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

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CHAPTER 8

Conclusions and outlook to the future

Pectinases are enzymes naturally secreted by *Aspergillus niger* to degrade the plant cell wall polysaccharide pectin (de Vries et al., 2017). These enzymes are also used in several applications in the food and feed industry, and in the industrial hydrolysis of plant biomass for subsequent production of biofuel and high-value biochemicals. In order to produce large amounts of pectinases to be used industrially, *A. niger* has been used as a work horse (Kashyap et al., 2001; Edwards and Doran-Peterson, 2012; Khan et al., 2013; Toushik et al., 2017). The expression of the genes encoding pectinases is induced in response to the presence of D-galacturonic acid (GA), the main sugar acid in pectin (Martens-Uzunova and Schaap, 2008). The requirement of the presence of the inducer in the fungal cultivation medium hampers the use of cheap and sustainable feedstock. Therefore, it is important to investigate the transcriptional mechanism that regulates the expression of the genes encoding pectinases in *A. niger* and find out ways to constitutively (i.e. independently of the inducer) produce pectinases.

The promoter element GARE required for the transcriptional activation of pectinases was known in both *A. niger* and *Botrytis cinerea*, which allowed the identification of the first fungal GA-responsive Zn_2Cys_6 type transcription factor BcGaaR in *B. cinerea* via the yeast-one-hybrid technique (Niu et al., 2015; Zhang et al., 2016). *A. niger* GaaR was identified via its homology to BcGaaR, and was found to be required for growth on GA and polygalacturonic acid, which is the most abundant pectin substructure. GaaR was found to be essential for the GA-responsive expression of the genes encoding several pectinases, (putative) GA-transporters and GA catabolic pathway enzymes (Chapter 3).

A promoter-reporter strain containing the promoter of the pectinase gene *pgaX* upstream of the reporter gene *amdS* (Niu et al., 2015) was utilized in a forward genetic screen to isolate mutants with constitutive expression of the genes encoding pectinases. One particular gene was found to be mutated in several mutants. This gene was called the repressor of GA utilization, *gaaX*. *gaaX* and *gaaR* are located side-by-side on the chromosome of *A. niger*, similar to the activator-repressor modules *qutA-qutR* and *qa-1F-qa-1S* involved in the utilization of quinic acid in *Aspergillus nidulans and Neurospora crassa*, respectively. In all systems, deletion of the repressor results in constitutive expression of target genes which requires the presence of the corresponding Zn_2Cys_6 type transcriptional activator (Lamb et al., 1996; Grant et al., 1988; Giles et al., 1985, Chapter 4). Moreover, *A. nidulans* strains expressing multiple copies of *qutA* showed constitutive expression of the genes involved in quinic acid utilization (Lamb et al., 1996). Similarly, overexpression of *gaaR* in *A. niger* resulted in constitutive expression of the genes involved in the breakdown of pectin and utilization of GA (Chapter 6). These results indicate that the activity of GaaR is inhibited by GaaX under non-inducing conditions via protein-protein interaction, and that GA-responsive gene expression only requires the presence of active GaaR relieved/escaped from GaaX inhibition. This suggests that the

inducer binds to GaaX resulting in a GaaX-unbound and active form of GaaR under inducing conditions. Because overexpression of the activators involved in GA or quinic acid utilization have similar effects, it is likely that activation mechanism of both QutA/QutR and GaaR/GaaX are conserved and comparable. Whether overexpression of *gaaX* results in a superrespressed phenotype like overexpression of *qutR* does (Lamb et al., 1996) requires further investigation.

A. niger catabolizes GA into pyruvate and glycerol via a four step catabolic pathway involving four enzymes (GaaA-GaaD) (Martens-Uzunova and Schaap, 2008). Comparative analysis of GA catabolic pathway deletion mutants (*ΔgaaA*, *ΔgaaB*, *ΔgaaC*, and *ΔgaaD*) revealed that 2-keto-3-deoxy-L-galactonate is the physiological inducer of the GA-responsive genes. *ΔgaaB* mutant accumulated the preceding metabolite L-galactonate, could not grow on GA and lacked the induced expression of GA-responsive genes, indicating that it cannot produce 2-keto-3-deoxy-L-galactonate. 2-keto-3-deoxy-L-galactonate accumulated in the *ΔgaaC* mutant that is not able to catabolize it further and could not grow on GA. This accumulation resulted in a significant increase in the expression of the genes encoding pectinases (Chapter 5). In addition, 2-keto-3-deoxy-L-galactonate was found to be more potent than GA in inducing the expression of pectinase gene *pgaX* (Chapter 7). This latter observation also supports the idea that 2-keto-3-deoxy-L-galactonate is the physiological inducer.

GaaX of *A. niger*, QutR of *A. nidu*lans and qa-1S of *N. crassa* are all multidomain proteins with sequence similarity to the three C-terminal domains of AROM, a large pentafunctional protein involved in the shikimate pathway. This indicates that these repressors share a common evolutionary origin. The last three domains of the AROM protein encode the shikimate kinase, 3-dehydroquinate dehydratase and shikimate dehydrogenase enzymes (Figure 1) (Lamb et al., 1996). In *A. nidulans*, dehydroquinic acid can be converted to dehydroshikimic acid via different 3-dehydroquinate dehydratase isoenzymes, AROM 3-dehydroquinate dehydratase or QutE, in the biosynthetic shikimate pathway or the quinic acid catabolic pathway, respectively (Hawkins et al., 1993). In *N. crassa*, dehydroquinic acid was suggested to be the physiological inducer of the quinic acid catabolic pathway genes, as mutants that accumulate or cannot produce dehydroquinic acid showed induced or diminished expression of these genes, respectively (Giles et al., 1967). Huiet and Giles identified two missense mutations in non-inducible *N. crassa* strains in the qa-1S domain showing homology with the AROM shikimate dehydrogenase, indicating that this domain might interact with the inducer (Huiet and Giles, 1986). As the outcome of these experiments are not in full agreement with each other, direct interaction studies are required to determine which part (or parts) of the repressor interacts with the inducer. Hawkins et al. have proposed that the QutR domain homologous to the AROM 3-dehydroquinate dehydratase can recognize and bind to dehydroquinic acid produced from quinic acid, but does not react on it enzymatically in *A. nidulans* (Hawkins et al., 1993). The GA catabolic pathway enzyme GaaC acting on the inducer 2-keto-3-deoxy-L-galactonate in *A. niger* is an aldolase, thus not a 3-dehydroquinate dehydratase. However, both the aldolase domain of GaaC and the 3-dehydroquinate dehydratase domain of GaaX belong to the same Aldolase_Class I_superfamily (CD-search, https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Marchler-Bauer et al., 2015). These findings and propositions suggest that 2-keto-3-

deoxy-L-galactonate binds to the GaaX domain homologous to the AROM 3-dehydroquinate dehydratase, and frees GaaR under inducing conditions.

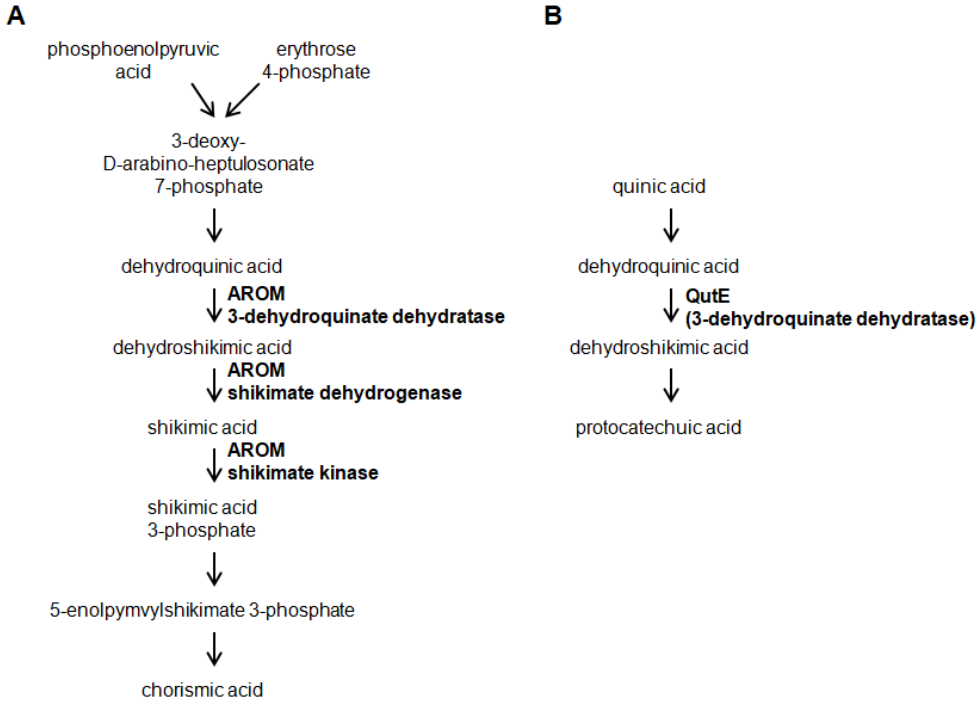

Figure 1. Comparison of the biosynthetic shikimate pathway **(A)** and the quinic acid catabolic pathway **(B)** in *A. nidulans*. Dehydroquinic acid is converted to dehydroshikimic acid in both pathways via two distinct classes of 3-dehydroquinate dehydratases (Hawkins et al., 1993).

Mutants with constitutive expression of the genes encoding pectinases obtained via the forward genetic screen contained loss-of-function mutations, including nonsense, frameshift or missense mutations, in all three domains of *gaaX* (Chapter 7). As it is not possible to predict whether the missense mutations result in a general loss of function or in a loss of the suggested interaction between the activator and repressor, it is difficult to predict the domain of GaaX interacting with GaaR. In *A. nidulans*, the 88 N-terminal amino acids of QutR were found to be sufficient to interact with QutA (Watts et al., 2002), and deletion of the QutR domains homologous to the AROM shikimate kinase, 3-dehydroquinate dehydratase or shikimate dehydrogenase still resulted in a functional repressor (Levett et al., 2000). This indicates that the mechanism of interaction of GaaR and GaaX might be different than that of QutA and QutR. The same forward genetic screen in *A. niger* also yielded to one mutant with the W361R mutation in GaaR. This mutation in the fungal-specific transcription factor domain of GaaR was found to be responsible for the constitutive expression of the genes encoding pectinases, and possibly disrupts the interaction between GaaX and GaaR (Chapter 7). Watts et al. reported that in *A. nidulans* QutR binds to QutA amino acids 449-468 (Watts et al., 2002), which are

located in the fungal-specific transcription factor domain of QutA. This comparison suggests that the fungal-specific transcription factor domains of QutA and GaaR are important for the interaction with their corresponding repressors. Future experiments, such as surface plasmon resonance measurements and structure elucidation of GaaR and GaaX proteins via crystallography would reveal the nature of interactions between GaaR, GaaX and 2-keto-3-deoxy-L-galactonate.

The subcellular localization of GaaX and GaaR was also studied in this thesis. GaaX is transcriptionally induced under inducing conditions and is localized in both the cytosol and the nucleus roughly at the same level (Chapter 4; Chapter 6). RNA seq analysis has shown that the expression of *gaaR* is not dramatically induced under inducing conditions (Chapter 3; Chapter 4). The presence of both N- or C-terminally eGFP-tagged GaaR in a *ΔgaaR* strain results in the complementation of growth on GA. However, the strain expressing the N-terminally tagged version (*eGFP-gaaR*) showed a reduced growth on GA compared to the reference strain, indicating that the N-terminal eGFP-tag might affect GaaR function. Both eGFP-GaaR and GaaR-eGFP localize mainly in the nucleus under inducing conditions, where they activate the transcription of GA-responsive genes. However, while eGFP-GaaR is localized mainly in nucleus also under non-inducing conditions, GaaR-eGFP is not. This indicates that C-terminal eGFP-tagging of GaaR restrains its nuclear accumulation under non-inducing conditions. Supporting evidence that GaaR-eGFP is restrained to accumulate in the nucleus under non-inducing conditions came from the observation that C-terminal eGFP-tagging of the constitutively active $GaaR^{W361}$ abolishes inducer-independent expression of the genes encoding pectinases, and that $GaaR^{W361}$-eGFP also does not accumulate in the nucleus under non-inducing conditions (Chapter 6; Chapter 7). The nuclear localization of the eGFP-GaaR under non-inducing conditions indicates that GaaX exhibits its repressing activity also in the nucleus. Use of more advanced techniques, such as bimolecular fluorescence complementation, would help to investigate the subcellular localization of the interaction of GaaX and GaaR.

To sum up, an important milestone in our current understanding of the transcriptional regulation of pectinases in *A. niger* was the identification and functional characterization of the transcriptional activator GaaR. After the identification of GaaR further discovery of the elements constituting the transcriptional regulatory system of pectinases, and the usage of this information to construct strains with improved pectinase production proceeded hand in hand (Figure 2). During the investigation of the physiological inducer of GA-responsive genes, the *ΔgaaC* mutant was constructed, which shows hyper-induced expression of the genes encoding pectinases on GA. The use of the *ΔgaaC* mutant to increase pectinase production has not been fully exploited yet. It is reasonable to suggest that cultivation of the *ΔgaaC* strain on GA and an additional C-source, could lead to further increased expression of the genes encoding pectinases. It is important to keep in mind that the GA-responsive genes are sensitive to CCR. Therefore, the *ΔgaaC* mutation should be combined with deletion of *creA.* The repressor of GA-utilization GaaX was identified as a result of a screen conducted to isolate mutants with constitutive expression of the genes encoding pectinases. Achievement of constitutive pectinase production via overexpression of *gaaR* provided further evidence that the presence of the inducer is not obligatory for GaaR activity, and that GaaX-unbound GaaR can activate the expression of GA-responsive genes. Finally, identification of the constitutively active *gaaR*

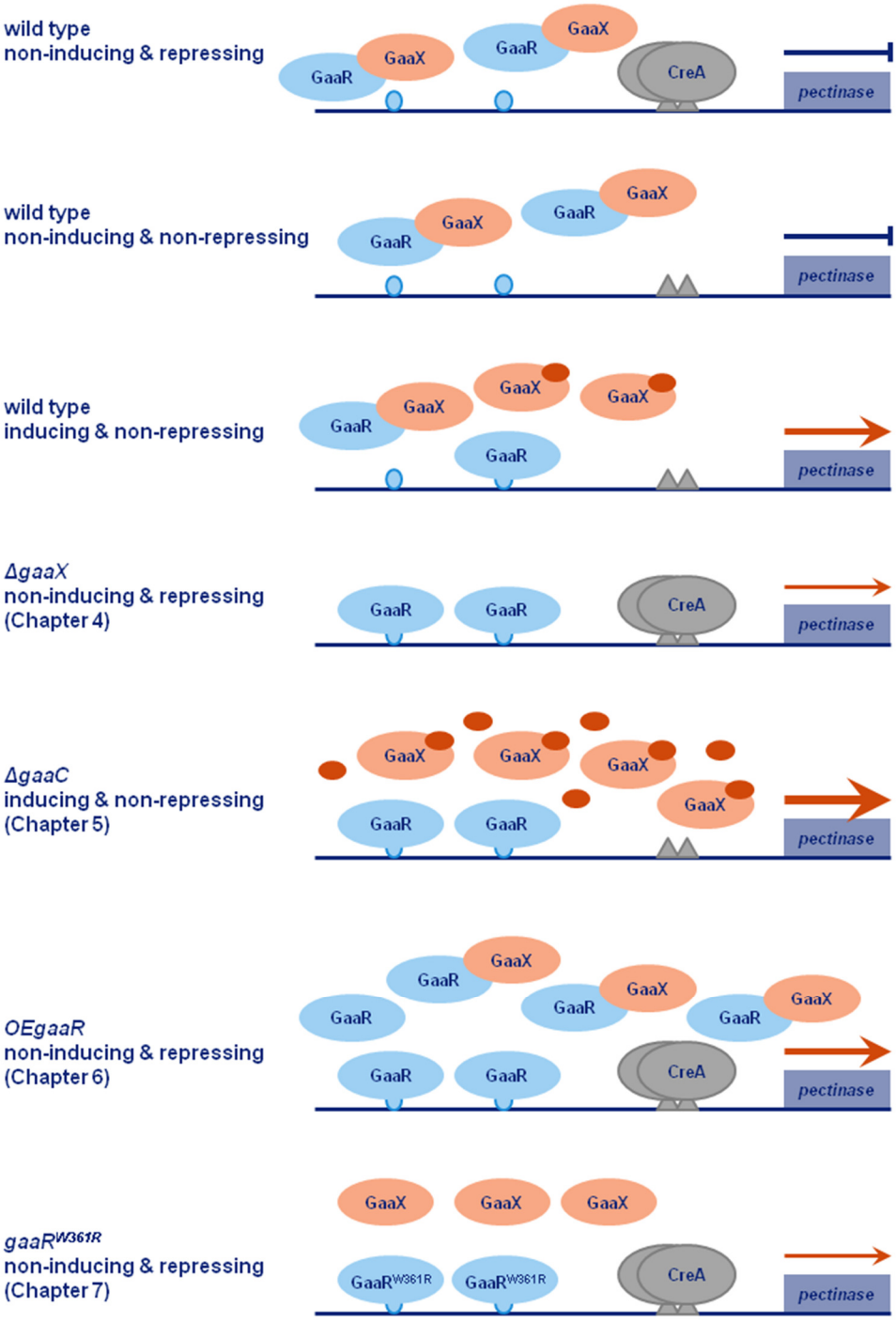
wild type
non-inducing & repressing
GaaX
GaaR
CreA
pectinase
wild type
non-inducing & non-repressing
wild type
inducing & non-repressing
ΔgaaX
non-inducing & repressing
(Chapter 4)
ΔgaaC
inducing & non-repressing
(Chapter 5)
OEgaaR
non-inducing & repressing
(Chapter 6)
gaaR^W361R
non-inducing & repressing
(Chapter 7)
GaaR^W361R

Figure 2. Current model describing the transcriptional regulation and approaches leading to increased or inducer-independent expression of most of the genes encoding pectinases in *A. niger*. Note that the amounts of the GaaR, GaaX and CreA proteins and the inducer (the small orange circle) in this scheme may not represent the real (relative) levels. Transcription factor binding sites are indicated by the blue circle and the gray triangle, and represent the binding sites of GaaR and CreA, respectively. Relative expression level of the genes encoding pectinases under each condition is roughly indicated by the thickness of the orange arrow. Deletion of CreA further increases the level of pectinase gene expression in the *ΔgaaX* and *OEgaaR* strains under repressing conditions (Chapter 4; Chapter 6).

allele which contains the W361R mutation made it possible to suggest that W361 is important for the interaction of GaaR and GaaX while it does not affect the transcriptional activity of GaaR. In this thesis, we also show the use of the state of the art CRISPR-Cas9 technology to introduce point mutated or eGFP-tagged versions of GaaR at the endogenous *gaaR* locus (Chapter 7). The main repressor CreA involved in carbon catabolite repression was found to repress the expression of different genes encoding pectinases at different degrees. Deletion of *creA* was not enough for constitutive expression of the genes encoding pectinases but further enhanced the pectinase production capacity of strains lacking *gaaX* or overexpressing *gaaR* (Chapter 4; Chapter 6). Combining the above mentioned techniques, an industrial *A. niger* strain overexpressing the constitutively active $gaaR^{W361R}$ and lacking *gaaX* and *creA* can be constructed for boosted and constitutive expression of the genes encoding pectinases.

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