Discovery and exploitation of the transcriptional regulatory system of pectinases in Aspergillus niger

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CHAPTER 1

Aim and outline of the thesis

Besides animals and plants, fungi represent one of the eukaryotic kingdoms of life. The fungal kingdom is thought to consist of over 1.5 million species and harbors a very diverse group of microorganisms varying from single celled yeasts to multicellular filamentous fungi (molds) and mushroom fungi (Stajich et al., 2009). Example species to each of these groups include Baker's Yeast (*Saccharomyces cerevisiae*), *Aspergillus* and *Penicillium* species, and edible mushrooms like the *Agaricus bisporus*, respectively. *Aspergillus niger* is a filamentous fungus that forms colonies on solid substrates. Depletion of the carbon source causes differentiation of the vegetative mycelium and induces the formation of spore-containing structures called conidiophores. The asexually derived spores, called conidia, can germinate and extend to form hyphae (Figure 1). In liquid medium, *A. niger* shows either dispersed or pelleted growth (Krijgsheld et al., 2013). In our daily life, we often encounter the black conidia of *A. niger* on the outside skin of onions (Gherbawy et al., 2015).

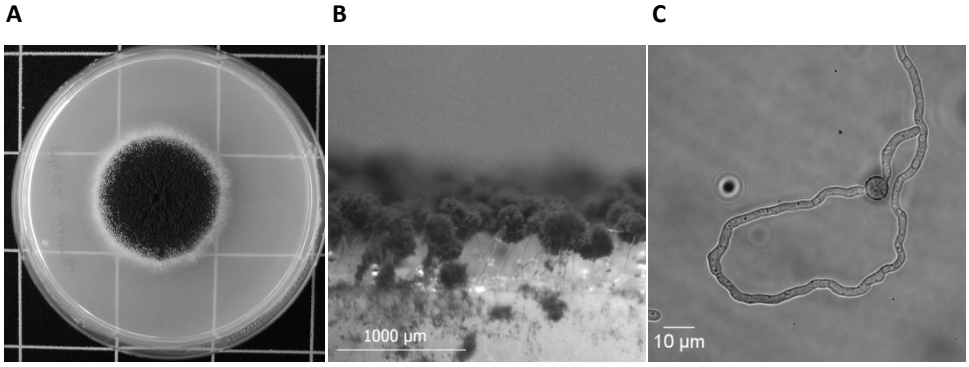

Figure 1. Pictures of *A. niger* strain N402 (or its derivatives). **(A)** Top view of a sporulating colony formed on solid agar plate. **(B)** Side view of conidiophores formed on solid agar plate. Chains of conidia are seen on top of the stalk. **(C)** Hyphae emerging from a single conidium in liquid medium.

A. niger is a saprobe, growing on decaying plant matter in nature. It secretes enzymes to degrade the large plant polysaccharides, and takes in and catabolizes the resulting di- or monomeric sugars to generate energy for life. These di- or monomeric sugars (or derivatives thereof) act as inducer molecules resulting in the activated and abundant expression the genes encoding plant polysaccharide degrading enzymes via substrate-specific transcription factors (TFs) (Culleton et al., 2013). These genes are often transcriptionally down-regulated via carbon catabolite repression (CCR) when a more energetically favorable carbon source, such as glucose, compared to plant polysaccharides is available as substrate (Adnan et al., 2018).

Being a generally regarded as a safe (GRAS) production organism, *A. niger* is also utilized industrially to produce large amounts of plant biomass degrading enzymes. These enzymes are subsequently used for food applications or for the hydrolysis of plant biomass to produce bioethanol and high-value biochemicals (Gupta et al., 2016; Kowalczyk et al., 2014). The requirement for the presence of specific inducers for the activated expression of particular enzyme networks hampers the use of cheap and sustainable feedstock for fungal fermentation. Approaches to modulate transcriptional regulation in order to rationally design fungal strains with increased or constitutive (i.e. inducer-independent) production of plant biomass degrading enzymes are reviewed in **Chapter 2**. Overexpression and constitutive activation of transcriptional activators, accumulation of inducers, and deletion of substrate-specific repressors or wide domain repressors involved in CCR are the main approaches discussed.

Plant biomass degrading enzymes are named based on the plant polysaccharide they act on. For example, enzymes that degrade the complex heteropolysaccharide pectin are called pectinases. Pectin is mainly made of polygalacturonic acid which is a linear polymer of D-galacturonic acid (GA) (Caffall and Mohnen, 2009). *A. niger* can secrete a large number of enzymes acting on pectin (Martens-Uzunova and Schaap, 2009; de Vries et al., 2017). The objective of the research described in this thesis is to understand the transcriptional regulation of pectin degrading enzymes in *A. niger* and to use this knowledge to construct strains constitutively expressing the genes encoding pectinases. The transcription of the genes encoding pectinases is induced when grown on GA and requires a common GA-responsive element in the promoter regions of these genes (Martens-Uzunova and Schaap, 2008; Niu et al., 2015). These observations indicated that there is a GA-responsive TF coordinating the expression of the genes encoding pectinases. In **Chapter 3**, the GA-responsive transcriptional activator GaaR in *A. niger* is identified by homology to BcGaaR in *Botrytis cinerea* (Zhang et al., 2016). In **Chapter 5**, the physiological inducer of GA-responsive genes is found to be the GA catabolic pathway intermediate 2-keto-3-deoxy-L-galactonate.

A forward genetic screen was conducted in search for mutants that constitutively express pectinases. As described in **Chapter 4**, this search led to the identification of the repressor protein GaaX. The same screen also revealed that the W361R mutation in GaaR results in constitutive expression of the genes encoding pectinases (**Chapter 7**). In **Chapter 7**, we also show the use of the CRISPR-Cas9 technology to construct strains expressing point mutated or eGFP-tagged versions of *gaaR* from the endogenous locus. Furthermore, **Chapter 6** describes that overexpression of *gaaR* also brings about inducer-independent expression of the genes encoding pectinases in *A. niger*.

In this thesis, the key players in the transcriptional regulatory system of pectinases in *A. niger*, such as the transcriptional activator GaaR, the repressor GaaX and the physiological inducer 2-keto-3-deoxy-L-galactonate, are discovered. Several approaches to exploit this system for increased or constitutive expression of the genes encoding pectinases are also presented, such as overexpression and constitutive activation of *gaaR*, accumulation of 2-keto-3-deoxy-L-galactonate, and deletion of *gaaX* or *creA*, the main transcriptional repressor involved in CCR. An overall conclusion and outlook to the future is given in **Chapter 8**.

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