Transcriptional control of pectin degrading enzymes in Aspergillus niger

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Chapter 7

General discussion

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With the rapid advances of several high throughput technologies, a variety of functional genomics approaches have been developed and became available to study transcriptional regulation in *A. niger*. These approaches include: (i) those for genetic characterization of mutant genes from strains selected in forward genetic screens (e.g. via genetic linkage based methods in combination with next generation sequencing based methods), (ii) those for studying gene functions (e.g. efficient gene deletion approaches, CRISPR-Cas9 genome modification approaches), (iii) those for surveying transcriptomics (RNA-seq) and (iv) proteomics (LC-MS_MS) approaches data, and (v) those for identification of transcription factor binding sites (CHIP-seq). In the last twenty years these approaches have led to the identification of several transcription factors involved in the plant biomass degradation, both in *Neurospora crassa* (Sun *et al.* 2012; Xiong *et al.* 2017) and several *Aspergilli*. Examples of *Aspergillus* transcription factor involved in plant cell wall biomass deconstruction in include: XlnR responsible for xylan degradation and xylose utilization (Van Peij *et al.* 1998; Klaubauf *et al.* 2014); RhaR which is responsible for L- rhamnose utilization (Gruben et al., 2014); GalX/GalR which regulates the D-galactose utilization system in *A. nidulans* (Kowalczyk et al., 2015); and Clr-2/ClrB/ManR are involved in the cellulose utilization (Craig *et al.* 2015; Raulo *et al.* 2016). More information on additional transcription factors in *A. niger* as well as in other filamentous fungi can be found in a recent review by (Benocci *et al.* 2017).

As an important industrial enzyme producer, *A. niger* can secrete a variety of enzymes to degrade plant polysaccharides, such as starch, inulin, cellulose, hemicellulose (xylan and arabinan), galactomannan and pectin (De Vries and Visser 2001). The expression of the enzyme-encoding genes is subjected to tight regulatory mechanisms that involve an interplay between both inducing and repressing transcriptional factors (TFs) (Niu *et al.* 2015; Benocci *et al.* 2017). In the introductionary Chapter of my Thesis, I have focused attention to XlnR, which serves as a transcriptional activator of xylan and cellulose degrading enzymes (Van Peij *et al.* 1998), AmyR which is the transcriptional activator of starch degrading enzymes (Petersen *et al.* 1999), and CreA which acts as a carbon catabolite transcriptional repressor of enzymes involved in degradation of different carbon sources, including starch, xylan and pectin (De Vries *et al.* 2002; Tamayo *et al.* 2008; Ichinose *et al.* 2014). Despite all new and available tools and technologies, TFs related to polygalacturonic acids (PGA), the main substructure of pectin, were not identified when the research described in this thesis started.

To identify TFs involved in the regulation of pectin degradation enzymes, both targeted and non-targeted approaches were used for screening. For the targeted approach, a large set of 240 transcription factor mutants was screened for reduced growth on pectinolytic substrates such as D-galacturonic acid (GA), polygalacturonic acid (PGA), and pectins (apple pectin and sugar beet pectin). In the non-targeted approach, we designed a forward genetic screen to isolate mutants with constitutive expression of enzymes related to PGA degradation. In **Chapter 2**, we describe an approach which allows construction of gene deletion strains with high efficiency. This approach combines transformation of split marker fragments with non-homologous end joining (NHEJ) mutants. Typically, a gene deletion construct comprises a selection marker flanked by 5' and 3' sequences of the gene of interest (GOI). In the split marker approach, the gene deletion construct is split into two parts, each containing a flanking region and a truncated form of the selection marker (Fairhead *et al.* 1996; Nielsen *et al.* 2006; Goswami 2012). This approach involves only two rounds of PCR and does not require subcloning (Goswami 2012), thus allowing for rapid assembly of deletion constructs. Moreover, as only transformants in which the two overlapping marker fragments are successfully recombined can grow in selective medium, this approach increases the efficiency of obtaining gene deletion mutants.

After the gene deletion construct is introduced into the fungi by PEG-mediated protoplast transformation, targeted gene deletion is achieved by homologous recombination (HR) between the DNA sequences flanking the selection marker and target gene. However, as DNA recombination in filamentous fungi preferably happens via the NHEJ pathway, DNA will integrate randomly resulting in low HR frequencies and hence low efficiencies in getting targeted gene deletion mutants. Therefore, NHEJ mutants are used to improve the frequencies of HR. Combining the split marker approach with NHEJ mutants has proven to be able to greatly reduce time and effort for generation of gene deletion mutants. In principle, this approach is suitable to generate whole genome gene deletion collections. In our research, this method has been used in a pilot experiment to construct 240 TF mutants in *A. niger* (Mark Arentshorst, unpublished results). Whereas *A. niger* contains over 700 transcription factor genes, only a subset of TF mutants was selected for this pilot. The collection of 240 TF mutants was screened for mutants with reduced growth on pectinolytic substrate. Unfortunately this effort did not lead to successful identification of TFs involved in pectin degradation. However, we showed that the approach is in principle suitable to generate whole

genome knockout collections and has provided an interesting collection of TF mutants which can be screened to identify TF related to other regulatory processes.

As targeted deletion of the 240 transcription factors failed to identify a specific transcription factor involved in pectin degradation, one possibility is that the candidate TF is among the remaining 460 TFs present in the genome that have not been disrupted. Alternatively, since pectin is a complex polysaccharide composed of various different monosaccharides, several transcription factors with partially overlapping functions could be involved in pectin degradation and that single deletion of one TF was not sufficient to reduce growth sufficiently to be detected by plate growth assays. Thus, deletion of multiple transcription factors could be required to generate a pectin degradation deficient mutant. In **Chapter 3,** we construct a set of auxotrophic strains which allow an efficient way to create strains with multiple gene deletions. Four well–selectable auxotrophic markers (*pyrG*, *nicB*, *argB*, and *adeA*) were used to make a quadruple auxotrophic strain. Except for the *pyrG* marker, the other markers were introduced by targeted deletion. During this process, the *pyrG* marker was reused as a selection marker for deletion of other markers. Since *pyrG* marker is a bidirectional marker, mutations or loss of *pyrG* prevents the conversion of 5'-fluoroorotic acid (5'FOA) into a toxic compound, making a fungal strain resistant to 5'FOA-plates. So a $pyrG^{+}$ strain can be cured by growing on media containing 5'FOA. By introducing direct repeats around the *pyrG* selection marker, the *pyrG* gene is efficiently looped out during 5'FOA selection. In addition, two genes involved in colour pigmentation (*fwnA* and *olvA*) were combined with auxotrophic markers to construct isogenic strains which can be used to construct diploids for the isolation of haploid segregants from a diploid using the parasexual cycle.

Since identification of transcription factors involved in pectin degradation using the targeted approach by constructing gene deletion mutants in selected transcription factors was not successful, a non-targeted approach was chosen. To identify mutants affected in the regulation of pectinolytic genes, a forward genetic screen to isolate mutants with constitutive expression of pectinases was designed. In **Chapter 4**, we selected five genes that were specifically induced by galacturonic acid (GA) based on available genome-wide expression profiles, to construct promoter-reporter strains for studying gene expression related to PGA degradation. These genes consist of three exo-polygalacturonases (*pgaX*, *pgxB* and *pgxC*), a GA transporter (*gatA*), and an intracellular enzyme involved in GA metabolism (*gaaB*), each containing a putative conserved galacturonic acid-responsive element (GARE; 5'-

TCCNCCAAT-3') in their promoter regions. In **Chapter 4**, we show experimentally that the GARE is indeed required for GA-mediated induction by promoter deletion studies and site-directed mutagenesis.

To construct the promoter-reporter strains, the promoter regions of the five genes were selected and fused to the *amdS* reporter gene. Because expression of the *amdS* gene renders the strain the ability to utilize acetamide as the nitrogen source, the ability to grow on acetamide can be used as a direct measurement of promoter activity. Growth analysis of the reporter strains indicated that the promoters of four genes (*pgaX*, *pgxB*, *pgxC*, and *gatA*) were specifically induced by GA. It also showed the activation of these four promoters is under the control of carbon catabolite repression by glucose in a glucose concentration-dependent way. The major transcriptional repressor is CreA for carbon catabolite repression in *Aspergillus*. Except in the case of *pgxC*, deletion of *creA* in the other promoter-reporter strains abolished glucose repression. Deletion of *creA* in strain containing the P*pgxC_amdS* reporter was still repressed by glucose indicating an alternative repression mechanism for *pgxC*. For the other three reporter strains (P*pgaX*, P*pgxB*, and P*pgxC)* , in the *creA* mutant, GA was shown to be required as an inducer to support growth on medium containing acetamide reporter strains. Thus these promoter-reporter strains in *creA* null background can be used to perform forward genetic screens for inducer-independent mutants, which may contain a mutation responsible for constitutive activation in a GA-specific transcription factor. Such mutants can guide the search for transcription factors involved in the activation of GA-responsive genes and in the regulation of pectin-degrading enzymes in *A. niger*. The results in **Chapter 4** has led us to propose a model, in which a GA-specific transcription factor upon activation by GA or a GA-derivative could bind to the conserved motif GARE (possibly in combination with the HAP-complex) and drive GA-specific gene expression.

In Chapter 5, we identified a GA-specific transcription factor (GaaR*)* in *A. niger* by its homology to *Botrytis cinerea* GaaR (Zhang *et al.* 2016). GaaR in *B. cinerea* was identified via a yeast one hybrid (Y1H) screen using a GA-specific promoter containing the GA-motif and by expressing *B. cinerea* TF in *S. cerevisiae* (Zhang *et al*. 2016). For identification of transcription factors that can interact with a specific DNA sequence, the Y1H is widely recognized as a powerful and straightforward approach. The Y1H screening procedure relies on the interaction of a single protein (the prey) with a bait DNA sequence positioned upstream of a reporter gene. A cDNA library encoding prey proteins is needed for screening. The cDNA of the prey protein is fused to the sequence of a transcriptional activation domain.

Positive protein-DNA interactions result in recognition and binding of a transcription factor to the bait sequence, thereby activating downstream transcription of the reporter gene (Ouwerkerk and Meijer 2001; Ouwerkerk and Meijer 2011). In my study, we also used a different method and identified *gaaX* and *gaaR* as relevant regulatory factors by using a reporter strain to do forward genetic screening (**Chapter 4 and 6**).

A. niger GaaR is about 50% identical to *B. cinerea* GaaR on the amino acid level. Deletion of *gaaR* in *A. niger* showed a strongly reduced growth on GA and PGA, and a little reduced growth on sugar beet pectin (SBP) compared to parental strain. The growth phenotype indicates that GaaR is required for the utilization and release of GA from pectin. Further transcriptomic analysis of the *gaaR* deletion strain by RNA sequencing showed genes encoding 25 pectinolytic enzymes, are not induced on GA in the *ΔgaaR,* indicating that GaaR is required for the induction of these genes on GA. Other genes involved in the degradation pectin side chains and involved in catabolism of L-rhamnose, L-arabinose, and D-xylose were still expressed in *ΔgaaR*, indicating that the degradation and metabolism of pectin sugars other than GA support the growth of *ΔgaaR* on SBP.

GaaR is a member of the family of Zn(II)2Cys6 transcription factor. Both in *A. niger* and *B. cinerea* it controls expression of genes involved in GA utilization. The GaaR encoding gene in *A. niger gaaR* is 2476 bp long and contains 5 introns resulting in a 740-amino acid long protein. GaaR contains a fungal specific DNA-binding domain Zn(II)2Cys6 with the pattern of $CX_2CX_6CX_6CX_2CX_6C$ at the residues 26-56 and a fungal transcription factor regulatory middle homology region (fungal_TF_MHR) at residues 139-518. Orthologs of GaaR were found across all 20 *Aspergillus* except *Aspergillus glaucus* based on the information from the *Aspergillus* genome database (http://www.aspgd.org/). *A.glaucus* is not able to grow on medium with GA as single carbon source (http://www.fung-growth.org/) corresponding with the absence of *gaaR* in *A. glaucus*, in agreement with the fact that *gaaR* is responsible for the utilization of GA.

In **Chapter 6**, the P*pgaX-amdS* reporter strain mentioned in Chapter 4 was used to screen for inducer-independent mutants with constitutive expression of pectinases. For selection of mutants showing inducer-independent activation, we performed UV mutagenesis of the reporter strain. Mutants which can grow on a non-inducing carbon source with acetamide plates were selected. Including also spontaneous mutants obtained on selective acetamide plates, a total of 73 mutants were isolated that could grow on non-inducing conditions.

Supernatants of these mutants grown on liquid non-inducing conditions were harvested and assayed on polygalacturonic acid plates to test pectinolytic activity. Finally, 65 out of the 73 mutants showed constitutive pectinolytic activity and were considered to be trans-acting mutants. In **Chapter 5,** GaaR was shown to be the transcriptional factor which regulates the expression of genes involved in GA utilization. Considering many studies which showed that mutations in transcription factor can cause constitutive expression, we were wondering if the constitutive phenotype of these mutants could also be ascribed to mutations in *gaaR*. Therefore, the *gaaR* gene of 15 constitutive mutants was sequenced. Unfortunately, there were no mutations found in *gaaR* of these 15 constitutive mutants, meaning that mutations in other genes were responsible for the constitutive phenotype. In order to find these other genes, we selected five constitutive mutants, as well as parent strain to conduct whole genome sequencing. The sequencing results revealed allelic mutations in one particular gene encoding a previously uncharacterized protein (NRRL3_08194). The gene was named *gaaX*. Sequencing of the remaining constitutive mutants showed that all but two of the mutants contain a mutation in *gaaX*. One mutant contains a mutation in GaaR causing the constitutive expression of pectinolytic genes and one mutant did not show mutation in either GaaR or GaaX. Both mutants will be characterized and studied in more detail in future studies.

Subsequently, we also performed targeted deletion and transcriptomic analysis of the mutant strain to study the function of *gaaX*. Growth assay showed that the *ΔgaaX* mutant grow normally on a variety of C-sources including GA, PGA and pectin. Deletion of *gaaX* was also shown to result in constitutive pectinolytic activity in plates assay. RNAseq analysis revealed that 37 genes were upregulated in *ΔgaaX* mutant (FDR<0.001, FC>4.0). Gene Ontology (GO) enrichment analysis using FetGOat (Nitsche et al. 2012) and manual inspection of the genes upregulated in the *ΔgaaX* mutant indicated that genes involved in pectin catabolism were highly enriched. Of the 37 genes, 16 are predicted to encode extracellular enzymes acting on the GA backbone of pectin or acting on pectin side chains. Nine genes of the 37 upregulated genes are predicted to encode intracellular proteins. Four of these nine genes (*gaaA–gaaD*) are required for the conversion of GA into pyruvate and glycerol (Martens-Uzunova and Schaap 2008). The exact role of the other five genes and their possible role in pectin catabolism is currently unknown.

Genomic localization of *gaaX* (NRRL3_08194) showed the *gaaX* gene is next to the GA-specific transcriptional factor *gaaR* (NRRL3_08195). Among the 19 *Aspergillus* species mentioned in **Chapter 4,** *gaaR* and *gaaX* orthologs showed a strongly conserved genomic

clustering pattern: either next to each other or separated by only one or a few genes. Like *gaaR*, also *gaaX* are absent in *A. glaucus*. The *A. niger* GaaX protein is predicted to be 697 aa long. Sequence alignment and BLASTP searches displayed similarity of GaaX to the last three domains in the C-terminal half of the AROM protein, which is a large (1586 aa in *A. niger*) pentafunctional protein composed of five domains involved in different enzymatic steps of the prechorismate shikimate pathway (Duncan *et al.* 1987; Hawkins and Smith 1991). Like GaaX, the previously identified quinate repressor protein QutR is also highly similar to the last three C-terminal domains of AROM (Lamb *et al.* 1996a), Thus GaaX is homologous to the quinate repressor protein QutR (Grant *et al.* 1988). The genomic clustering of *gaaX* and *gaaR* is analogous to that of the quinic acid utilization transcriptional activator (QutA/Qa-1F) and repressor (QutR/ Qa-1S) in *A. nidulans* and *N. crassa*, respectively (Geever *et al.* 1989; Levesley *et al.* 1996). The amino acid homology to the quinate repressor, the similarity in chromosomal organization of *gaaR/gaaX* compared to the known activator/repressor genes *qutA*/*qutR*, and the constitutive phenotype of the isolated *gaaX* mutants point to the possibility that *gaaX* encodes a repressor protein that controls the activity of GaaR and keeps GaaR inactive under non-inducing conditions, similar as what was predicted for QutA/QutR (Lamb *et al.* 1996b; Levett *et al.* 2000; Watts *et al.* 2002).

Recent evidence from our group has shown that the GA-metabolic pathway intermediate 2-keto-3-deoxy-L-galactonate (2-KDG) is likely to act as the inducer (Alazi *et al.* 2017). We propose a model (Figure 1) in which it is postulated that under inducing conditions the inducer, 2-KDG, will bind to repressor protein GaaX. We speculate that the interaction of the inducer with the repressor results in the release of active GaaR from GaaX. Whether this occurs in the cytosol or nucleus remains to be determined, but preliminary experiments suggests that GaaR is present in the nucleus under inducing and non-inducing conditions (E. Alazi, personal communication) suggesting that the interaction takes place in the nucleus. Active GaaR induces not only the expression of GA-responsive genes involved in GA release, uptake and metabolism, but also induces the expression of *gaaX* (Martens-Uzunova and Schaap 2008; Niu *et al*. 2017). Under inducing conditions, a sufficient amount of the inducer is likely to keep GaaX dissociated from GaaR and consequently the GaaR transcription factor will be active. When the concentration of inducer decreases (non-inducing conditions), the amount of inducer is insufficient to bind to the repressor protein thereby allowing interaction between GaaR and the repressor GaaX to inactivate the GaaR transcriptional activator. The exact mechanism by which GaaX controls GaaR activity is unknown. However, such a

mechanism allows an elegant way which enables the cell to activate GA-induced expression when the inducer is present in the cell and allows to turn off expression of GA-responsible genes when inducer levels decrease. It also ensures the rapid response to the presence of GA as no *de novo* synthesis of GaaR is required.

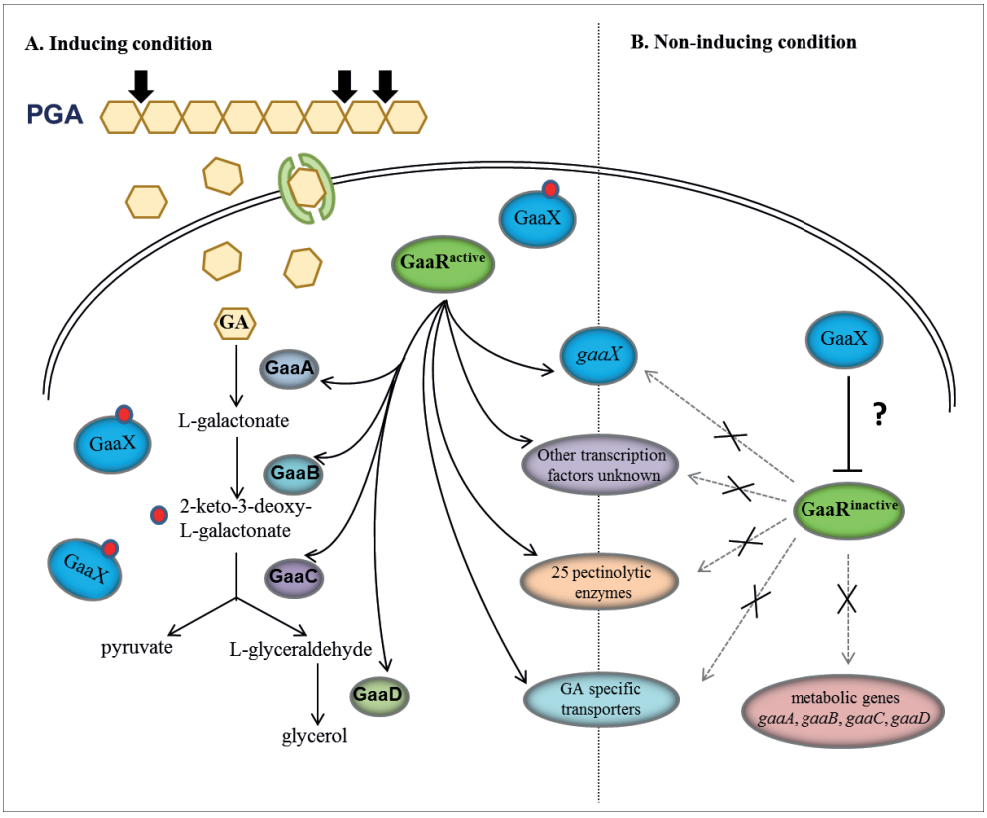

Figure 1. A) Model describing GaaR/GaaX controlled gene expression in response to galacturonic acid (GA). Under inducing conditions (presence of (poly) galacturonic acid), the inducer 2-keto-3-deoxy-L-galactonate (red dot) is predicted to bind to GaaX. The binding of the inducer to GaaX is expected to cause dissociation of the GaaX /GaaR complex resulting in non-GaaX-bound GaaR which can drive the expression of GA-responsive genes. These genes include: *gaaX* (Martens-Uzunova and Schaap 2008); other transcription factors unknown (Niu *et al.* 2017) (Supplementary Table 3, among the differentially expressed genes between WT and *ΔgaaX* , at least NRRL3_00899 and NRRL3_01451 are supposed to be fungal specific transcription factors); 25 pectinolytic genes which involved in GA release (Alazi *et al.* 2016; Niu *et al.* 2017) ; GA specific transporters which take up GA into the cell; and GA intracellular metabolism genes *gaaA*, *gaaB*, *gaaC*, *gaaD* (Alazi *et al.* 2016; Niu *et al.* 2017). Under inducing condition, *gaaX* is induced but the presence of the inducer keeps GaaX inactive. B) Under non-inducing conditions (absence of pectin or (poly)galacturonic acid), no inducer is present to bind to the repressor protein thereby allowing interaction between the repressor GaaX and the transcription activator GaaR to inactivate GaaR, preventing the expression of GA-responsible genes.

The proposed model on how GaaR/GaaX regulate gene expression is not only similar to what is shown for QutR/Qa-1S) but also shows similarities to the well-known galactose regulatory system from *S. cerevisiae*. In this system, three proteins, Gal3p/Gal4p/Gal80p, are involved in the regulation of galactose utilization. Gal4p works as a transcriptional activator, Gal80p works as a transcriptional repressor, and Gal3p possibly works as a galactose sensor. Gal80p can bind to both of Gal4p and Gal3p. In the absence of galactose, Gal80p binds to Gal4p preventing *GAL* gene expression. In the presence of galactose, galactose triggers an association between Gal3p and Gal80p, by binding of galactose to Gal3p relieving Gal4p from Gal80p (Platt *et al.* 2000; Timson *et al.* 2002; Diep *et al.* 2008; Jiang *et al.* 2009). The differences between the GA utilization system and Gal system are that in the GA case there are only two genes found to be involved in the GA utilization system so far. At this point we cannot exclude that there is a third gene involved in the GA utilization system. However, GaaX does not show homology to Gal80p or Gal3p making it unlikely that the two systems are evolutionary related. To further elucidate the regulation mechanism of the GA utilization system, future research should be aimed at understanding the biophysical and biochemical interactions between GaaR, GaaX and the inducer. Protein-protein interaction between GaaX and GaaR could be studies by co-immunoprecipitation experiments under inducing and non-inducing condition or via biomolecular fluorescence complementation (BiFC). As we have shown that GaaX can be labeled with GFP at either the N- or the C-terminus and likewise GaaR can be labeled with GFP either at the N- or C-terminus resulting in functional proteins (Chapter 6, and E. Alazi, personal communication). Constructs to perform BiFC can be designed accordingly.

Another intriguing remaining question is the binding of GaaR and/or GaaX to DNA. Although GaaX does not contain known DNA binding motifs, DNA binding can not be excluded. GaaR is expected to bind to the GARE motive, but for *A. niger* this has not been experimentally confirmed. Chip-seq experiments could be performed to identify the promoter sequences that are actually directly bound by GaaR and/or GaaX.

Finally, based on the knowledge available for the QutR/QutA regulatory system, it would be of interest to dissect the functional domains in both GaaX and GaaR involved in activation, repression, protein-protein interactions, protein-inducer interactions, and DNA-protein interactions.

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