *et al.*, 2009). Under nutrient-rich conditions, autophagy is blocked by Tor-mediated hyperphosphorylation of Atg13, which reduces its affinity for Atg1. Inhibition of Tor by nutrient starvation conditions reduces the phosphorylation state of Atg13, enabling it to form a complex with Atg1 and thereby initiating the autophagy

process. Both Atg1 and Atg13 are direct substrates for PKA, but its phosphorylation sites are distinct from those targeted by Tor (Stephan *et al.*, 2009). The concerted regulation of autophagy by Tor and PKA is rather complex and currently not fully understood. Responses are likely dependent on the nature of the limiting nutrients, providing the cell with more flexibility to adapt to changing environments (Dechant and Peter, 2008).

To date, more than 30 *atg* genes have been identified in yeast and filamentous fungi, which control the autophagic machinery (Feng *et al.*, 2014). Genome-wide transcriptional profiling of *A. niger* and *Paxillus involutus* has shown that the majority of these genes is upregulated under carbon starvation conditions (Nitsche *et al.*, 2012; Ellström *et al.*, 2015). In addition, extensive transcriptome analysis demonstrated the transcriptional induction of specific *atg* genes in *A. nidulans* (Szilágyi *et al.*, 2013). Analysis of *atg* deletion mutants in *Aspergillus* and *Penicillium* showed that phenotypic effects vary among different species. While the deletion of *atg1* severely reduced conidiation in *A. fumigatus* (Richie *et al.*, 2007) and *P. chrysogenum* (Bartoszewska *et al.*, 2011), conidiospore formation was only mildly reduced in the *A. niger* Δ*atg1* mutant (Nitsche *et al.*, 2013). Similarly, the effect on radial growth and conidiation upon disruption of *atg8* was less pronounced in *A. niger* (Nitsche *et al.*, 2013) than in *A. oryzae* (Kikuma *et al.*, 2006). In *A. nidulans*, the deletion of either *atg1, atg4, atg5, atg7* or *atg9* resulted in a deficiency in conidiation at 42°C (Pinar *et al.*, 2013). During submerged carbon starvation, *A. niger* Δ*atg1* and Δ*atg8* mutants showed growth profiles similar to wild-type, but the emergence of empty compartments and the formation of thin hyphae was clearly accelerated (Nitsche *et al.*, 2013). This suggests that autophagy promotes survival during carbon starvation by endogenous recycling of nutrients and protecting older hyphal compartments from cell death.

Recycling responses: GH specific for cell wall degradation

During carbon starvation, the fungal cell wall is thought to undergo drastic remodelling or degradation. Fungal carbohydrate-active enzymes are key effectors in these cell wall changes. The production of such hydrolytic activities in response to carbon starvation has been observed in a number of fungi (Jaroszuk-Scisel *et al.*; Lahoz *et al.*, 1976; Perez-Leblic *et al.*, 1982; McIntyre *et al.*, 2000; White *et al.*, 2002). Recent investigations of the transcriptome and proteome of carbon starving *A. niger* (Nitsche *et al.*, 2012; van Munster *et al.*, 2015b) and *A. nidulans* (Szilágyi *et al.*, 2013) cultures have provided an overview of the response of fungal cell wall active carbohydrate hydrolases.

The cell wall is a rigid, complex structure, which maintains the shape of the cell and is responsible for counteracting turgor pressure and for protecting the plasma membrane against mechanical damage. The fungal cell wall accounts for a considerable percentage of cellular biomass (about 20 %) and thus it represents a rich carbon source during carbon starving conditions. The fungal cell wall consists mainly of polysaccharides. For almost all

fungi, the central core of the cell wall is a ß-1,3-glucan with ß-1,6 branch points. Chitin is covalently bound to the non-reducing end of ß-1,3-glucan side chains via ß-1.4-linkages. The *Aspergillus* cell wall also contains α-1,3-glucan, galactomannan (α-1,2/α-1,6-linked mannan with short β-1,5-galactofuranose side chains), and galactosaminogalactan (a linear heterogenous polymer containing α-1,4-galactose/galactosamine) (Latgé, 2007). Cell walls also contain several heavily glycosylated proteins including GPI-modified cell wall proteins or hydrophobins (De Groot *et al.*, 2005).

Cell wall homeostasis undergoes a dramatic change during carbon starvation. For example, processes responsible for the synthesis of cell wall components are down-regulated (Szilágyi *et al.*, 2013). In *A. nidulans*, *fksA* encoding the catalytic subunit of ß-1,3-glucan synthase (Kelly *et al.*, 1996); *chsB* and *chsF* coding for III type chitin synthases (Horiuchi, 2009) and *agsB* encoding α-1,3-glucan synthase (Fujioka *et al.*, 2007) were down-regulated in carbon starving cultures (Szilágyi *et al.*, 2013). In both *A. niger* and *A. nidulans*, genes coding for GPI-anchored enzymes, which may function in the remodeling of rigid cell wall to a looser structure during growth–*e.g. chiA* in the case of chitin (Yamazaki *et al.*, 2008) or *eglC* in the case of ß-1,3-glucan (Choi *et al.*, 2005)–were also down-regulated during carbon starvation (Szilágyi *et al.*, 2013; van Munster *et al.*, 2015b).

In contrast to the down-regulation of cell wall synthesis, cell wall-degrading processes are upregulated in carbon starving cultures (Szilágyi *et al.*, 2013). In *A. nidulans* and *A. niger*, elevated levels of extracellular protease, ß-1,3-glucanase, ß-glucosidase, α-1,3-glucanase and chitinase were detected during carbon starvation (Zonneveld, 1974; Emri *et al.*, 2004; Szilágyi *et al.*, 2010a; Nitsche *et al.*, 2012) and genes encoding such autolysis-related hydrolytic enzymes were up-regulated (Nitsche *et al.*, 2012; Szilágyi *et al.*, 2013; van Munster *et al.*, 2015b). However, only a number of these enzymes have been investigated in detail.

The *A. nidulans* EngA plays a role in biomass decrease during autolysis (Szilágyi *et al.*, 2010a). Its ortholog in *A. fumigatus* has been characterized as an endo-acting β-1,3-glucanase, and was found to be associated with the fungal cell wall (Fontaine *et al.*, 1997; Mouyna *et al.*, 2002). *A. nidulans* EngA also has ß-1,3-endoglucanase activity, and its activity and stability at a broad pH range means that EngA preserves its activity even at alkaline pH values typical of late carbon starving cultures. EngA is the main secreted ß-1,3-glucanase in carbon starving cultures of *A. nidulans* and the deletion of *engA* confirmed the prominent role of EngA in cell wall degradation and autolysis. However, gene deletion did not abolish the ß-1,3-glucanase enzyme production (Szilágyi *et al.*, 2010a). Nuero et al. purified three enzymes with ß-1,3-glucanase activity from autolyzing cultures of *A. nidulans* confirming the existence of several enzymes with ß-1,3-glucanase activity in carbon-depleted cultures of *A. nidulans* (Nuero *et al.*, 1993).

High α-1,3-glucanase activities are characteristic for carbon starving cultures of *A. nidulans* (Zonneveld, 1974). Correspondingly, genes encoding enzymes with putative α-1,3-glucanase activity were up-regulated during carbon starvation in *A. niger* and *A. nidulans* (Nitsche *et al.*, 2012; Szilágyi *et al.*, 2013). One of these, *A. niger* AgnB, was characterized as α-1,3-glucanase which releases glucose from α-1,3-glucan substrates. However, no direct role for this enzyme in cell wall degradation could be identified (van Munster *et al.*, 2015a). In *A. nidulans*, which in contrast to *A. niger* has a sexual cycle, the α-1,3-glucanase MutA hydrolyzes cell wall material during sexual reproduction, possibly to generate energy that is needed for the production of the cleistothecia (fruiting bodies) (Zonneveld, 1972; Wei *et al.*, 2001). The complete hydrolysis of glucans is achieved by the synergistic action of endo- and exo-glucanases. The up-regulation of high affinity glucose transporters also supports the hypothesis that glucan monomers are taken and utilized by the surviving cells upon carbon starvation (Szilágyi *et al.*, 2013).

Chitinase ChiB is rate-limiting in biomass degradation during carbon starvation in *A. nidulans.* Deletion of *chiB* resulted in the loss of extracellular endochitinase producing capability and caused a non-autolysing phenotype, which was characterized by the lack of hyphal fragmentation and pellet disorganization in ageing carbon starving cultures (Yamazaki *et al.*, 2007; Pócsi *et al.*, 2009). Deletion of the homologous *A. niger cfcA* and *A. fumigatus chiB1* – also encoding the main chitinases induced during starvation – does not result in a change in biomass loss during starvation (Jaques *et al.*, 2003; van Munster *et al.*, 2015a); the physiological role of these enzymes thus appears to be somewhat different. However, the *A. niger* enzyme is responsible for cell wall degradation as deletion of the corresponding gene reduces hyphal fragmentation during starvation. *A. fumigatus* ChiB and *A. niger* CfcA hydrolyze chitin and chitin oligosaccharides by cleaving mainly chitobiose from the non-reducing substrate end, but can also perform a transglycosylation reaction (Escott *et al.*, 1998; Xia *et al.*, 2001; Jaques *et al.*, 2003; Lü *et al.*, 2009; van Munster *et al.*, 2015a). The *A. nidulans* ChiB appears to have similar activity (Erdei *et al.*, 2008). Chitobiose can be further hydrolysed to monomers by *N*-acetyl-ß-D-glucosaminidase enzymes. The *A. niger* and *A. nidulans nagA,* both encoding an *N*-acetyl-ß-D-glucosaminidase, were induced by carbon starvation (Pusztahelyi *et al.*, 2006; Nitsche *et al.*, 2012; Szilágyi *et al.*, 2013). The *A. nidulans nagA* disruptant strain was not able to grow on medium containing chitobiose as sole carbon source (Kim *et al.*, 2002), but there was no effect on autolysis. Instead, deletion reduced cell death (Shin *et al.*, 2009). Similar viability effects were observed for the *ΔengAΔchiB* strain (Szilágyi *et al.*, 2012), showing a possible link between cell wall-hydrolyzing enzymes to the initiation of cell death processes in old carbon starving cultures.

High extracellular hydrolase activities were demonstrated to have a strong antifungal activity against the producer itself (Szilágyi *et al.*, 2012). It means that living fungal cells need to protect themselves against the deleterious effects of these enzymes. To shed light

on the details of such self-protection remains a challenge for future research in this field. The transcriptionally regulated *mpkA* and *rlmA* genes, which encode key elements of the cell wall integrity stress signaling pathway (Fujioka *et al.*, 2007), were not up-regulated during carbon starvation. The clear overall repression of cell wall biosynthetic genes also rejects the idea that the cell wall integrity pathway would have a prominent role in self-protection (Szilágyi *et al.*, 2013). An alternative way for protection could be an increased production of the pigment melanin in the surviving cells. This could be an effective response against the deleterious effects of cell wall hydrolysing enzymes as melanin itself can inhibit autolytic chitinase and glucanase activities and, as a consequence, the enzymatic lysis of cells in *A. nidulans* (Kuo and Alexander, 1967).

Recycling responses: GH specific for conidiation

During periods of stress, such as during prolonged starvation, *Aspergillus* can survive by forming stress resistant spores. The formation of these spores requires energy and building blocks. In case of nutrient limitation, fungal autolysis and autophagy may make these nutrients available by recycling the cell wall or intracellular components. The regulatory machinery responsible for the initiation of sporulation is linked to the regulation of autolysis and is discussed in the following section on genetic control of the responses to carbon starvation.

Extensive morphological changes are taking place during sporulation, and the fungal cell wall needs to be adapted to enable these changes. This can be observed by changes in appearance (Adams *et al.*, 1998), as well as by the differences in carbohydrate composition of vegetative mycelium and spores (Maubon *et al.*, 2006). Spore walls of *A. niger* and *A. fumigatus* contain, compared to walls of vegetative hyphae, more mannose (25 % instead of 4-6 %) and galactose (12-14 % instead of 4-8 %), and less (N-acetyl)-glucosamine (6 % instead of 13 %) (Maubon et al., 2006; van Munster et al., 2013). In addition to a carbohydrate backbone, the spore cell walls contain an outer layer of melanin, which gives the spores their color and increases its resistance to environmental stress (Latgé *et al.*, 2005). CAZymes build and remodel this cell wall during sporulation. Sporulation-specific chitin synthases (Fujiwara *et al.*, 2000; Lee *et al.*, 2004) have been identified that contribute to these cell wall changes. Recently, sporulation-specific CAZymes that are predicted to be active on the fungal cell wall, were identified by comparing the expression profiles of glycoside hydrolases in wild-type and a strain carrying a deletion of the developmental regulator *brlA* (van Munster *et al.*, 2015b). These are a glycoside transferase from GT family 1, a protein containing only a CBM14 domain, the three β-glucan acting enzymes BgtD, An02g00850 and GelG, and the chitin acting CfcI, CtcB and an AA11 member.

Transcriptome analysis shows that the sporulation-specific glycoside hydrolases are strongly induced in sporulating aerial mycelium compared to vegetative mycelium (van Munster *et*

al., 2013). Using promoter fusion strains, activities of the promoters of *cfcI* and *ctcB* were located to the conidophores. These genes encode the chitinases CfcI and CtcB. Deletion of both genes resulted in accumulation of chitin in the spore cell wall, indicating that both enzymes have a function in modification of the cell wall during sporulation (van Munster *et al.*, 2013). CfcI is capable of hydrolyzing chitotriose and longer chitin oligosaccharides by cleaving off monomers, possibly in a processive mode. The enzyme acts on the reducing end of the oligosaccharide substrates. CtcB belongs to the phylogenetic subgroup of GH18 that only encodes endo-chitinases. Together these enzyme activities would thus be capable of degrading the chitin polymer to N-acetyl-glucosamine monomers (van Munster *et al.*, 2012, 2015c).

The *A. fumigatus* β-1,3-glucanosyltransferase Gel7, the ortholog of the sporulation-specific *A. niger* GelG, was found to be important during sporulation. Deletion of the *gel7* gene resulted in abnormal conidia formation. In addition, the enzyme is capable of compensating for cell wall effects generated by *gel1* and *gel2* deletion (Zhao *et al.*, 2014). Orthologs of the *A. niger* sporulation-specific An02g00850 have been characterized, the *A. fumigatus eng2* and *A. nidulans xgeA* hydrolyze soluble β-1,3-glucan with β-1,6-glucan branches as well as β-1,3:1,4-glucan (lichenan) (Bauer *et al.*, 2006; Hartl *et al.*, 2011). Deletion of *eng2* in *A. fumigatus* gave no phenotype during vegetative growth consistent with a possible (unexplored) role during sporulation (Hartl *et al.*, 2011).

Recycling responses: scouting for new C-sources

Next to recycling fungal cell components, another possibility for carbon-starved fungi is to find an external carbon source. One of the substrates that may be available is the recalcitrant cell wall from dead plants. Plant cell walls can contain the carbohydrates cellulose, hemicelluloses and pectins, as well as other components such as lignin and structural proteins. The exact plant cell wall composition is dependent on the plant species, but also varies between different plant structures and wall types. For example, the primary cell walls of grasses contain low amounts of pectin and relatively high amounts of the hemicelluloses glucuronoarabinoxylans and mixed-linkage glucans. For gymnosperms, the cell wall contains more xyloglucan, pectin and structural proteins (Vogel, 2008; Sarkar *et al.*, 2009). The structure of individual plant cell wall carbohydrates is also dependent on their origin (Scheller and Ulvskov, 2010). Plant cell walls thus constitute a carbon source that is very variable in composition, but in all cases, plant cell wall components are cross-linked and form a complex network that may be quite resistant to enzymatic degradation.

A. niger and *A. nidulans* are both metabolically versatile and are capable of growing on a large number of different carbon sources. Their genome encodes a large suite of CAZymes that are active on plant-derived polysaccharides, and a recent catalogue of the *A. niger* genome showed that its genome encodes enzymes that are predicted to be active on at

least 14 different plant cell wall polysaccharides (Andersen *et al.*, 2012). The response of *A. niger* to different lignocellulosic substrates seems to require a core set of enzymes, that is complemented by a set of genes that seem differentially expressed on different substrates (Pullan *et al.*, 2014), a strategy similar to that observed in *N. crassa* (Benz *et al.*, 2014).

The expression of the genes encoding all these enzymatic activities requires a big investment by the fungus, and thus needs to be carefully regulated. This regulation is mediated by the coordinated action of transcriptional repression and inducer-dependent transcriptional activation.

In the presence of sufficient amounts of easily metabolisable carbon sources such as glucose or xylose, carbon catabolite repression (CCR) inhibits transcription of genes that encode enzymes required for complex carbon sources (Ruijter and Visser, 1997; de Vries *et al.*, 1999). Similar to other filamentous fungi and yeast, in *A. niger* and *A. nidulans* CCR is mediated by the global transcriptional repressor CreA. In nutrient rich conditions, glucose is phosphorylated after uptake by the kinases GlkA and HxkA (Flipphi *et al.*, 2003). In *A. nidulans*, similar to in *S. cerevisiae*, a kinase can phosphorylate CreA, causing it to shuttle from the nucleus to the cytoplasm under de-repressing conditions. The presence of phosphorylated sugar inhibits this kinase via RAS-signaling, allowing non-phosphorylated CreA to remain in the nucleus under repressive conditions (Brown *et al.*, 2013). There, CreA can bind to two palindromic consensus sequences (5'-SYGGRG-3) in the promoter of genes (Cubero and Scazzocchio, 1994; Takashima *et al.*, 1996) and represses gene transcription. CreA influences other transcription factors, for example the XlnR transcriptional activators of genes encoding plant polysaccharide active CAZymes (de Vries *et al.*, 1999), as well as the transcription of individual CAZyme encoding enzymes.

To be transcribed to a high level, genes encoding plant polysaccharide active enzymes require activation by inducer-dependent transcriptional regulators, such as XlnR for hemicellulase and cellulase genes, or ClbR, ClrA, ClrB for cellulase genes (Glass *et al.*, 2013).

In carbon starvation conditions, low level transcription of a set of genes that encode plant polysaccharide-degrading enzymes has been detected in *A. niger* as well as in other fungi (Foreman *et al.*, 2003; Martens-Uzunova and Schaap, 2009; Coradetti *et al.*, 2012; Szilágyi *et al.*, 2013; van Munster *et al.*, 2014). These genes include for example *A. niger* endo-α-1,5-arabinanase encoding *abnA* and *abnC*, α-arabinofuranosidase encoding *abfB* and *abfC*, exo-polygalacturonase encoding *pgaX*, *pgxB*, several genes encoding beta-glucosidases and the exo-inulinase encoding *inuE*. The encoded enzyme activities are mainly predicted to be active on terminal residues of plant polysaccharides, or their corresponding oligosaccharides. The regulatory mechanism for the up-regulation of genes during carbon starvation is not known completely. However, for the cellobiohydrolase encoding *cbhB*, its

neighboring gene encoding a GH5 cellulase and also the α-arabinofuranosidase encoding *abfB*, low level up-regulation was detected not only under carbon starvation conditions, but also in a *creA* deletion mutant grown on glucose (Delmas *et al.*, 2012). This suggests that carbon catabolite derepression via CreA is responsible for the observed up-regulation.

In the culture filtrate of carbon-starved cultures, proteins corresponding to such transcripts have also been found (Braaksma *et al.*, 2010; Nitsche *et al.*, 2012; van Munster *et al.*, 2014, 2015b). The concerted action of these enzymes on plant polysaccharides results in the release of products that include monomers as well as small linear and branched oligosaccharides. These reaction products include known inducers for lignocelluloses degrading enzymes such as arabinose (van Munster *et al.*, 2014). This supports a model where carbon starvation allows low-level up-regulation of a subset of genes, which encode enzymes that are thought to perform a scouting role. These enzymes are thought to release inducers from any substrates detected in the environment, either alone or in coordination with any constitutively expressed plant polysaccharide-degrading enzymes. These inducers may be taken up by the fungus and induce the transcription of a full set of plant carbohydrate degrading enzymes through dedicated transcription factors. Correspondingly, the early responses of *A. niger* and *N. crassa* to carbon starvation overlap with their early response to wheat straw and Avicel respectively (Coradetti *et al.*, 2012; van Munster *et al.*, 2014). This indicates that an initial phase of carbon starvation is required, during which scouting enzymes may generate inducers and dedicated transcription factors are allowed to respond to inducers. Subsequently, gene transcription is activated to generate the full set of enzymes required for the degradation of lignocellulose.

Co-regulation of conidiogenesis and cell wall hydrolase production

In the previous sections we have shown that carbon stress induces complex morphological, developmental and physiological changes both in *A. nidulans* and *A. niger*. Many of these stress responses (*e.g.* hyphal fragmentation and cell wall hydrolase production on one hand and conidiophore formation and survival on the other) seem to be contradictory. The diversity of the stress responses as well as their antagonistic nature suggests the existence of a complex regulatory network in which overlaps and intensive cross talks among the different signaling pathways are responsible for their proper orchestration in these multicellular organisms.

A key step of conidiation in *A. nidulans* is the activation of *brlA* (Adams *et al.*, 1998; Park and Yu, 2012). BrlA is a transcription factor which is responsible for the induction of AbaA which further induces a third transcription factor WetA (Adams *et al.*, 1998; Park and Yu, 2012). These three transcription factors (the so-called central regulatory pathway of conidiogenesis) coordinates all the conidiation-specific gene expressions during the development of conidiophores and the maturation of spores (Adams *et al.*, 1998; Park

and Yu, 2012). BrlA (but not AbaA or WetA) is necessary for induction of *engA* and *chiB* during carbon stress (Pócsi *et al.*, 2009; Szilágyi *et al.*, 2010a). Deletion of or loss-of-function mutations in *brlA* inhibits the accumulation of extracellular chitinase and ß-1,3-glucanase activities in the fermentation broth and hinder hyphal fragmentation in carbon starved cultures (Pócsi *et al.*, 2009; Szilágyi *et al.*, 2010a). It is assumed that in some cells induction of *brlA* is followed by the activation of the whole central regulatory pathway of conidiogenes resulting in conidiophore development and conidia formation. In other cells, BrlA does not induce *abaA* (as was observed in submerged carbon starved cultures (Pócsi *et al.*, 2009)) and these cells are responsible for cell wall hydrolase production. Identification of factors preventing the complete activation of the central regulatory pathway after *brlA* induction needs further investigations. Successful escape of hyphae into the air with the help of hydrophobins induced by BrlA is one possible regulator of conidiogenesis versus cell wall hydrolase production at least in surface cultures.

Two heterotrimeric G-protein mediated signaling pathways, the FadA and GanB pathways, negatively influence *brlA* expression (Yu, 2006; Park and Yu, 2012). Activation of the FadA(Gα)-SfaD(Gβ)-GpgA(Gγ) heterotrimeric G protein modulate cAMP-protein kinase A signaling, repress *brlA*, negatively regulate secondary metabolism and maintain vegetative growth (Shimizu and Keller, 2001; Yu, 2006; Park and Yu, 2012). Dissociation of the GanB(Gα)-SfaD(Gβ)-GpgA(Gγ) complex also mediates cAMP-protein kinase A signaling, negatively regulates the central pathway of conidiogenesis and positively regulates germination and stress responses (Yu, 2006; Park and Yu, 2012). FluG is proposed to be a key positive activator of *brlA* in *A. nidulans* (Lee and Adams, 1994). Its activity somehow belongs to the extracellular formation of a diorcinol – dehydroaustinol adduct which triggers the activation of the pathway (Lee and Adams, 1994; Rodríguez-Urra *et al.*, 2012). Accumulation of FluG beyond a certain level inhibits the negative regulation imposed by SfgA on Flb (including FlbA) proteins and as a consequence activates *brlA* (Park and Yu, 2012). FlbA is a key regulator of the FadA pathway (Adams *et al.*, 1998; Park and Yu, 2012; Pusztahelyi and Pócsi, 2013), therefore activation of FluG signaling not only induces *brlA* but also decreases the negative effect of FadA signaling on *brlA* transcription (Adams *et al.*, 1998; Park and Yu, 2012; Pusztahelyi and Pócsi, 2013). Activity of FadA and GanB G-protein signaling is proposed to depend on nutritional conditions therefore, carbon starvation decreases their activity, hinders vegetative growth and allows *brlA* to be induced. Carbon stress-dependent activation of *brlA* cannot be explained exclusively by the decreased activity of heterotrimeric G-protein pathways. Mutations in the genes involved in FadA or GanB signaling had only negligible effect on chitinase production and hyphal fragmentation in carbon stressed cultures (Molnár *et al.*, 2004, 2006). Since loss of function mutation in *fluG* (but not in *flbA*) inhibited the carbon starvation induced activation of *engA* and *chiB* (Molnár *et al.*, 2004; Emri *et al.*, 2005; Pócsi *et al.*, 2009; Szilágyi *et al.*, 2010a) a carbon stress-dependent behavior of FluG signaling is assumed.

Elements of the sexual development are also known to influence *brlA* expression, *e.g.* NsdD; an activator of sexual development (Lee *et al.*, 2014) and MpkB MAP kinase; an upstream regulator of NsdD (Kang *et al.*, 2013), both negatively regulate *brlA* expression. At the same time, BrlA has also been reported to represses *steA,* which encodes another activator of sexual development (Vallim *et al.*, 2000), indicating that BrlA has a central role in the developmental choices. Moreover, Kang *et al.,* (Kang *et al.*, 2013) showed the negative effect of MpkB not only on conidiogenesis but also on cell wall hydrolase production during long cultivation times which might also be controlled via BrlA.

Atoui et al. (Atoui *et al.*, 2010) demonstrated that appropriate glucose concentration can modify the light-dependent intracellular localization of VeA which is an important element of the light dependent regulation of conidiogenesis (including *brlA* and *fluG* transcription), sexual development, and secondary metabolism (Etxebeste *et al.*, 2010; Bayram and Braus, 2012; Park and Yu, 2012). This observation suggests a further possible way for carbon source-dependent modulation of differentiation, secondary metabolism and cell wall hydrolase production.

RlmA is also a negative regulator of *brlA* and represses *engA* and *chiB* transcription right after glucose depletion (Pusztahelyi and Pócsi, 2013). Since RlmA is supposed to be involved in cell wall stress response (Fujioka *et al.*, 2007), this connection between hydrolase production and cell wall stress may represent a negative feedback regulation of cell wall hydrolase production preventing overproduction of these enzymes which would be dangerous for the producer cells.

Regulation of cell wall hydrolase production depends not only on BrlA, but other regulatory mechanism including the carbon catabolite repressor CreA (Emri *et al.*, 2006). Positive regulatory effect of mono- and oligosaccharides released during cell wall hydrolysis is also emphasized (Szilágyi *et al.*, 2010a). The CreA-dependent repression of certain cell wall hydrolases (e.g. *chiB*, but not *engA*) in the presence of repressive carbon sources during carbon starvation may explain why cell wall hydrolase production depends strongly on carbon stress while in case of conidiogenesis nutritional stress is only one and probably not the most important signal for induction (Park and Yu, 2012).

Regulation of extracellular protease formation during carbon starvation

The induction of proteases has also been proposed as a key event in aging fungal cultures (White *et al.*, 2002). The production of extracellular proteases is a part of a general stress response aimed at scavenging new energy sources for survival in carbon starving cultures. The proteases may be involved in the degradation of extracellular proteins and/or the protein content of the cell wall. For industrial relevant fungi, such as *A. niger*, this is relevant in relation to the production of (recombinant) protein in this fungus. High protease activity

during starvation has a negative effect on the yield of product formation because of the unwanted proteolytic degradation of the product (Braaksma *et al.*, 2009). In *A. niger*, Nitsche et al., identified several proteases that are highly induced during starvation (Nitsche *et al.*, 2012). These proteases included *pepA* and *pepB* which encode the major extracellular proteases. Regulation of protease expression in *A. niger* involves the Zn(II)2Cys6 transcription factor PrtT (Punt *et al.*, 2008), but the expression of protease encoding genes is also under control of carbon catabolite repression (van den Hombergh *et al.*, 1997a). The expression of the PrtT transcription factor is also strongly induced upon starvation (Nitsche *et al.*, 2012). PrtT-homologues are only present in a few other *Aspergilli*. Interestingly, *A. nidulans* does not have a PrtT homolog and the protease regulation seems to be fundamentally different in comparison with *A. niger.*

The genome of *A. nidulans* contains several genes encoding putative extracellular proteases which are up-regulated by carbon starvation (Szilágyi *et al.*, 2013; Katz *et al.*, 2013). These include *prtA* and *pepJ* encoding the two major extracellular proteases of this fungus (Katz *et al.*, 1994; Emri *et al.*, 2009; Szilágyi *et al.*, 2011). The p53-like transcription factor XprG was identified as a major positively acting transcription factor of extracellular protease formation in carbon starved cultures of *A. nidulans* (Katz *et al.*, 1996, 2006). Microarray data demonstrates that XprG regulates (directly or indirectly) a large number of genes related to carbon stress including secondary metabolism, development or high-affinity glucose uptake (Katz *et al.*, 2013). It also activates hydrolases other than proteases: It proved to be important in the carbon starvation dependent activation of *engA* and *chiB* most likely via the activation of BrlA (Katz *et al.*, 2013), regulates the carbon stress dependent induction of *agdB* encoding a putative α-glucosidase (Katz *et al.*, 2013) and the production of acidic phosphatase in response to phosphate limitation (Katz *et al.*, 2006). These data suggest that XprG can be a key regulator of responses to starvation (Katz *et al.*, 2013). Little is known of the regulation of XprG. It is well documented that carbon starvation induces it (Szilágyi *et al.*, 2013; Katz *et al.*, 2013). HxkC, HxkD and AtmA are negative regulators of XprG (Bernardo *et al.*, 2007). HxkC and HxkD are regulatory type hexokinases; HxkC is associated with mitochondria, while HxkD is a nuclear protein (Bernardo *et al.*, 2007). AtmA is a serine/threonine protein kinase, an ortholog of mammalian ataxia-telangiectasia mutated (ATM) kinase which acts as a redox sensor controlling mitochondrial function (Ditch and Paull, 2012). Deletion of *atmA* in *A. nidulans* has very pleiotropic effects. AtmA is involved in the DNA damage response and required for polarized hyphal growth (Malavazi *et al.*, 2006, 2007, 2008). Among carbon starving conditions AtmA is necessary for normal transcription of genes involved in autophagy (Krohn *et al.*, 2014), it may contribute in the selective clearance of damaged mitochondria which prevent accumulation of reactive oxygen species (Krohn *et al.*, 2014), genetically interacts with XprG inhibiting XprG functions (Krohn *et al.*, 2014) and it is necessary for induction of certain carbohydrate active enzymes (CAZy) but acts independently to CreA (Brown *et al.*, 2013). CreA also negatively regulates

extracellular protease formation during carbon starvation (Emri *et al.*, 2006; Katz *et al.*, 2008). It is suggested that CreA regulates *xprG* expression or interacts directly with the XprG protein (Katz *et al.*, 2008).

FluG is necessary for extracellular protease formation in carbon starved cultures; no extracellular protease activity was detected in a loss-of-function *fluG* mutant (Emri *et al.*, 2005; Szilágyi *et al.*, 2011). BrlA is also a positive regulator of protease formation, however its action depended on the experimental design: when growing cultures were transferred into carbon source free media, inactivation of *brlA* (or *fluG*) decreased markedly the extracellular protease activity. In contrast, when carbon starvation was induced simply by long time cultivation, *brlA* deletion had only a minor effect, while in the *fluG* loss-of-function mutant no proteinase activity was detected (Szilágyi *et al.*, 2011). These data suggest that FluG may act not only by regulating *brlA* transcription. Since mutations in FadA and GanB signaling have modulatory effect on protease production, it is possible that FluG, at least in parts, regulates the protease genes via FlbA and heterotrimeric G protein signaling (Molnár *et al.*, 2004, 2006). A connection between FluG and XprG is also possible but this hypothesis needs further investigation.

Although the presence of extracellular proteins is not needed for the induction of protease genes in carbon starving cultures (Katz *et al.*, 2008), the substrate of these enzymes can influence indirectly their production. High amount of organic nitrogen sources can repress the formation of proteases (as well as cell wall hydrolases) which depends on the MeaB transcription factor (Szilágyi *et al.*, 2010b; Spitzmüller *et al.*, 2015). The alkalization of pH by the release of ammonia during carbon starvation also supports the role of protein degradation in carbon-starving cultures (Emri *et al.*, 2004). It is worth mentioning that PrtA, PepJ, EngA and ChiB preserves their activity in alkaline conditions that shows that the fungus is adapted to the alkaline environment during carbon starvation (Katz *et al.*, 1994; Erdei *et al.*, 2008; Emri *et al.*, 2009; Szilágyi *et al.*, 2010a). Moreover alkaline pH is important in the up-regulation of extracellular protease and secondary metabolite production partly by the activity of PacC (Brakhage *et al.*, 2004; Szilágyi *et al.*, 2011).

Conclusions and future trends

The lack of available carbon source can be life-threatening for fungi as maintenance of crucial cellular processes such as ion homeostasis requires ATP. A lack of ATP could consequently result in rapid cell death. Endogenous recycling (autolysis) and extracellular hydrolysis are ways to liberate energy-rich building blocks to generate ATP under carbon starvation conditions. In filamentous fungi, the energy obtained from recycling processes is not only used for maintenance, but also used to fuel asexual development (Figure 1).

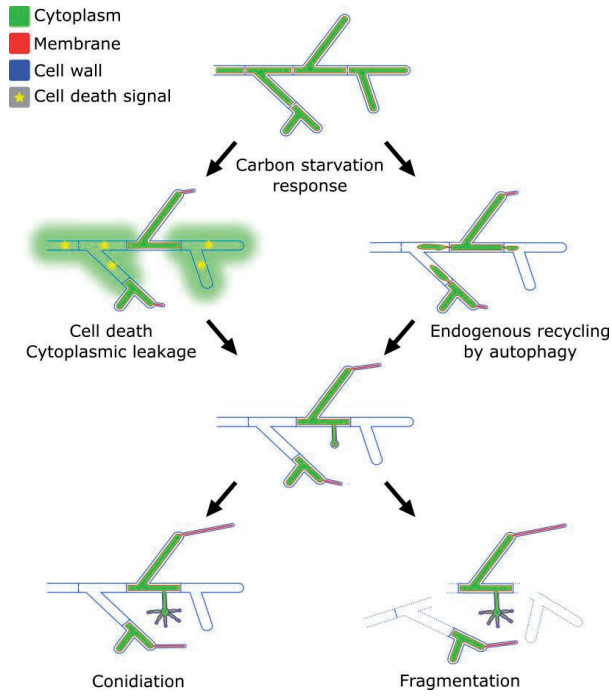

Figure 1 | Model for carbon starvation responses in filamentous fungi. Schematic representation of morphological starvation responses during submerged carbon starvation. Upon carbon starvation, empty hyphal compartments emerge as a consequence of cell death and/or endogenous recycling of neighbouring compartments by autophagy. Cell death of compartments results in the leakage of cytoplasm into the culture broth, whereas this is prevented when cellular contents are endogenously recycled by autophagic processes. Liberated carbon sources are reused to fuel the outgrowth of thin hyphae. Towards late phases of carbon starvation, only a few surviving compartments are left in the culture broth, often bearing reproductive structures and elongated thin hyphae. Depending on the species and the cultivation conditions, the largely empty mycelial network either remains intact or is being fragmented by the activity of glycosyl hydrolases weakening the cell walls (amended and adopted from Nitsche *et al.* 2012).

The formation of conidiospores might assist in the propagation of the fungal species as conidia are much more resistant to unfavourable conditions (including starvation) and allow spreading of the species. Many authors have referred to carbon starvation response as autolysis (White *et al.*, 2002). However, autolysis suggests a rather uncontrolled and destructive process. In fact, the carbon starvation response seems to be a sophisticated manner to deal with the limitation and to search for alternative sources and to maintain viability of the species via reproduction. Several studies have indicated that the process of autophagy plays an important role in the starvation response. Preliminary studies have shown that autophagy is important to delay cell death and as such allows adaptation of the fungus to the carbon starvation condition (Nitsche *et al.*, 2013). However, the carbon

starvation-related phenotypes of autophagy defective mutants are subtle, indicating that additional processes or pathway are also contributing.

An important challenge for future research will be to understand the molecular mechanisms driving the multi-cellular nature of a carbon starved mycelium. When exposed to carbon starvation, the fungal mycelium starts to differentiate and initially thin hyphae are formed (Figure 1). These hyphae might be considered as scouting hyphae and might produce new enzymes with interesting biochemical properties to look for alternative carbon sources. During starvation, some compartments in the mycelium are protected from cell death, while other compartments seem to be sacrificed. It is currently unknown whether the cell content of empty compartments leaks into the medium or that neighbouring compartments can directly take up the cell content via the septal pore. Further understanding the destination and function of different cell types (thin hyphae, sacrificed compartment, surviving hyphae and hyphae that form conidiospores) may be assisted by recent tool developments for single cell/compartment transcriptomics and by identifying molecular signals and proteins related to programmed cell death or cell surviving. Identification of these molecular signals to promote fungal cell death or protect cells from dying in response to starvation conditions are also very interesting targets for antifungal drug development.

Acknowledgements

The authors would like to thank Thomas Jørgensen and Benjamin Nitsche for sharing unpublished results and helpful discussions. We thank Paul Daly for proof-reading the manuscript. AB and AR were supported by grants of the SenterNovem IOP Genomics project (IGE07008) and by the Kluyver Centre for Genomics of Industrial Fermentation and the Netherlands Consortium for Systems Biology, which are part of the Netherlands Genomics Initiative/Netherlands Organization for Scientific Research (KC4.3). JvM was supported by grant IGE07008 from the Dutch SenterNovem IOP Genomics project and by grant BB/G01616X/1 from the UK Biotechnology and Biological Sciences Research Council (BBSRC) Sustainable Bioenergy Centre (BSBEC) under the Lignocellulosic Conversion to Ethanol (LACE) program. IP and TE were supported by the Hungarian Scientific Research Fund (OTKA K100464, K112181) and by the SROP-4.2.2.B-15/1/KONV-2015-0001 project. The project has been supported by the European Union, co-financed by the European Social Fund.