Transcriptional control of pectin degrading enzymes in Aspergillus niger

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Chapter 5

The transcriptional activator GaaR of *Aspergillus niger* is required for release and utilization of D-galacturonic acid from pectin

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Published in FEBS Lett. 2016 Jun; 590(12): 1804-1815

Abstract

We identified the D-galacturonic acid (GA) responsive transcriptional activator GaaR of the saprotrophic fungus *Aspergillus niger*, which was found to be essential for growth on GA and polygalacturonic acid (PGA). Growth of the *ΔgaaR* strain was reduced on complex pectins. Genome-wide expression analysis showed that GaaR is required for the expression of genes necessary to release GA from PGA and more complex pectins, to transport GA into the cell and to induce the GA-catabolic pathway. Residual growth of *ΔgaaR* on complex pectins is likely due to expression of pectinases acting on rhamnogalacturonan and subsequent metabolism of the monosaccharides other than GA.

Keywords

polygalacturonic acid, pectinase, Zn_2Cys_6 transcription factor, gene regulation, transcriptomics

Abbreviations

AP apple pectin
CM Complete medium
CP citrus pectin
GA D-galacturonic acid
MM minimal medium
PGA polygalacturonic acid
RG rhamnogalacturonan
SBP sugar beet pectin
TF transcription factor
XGA xylogalacturonan

Introduction

Pectins are complex heterogeneous polysaccharides found in plant cell walls. Four sub-structures of pectin have been identified and include polygalacturonic acid (PGA) also known as homogalacturonan, xylogalacturonan (XGA), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II) (Mohnen 2008). The backbones of PGA, XGA and RG-II are made up of α-1,4-linked D-galacturonic acid (GA) residues. PGA, a linear polymer of GA, is the most abundant polysaccharide present in pectin (Mohnen 2008). In XGA, β-D-xylose residues are β-1,3-linked to GA residues of the PGA backbone. The backbone of RG-I is made up of alternating GA and L-rhamnose residues (Mohnen 2008; Leijdekkers *et al.* 2015). Side chains of RG-II contain at least 12 different types of monosaccharides, whereas the side chains of RG-I are mainly arabinan and arabinogalactan comprising of L-arabinose and D-galactose residues (Mohnen 2008).

In nature, pectin is an important carbon source for many saprotrophic fungi, such as *Aspergillus niger*. Previous studies demonstrated that *A. niger* can produce more pectin degrading enzymes than other more specialized fungi such as *Podospora anserina* or *Neurospora crassa* (Espagne *et al.* 2008; Coutinho *et al.* 2009; Martens-Uzunova and Schaap 2009). GA is the main product of pectin degradation. In *A. niger*, GA is transported into the cell by a GA-induced sugar transporter named GatA (Sloothaak *et al.* 2014). GA is then catabolized into pyruvate and glycerol (Martens-Uzunova and Schaap 2008), through a pathway consisting of four enzymes: GaaA, D-galacturonate reductase, GaaB, L-galactonate dehydratase, GaaC, 2-keto-3-deoxy-L-galactonate aldolase, and GaaD, L-glyceraldehyde reductase (Martens-Uzunova and Schaap 2008). Deletion of *gaaA*, *gaaB* or *gaaC* abolished growth on GA as the sole carbon source (Mojzita *et al.* 2010b; Wiebe *et al.* 2010; Kuivanen *et al.* 2012). *gaaD*, also known as the L-arabinose reductase gene, *larA*, is involved in the L-arabinose catabolic pathway and the *ΔlarA* strain showed a reduced growth on L-arabinose as the sole carbon source (Mojzita *et al.* 2010a).

The production of extra- and intracellular enzymes in *A. niger* is regulated by a network of transcription factors (TFs) (Kowalczyk *et al.* 2014). Small sugar molecules (mono- and disaccharides) act as inducers and stimulate TFs which can bind to conserved motifs in the promoters of their target genes and activate or repress their expression. Expression of pectinase genes is highly controlled and depends on both induction and carbon catabolite repression (De Vries *et al.* 2002; Niu *et al.* 2015). Induction of the genes required for pectin degradation, GA transport and GA catabolism requires the presence of GA and it has been

shown that GA or a derivative of GA induces the expression of pectinase genes (De Vries *et al.* 2002; Wiebe *et al.* 2010; Kuivanen *et al.* 2012).

Coordination of the induction of genes encoding extracellular enzymes and sugar uptake systems in fungi are often mediated by Zn_2Cys_6 TFs that bind to conserved promoter elements in the co-regulated genes (Chang and Ehrlich 2013; Kowalczyk *et al.* 2014; Tani *et al.* 2014). TFs inducing the genes required for the utilization of L-rhamnose (RhaR), arabinan/L-arabinose (AraR), xylan/D-xylose (XlnR), D-galactose (GalX) and cellulose (XlnR, ClrA and ClrB) have been identified in *A. niger* (Van Peij *et al.* 1998; Battaglia *et al.* 2011; Gruben *et al.* 2012; Gruben *et al.* 2014; Raulo *et al.* 2016). Although L-rhamnose, L-arabinose, D-xylose, and D-galactose are also present in complex pectins, knock out mutants in these TFs display no signs of reduced growth on pectin (Battaglia *et al.* 2011; Gruben *et al.* 2012; Gruben *et al.* 2014), suggesting that the utilization of GA, the main component of this substrate, is not affected.

Martens-Uzunova and Schaap have previously identified a set of GA-induced genes in *A. niger*, containing several pectinases (*pgaX*, *pgxA*, *pgxB*, *pgxC*, *paeA*, *pelA* and *abfC*), sugar transporter encoding genes (*gatA*, An03g01620 and An07g00780) and the GA catabolic pathway genes (*gaaA-D)* (Martens-Uzunova and Schaap 2008). These genes were suggested as the GA-regulon and contain a common GA responsive element (GARE) in their promoter regions. The consensus element was defined as CCNCCAA (Martens-Uzunova and Schaap 2008). Deletion and mutational analysis of GARE showed that the element is required for GA-induced gene expression in both *A. niger* and *Botrytis cinerea* (Niu *et al.* 2015; Zhang *et al.* 2016). A yeast one-hybrid study using a GA-responsive promoter in *B. cinerea* recently identified a novel Zn_2Cys_6 TF (BcGaaR) required for GA utilization (Zhang *et al.* 2016). In this study, the GA-responsive transcriptional activator GaaR of *A. niger* was identified by homology to BcGaaR. Deletion analysis and transcriptomic profiling studies performed in this study showed that the *A. niger* GaaR ortholog is required for growth on GA and PGA and for the induction of the GA-regulon when grown on sugar beet pectin (SBP).

Materials and Methods

Strains, media and growth conditions

A. niger strains MA234.1 (*cspA1*, *kusA::DR-amdS-DR*) and N593.20 (*cspA1*, *pyrG⁻*, *kusA::amdS*) were used to create the *ΔgaaR* strains. N593.20 was made by transformation of N593 (Goosen *et al.* 1987) with a deletion construct (*kusA::amdS*) (Meyer *et al.* 2007) resulting in the deletion of *kusA*. Strain FP-1132.1 (*cspA1, pyrG⁻::AOpyrG, kusA::amdS)* was

obtained by transformation of N593.20 with *pyrG* from *Aspergillus oryzae.* MA234.1 was obtained by transformation of MA169.4 (*kusA*$^{-}$*, pyrG*$^{-}$) (Carvalho *et al.* 2010) with a 3.8 kb *Xba*I fragment containing the *A. niger pyrG* gene, resulting in the full restoration of the *pyrG* locus.

Complementation studies were performed with JN35.1 (*cspA1, kusA::DR-amdS-DR, gaaR::hygB*). To restore functionality of the *kusA* gene to allow ectopic integration of the complementing fragment, the *amdS* marker was looped out of JN35.1 by FAA counter-selection as described (Arentshorst *et al.* 2012) to give JN36.1. The *gaaR* complemented strain JN37.4 was created using JN36.1, by transformation of the *gaaR* gene including promoter and terminator regions (see below). All strains used are listed in Table S1.

Media were prepared as described (Arentshorst *et al.* 2012). For growth phenotype analyses, strains were grown on minimal medium (MM) with 1.5% (w/v) agar and various sole carbon sources: 25 or 50 mM glucose (VWR International), GA (Chemodex), L-rhamnose (Fluka), L-arabinose (Sigma-Aldrich) or D-xylose (Merck), and 1% (w/v) PGA (Sigma), SBP (Pectin Betapec RU301 Herbstreith & Fox KG), citrus pectin (CP) (Acros Organics) or apple pectin (AP) (Pectin Classic AU2022 Herbstreith & Fox KG). pH was adjusted to 5.8 with NaOH or HCl buffer. The plates were inoculated with 2 µl containing 1000 freshly harvested spores and cultivated at 30 °C for 4 days. For gene expression analyses, freshly harvested spores were inoculated with a final concentration of 10^6 spores/ml in 100 ml complete medium (CM) (pH 5.8) with 2% (w/v) D-fructose (Sigma-Aldrich) and were pre-grown for 16h. For Northern blot analysis, mycelium was harvested by filtration through sterile myracloth, washed twice with MM with no carbon sources (pH 4.5) and 1.5 g (wet weight) mycelium was transferred and grown in 50 ml MM (pH 4.5) with 50 mM GA or 50 mM D-fructose for 2, 4 and 6h. For RNA-seq analysis, 2.5 g of pre-grown mycelia were transferred to 50 ml MM (pH 4.5) with 25 mM GA and incubated for 2h or to 50 ml MM with 1% SBP and incubated for 2, 8 or 24h. All incubations were performed in rotary shaker at 30°C and 250 rpm.

Construction of gene deletion and complementation strains

Protoplast-mediated transformation of *A. niger*, purification of the transformants and genomic DNA extraction were performed as described (Arentshorst *et al.* 2012). To construct the deletion cassettes, 5' and 3' flanks of the *gaaR* gene were PCR-amplified using the primer pairs listed in Table S2 and N402 genomic DNA as template. To create JN35.1 strain, the split marker fragments with *hygB* selection were created using fusion PCR (Arentshorst *et al.*

2015) and transformed to MA234.1. To create FP-1126.1 strain, the flanking regions were fused with a fragment containing the *A. oryzae pyrG* gene using GoTaq® Long polymerase (Promega) and transformed into N593.20 strain. Parental strains and *gaaR* deletion mutants were deposited at the Centraal Bureau Schimmelcultures (CBS) under accession numbers indicated in Table S1. To complement the *gaaR* gene, the *gaaR* gene together with its 5' and 3' flanks was PCR-amplified using the primer pairs listed in Table S2, ligated into pJET1.2/blunt cloning vector (Fermentas), amplified in the *E. coli* strain DH5*α* and transformed in to strain JN36.1 together with plasmid pMA357. pMA357 contains the *A. nidulans amdS* gene, cloned behind the *A. nidulans gdpA* promoter (Mark Arentshorst, unpublished vector). Deletion and complementation of *gaaR* were confirmed via Southern blot analysis or diagnostic PCR.

Gene expression analysis

For Northern blot analysis, strains MA234.1 (reference strain) and JN35.1 (Δ*gaaR*) were pre-grown in CM with D-fructose. At the time of transfer (t = 0) and 2, 4 and 6h after the transfer to MM with GA or D-fructose, mycelium was harvested from cultures by filtration through sterile myracloth and frozen immediately in liquid nitrogen. Mycelium samples were stored at -80 °C. Total RNA was extracted from frozen mycelium samples after grinding in liquid nitrogen, using NucleoSpin RNA Kit (Macherey-Nagel) following the protocol provided by the supplier, including the rDNase treatment. Total RNA samples were stored at -80 °C. Quantification and purity assessment of total RNA was done by spectrophotometric method (NanoDrop 2000, Thermo Scientific). Standard molecular techniques were applied as described (Sambrook and Russell 2001). 3.5 µg RNA was loaded per sample and hybridized with [α-32P]-dCTP labelled probes after blotting (DecaLabel DNA Labelling Kit, Thermo Scientific). Probes were PCR-amplified using the N402 genomic DNA and the primer pairs are listed in Table S2. For RNA-seq analysis, the mycelium of FP-1132.1 (reference strain) and FP-1126.1 (Δ*gaaR*) was ground in Tissue Lyser II (Qiagen) and RNA was extracted using TRIzol reagent (Invitrogen) and purified with NucleoSpin RNA Clean-up kit (Macherey-Nagel) with rDNase treatment. RNA quantity of the samples was checked with a NanoDrop-1000 spectrophotometer and the quality by RNA gel electrophoresis. Single-read samples were sequenced using Illumina HiSeq^{TM} 2000 platform (http://illumina.com). Purification of mRNA, synthesis of cDNA library and sequencing reactions were conducted in the BGI Tech Solutions Co., Ltd. (Hong Kong). Transfer experiments and subsequent RNA-sequencing were performed in duplicates.

Bioinformatics

Raw reads were produced from the original image data by base calling. On average, ~13 million read of 51 bp per sample were obtained. After data filtering, the adaptor sequences, highly 'N' containing reads (> 10% of unknown bases) and low quality reads (more than 50% bases with quality value of < 5%) were removed. After data filtering, in average, ~97.5% clean reads remained in each sample. Clean reads were then mapped to the genome of *Aspergillus niger* NRRL3 (http://genome.jgi.doe.gov/Aspni_NRRL3_1) using Bowtie2 (Langmead *et al.* 2009) and BWA software (Li and Durbin 2009). In average, 63.8% total mapped reads to the genome was achieved. The gene expression level was measured in "fragments per kilobase of exon model per million mapped reads" (FPKM) (Trapnell *et al.* 2010) using RSEM tool (Li and Dewey 2011). Genes with expression value lower than 14 were considered low-expressed (approximately bottom 50%) and differential expression was identified by Student's t-test with a P-value cut-off 0.05. The RNA-seq data have been submitted to Gene Expression Omnibus (GEO) (Edgar *et al.* 2002) with accession number: GSE80227. Homology searches were performed using the blastp algorithm from NCBI against the non-redundant database and proteins with an E-value ≤ 1E-50 were defined as homologous (Altschul *et al.* 1990). Hierarchical clusters using the average expression values of genes were made via Genesis 1.7.7 (Sturn *et al.* 2002) with Pearson correlation and complete linkage. Low-expressed pectinases in all conditions were not included.

Results and Discussion

Identification of the *A. niger* GaaR by homology to *B. cinerea* BcGaaR

A putative *A. niger* GA-responsive transcriptional activator was identified by homology to the recently identified *B. cinerea* Zn_2Cys_6 TF (BcGaaR) (Zhang *et al.* 2016). The *A. niger* ortholog (named GaaR) is a 740 amino acid long protein encoded by *gaaR* (An04g00780/NRRL3_08195) and the bidirectional best blast hit of the 817 amino acid long BcGaaR (Bcin09g00170). Analysis of the presence of GaaR among 20 *Aspergillus* species using the *Aspergillus* genome database (http://www.aspgd.org/) revealed that all Aspergilli, except *Aspergillus glaucus* contain a GaaR ortholog in their genome (data not shown). Interestingly, *A. glaucus* is not able to grow on GA as the sole carbon source (http://www.fung-growth.org), indicating the requirement of GaaR for GA utilization. *A. niger* GaaR and BcGaaR show 50.3% identity on the amino acid level throughout the entire protein sequence (Figure S1). GaaR contains a typical Zn_2Cys_6 DNA binding domain with the pattern of $CX_2CX_6CX_6CX_2CX_6C$ close to its NH_2-terminal end (residues 26-56) and a

fungal specific TF domain (residues 139-518). Amino acid alignment and phylogenetic analysis of GaaR revealed no significant similarity (an E-value cutoff < 1E-50) of GaaR to other TFs involved in plant cell wall utilization such as XlnR, AraR, RhaR, GalX, ClrA and ClrB or to any other TF in *A. niger* (data not shown).

Deletion and complementation of *gaaR* and growth analysis of the Δ*gaaR* in *A. niger*

To assess the function of *gaaR* in *A. niger*, several deletion strains (*ΔgaaR*) were created and verified by Southern blot analysis (Figure S2 and data not shown). The growth phenotype of the *ΔgaaR* strains was analyzed on different monomeric and polymeric carbon sources (Figure 1A). Deletion of *gaaR* in the AB4.1 background (MA234.1, Figure S2) and N593 background (N593.20, Figure 1A) resulted in an identical phenotype. Disruption of *gaaR* resulted in a strongly reduced growth on GA and PGA and in a reduced growth and sporulation on SBP, CP and AP. No significant differences in growth and sporulation were observed on other carbon sources tested (Figure 1A, Figure S2). The strongly reduced growth of *ΔgaaR* on GA and PGA was fully complemented by reintroducing the *gaaR* gene ectopically (Figure S2).

GaaR is required for the induction of genes related to D-galacturonic acid utilization

The presence of GA has been shown to induce genes involved in PGA degradation (e.g. *pgxB, pgxC*), GA transport (*gatA*) and catabolism (*gaaA-D*) (Martens-Uzunova and Schaap 2008; Niu *et al.* 2015). As a first indication for the involvement of GaaR in the induction of a subset of these genes on GA, a Northern blot analysis was performed. The reference strain and *ΔgaaR* made the AB4.1 background were pre-grown in D-fructose medium and transferred to either GA or D-fructose medium. For the reference strain, transfer of mycelium to GA resulted in a rapid induction of *pgxB*, *pgxC*, *gatA*, *gaaB* and *gaaC*, whereas this induction was not observed in *ΔgaaR* (Figure 1B).

To analyze the expression of a larger number of genes involved in pectin degradation, GA transport and catabolism, a genome-wide gene expression analysis was performed using RNA-seq. The reference strain and *ΔgaaR* in the N593 background were again pre-grown in D-fructose medium and transferred to GA medium. RNA-seq analysis indicated that the GA-induced expression of all genes that were previously identified as part of the GA-regulon (Martens-Uzunova and Schaap 2008) is dependent on GaaR (Table 1 and Figure 2). The only exception is a putative GA transporter (An03g01620) that is expressed more than 3-fold less in *ΔgaaR* for which the p-value did not pass our significance level (0.05). In general, these

observations show that the genes in the suggested GA-regulon (Martens-Uzunova and Schaap 2008) showed a significant reduction in *ΔgaaR* compared to the reference strain on GA (Table 1) and that GaaR is required for the induction of those genes.

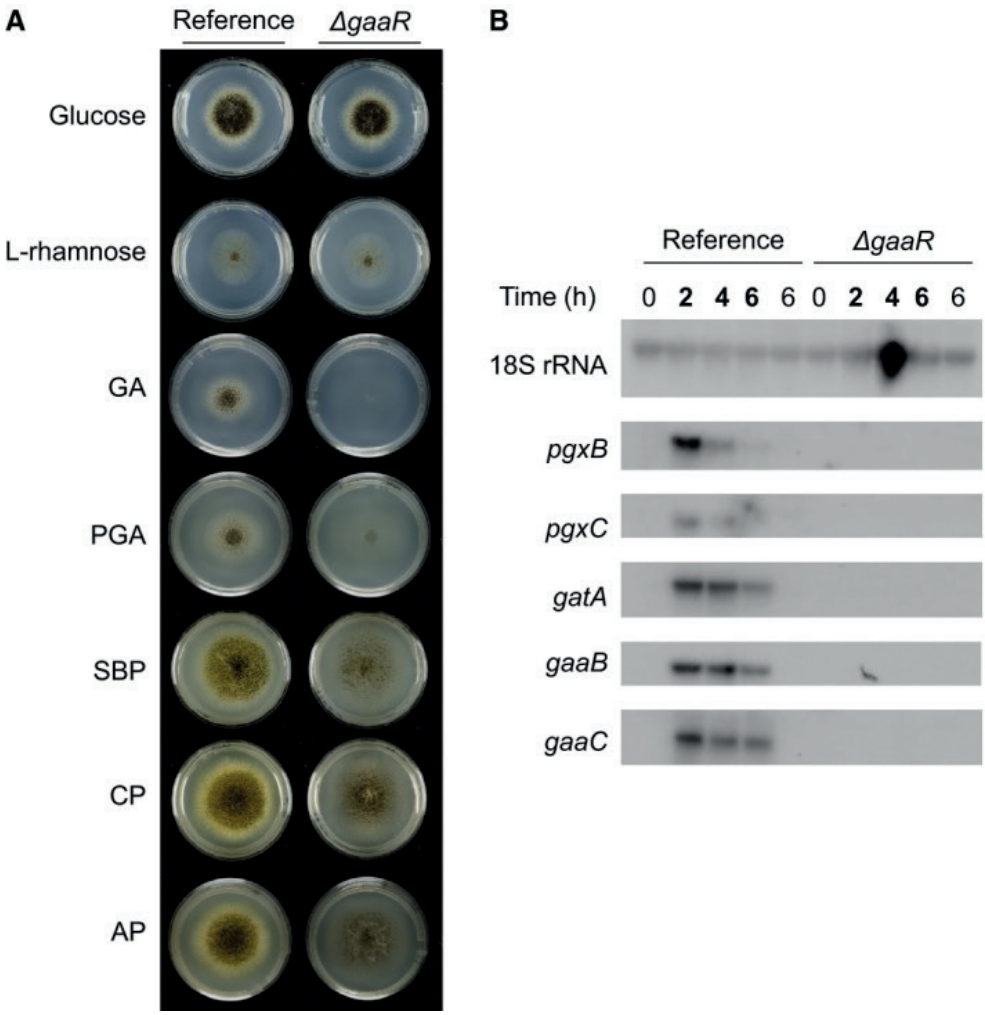


Figure 1 Phenotypic and gene expression analyses of *A. niger ΔgaaR* **A)** Growth profile of the reference strain (FP-1132.1) and *ΔgaaR* (FP-1126.1) on MM with 25mM monomeric and 1% polymeric carbon sources. Strains were grown for 4 days at 30°C. **B)** Northern blot analysis of selected GA-induced genes in the reference strain (MA234.1) and *ΔgaaR* (JN35.1). Mycelia were transferred from D-fructose (pre-culture) to GA or D-fructose. Total RNA was isolated at the time of transfer (0h) from mycelia grown in CM with 2% D-fructose and at different time points (2, 4 and 6h) after the transfer from mycelia grown in MM containing 50 mM GA (in bold) or D-fructose.

To identify additional pectinase genes controlled directly or indirectly by GaaR, the expression of all 58 pectinolytic genes (Martens-Uzunova and Schaap 2009) was examined (Table S3). An overview of the gene abbreviations and their (putative) function is given in Martens-Uzunova and Schaap, 2009 (Martens-Uzunova and Schaap 2009). This analysis resulted in the identification of several additional pectinase genes for which the expression on GA is dependent of GaaR (Table 1 and Figure 2, Figure S3). This difference could be caused by higher sensitivity of the RNA-seq analysis compared to the previously used Affymetrix microarrays. In general, these newly identified genes were lower expressed compared to the genes in the GA-regulon described previously (Martens-Uzunova and Schaap 2008). The gene encoding the putative pectin methylesterase C (*pmeC*) was missing on the Affymetrix chips, and therefore missed previously, but the RNA-seq study clearly indicated that induction of *pmeC* on GA is GaaR dependent. Inspection of the promoter regions of the newly identified members of the GA-regulon indicated the presence of putative GaaR binding sites in the promoter regions of most genes (Table 1), enabling us to expand the GA-regulon to a larger set of genes.

Table 1 RNA-seq analysis on GA of the genes that depend on GaaR for induction. Expression values (FPKM) are averages of duplicates. Fold changes ≥2 and p-values ≤0.05 are highlighted. GARE position is given with respect to the transcription start site. Genes identified as the GA-regulon by Martens-Uzunova and Schaap [7] are marked with an asterisk (*[1])

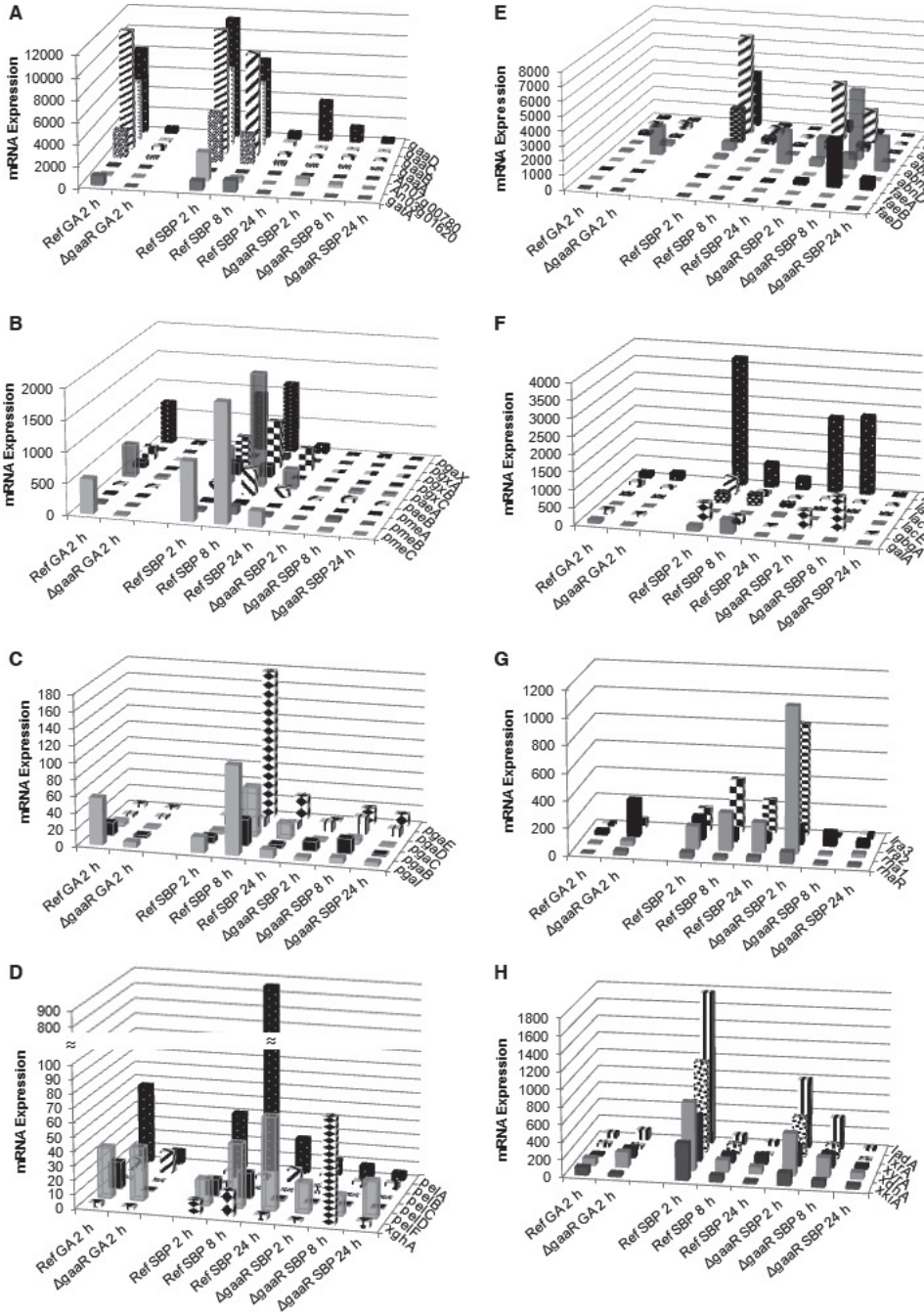
NRRL3 protein ID	CBS 513.88 gene ID	Gene name	Ref GA 2h	*ΔgaaR* GA 2h	Fold change Ref/*ΔgaaR* GA 2h	p-value	GARE (CCNCCAA) position
NRRL3_00958	An14g04280	*gatA**[1]	888.35	13.32	66.69	1.54E-03	+ strand -360
NRRL3_08663	An03g01620	GA transporter (putative)*[1]	106.09	30.34	3.50	1.25E-01	+ strand -673
NRRL3_04281	An07g00780	GA transporter (putative)*[1]	90.41	1.86	48.74	7.77E-03	- strand -42 and -994
NRRL3_05650	An02g07710	*gaaA* *[1]	2599.98	117.53	22.12	1.69E-04	+ strand -414 and -100
NRRL3_06890	An16g05390	*gaaB* *[1]	11309.00	344.03	32.87	1.88E-03	+ strand -326
NRRL3_05649	An02g07720	*gaaC* *[1]	5658.32	106.21	53.27	2.98E-04	- strand -292 and -606
NRRL3_10050	An11g01120	*gaaD* *[1]	8104.43	506.79	15.99	7.01E-03	- strand -538, -583, -801 and -813
NRRL3_03144	An12g07500	*pgaX* *[1]	698.90	24.27	28.80	1.19E-02	+ strand -388
NRRL3_09810	An11g04040	*pgxA* *[1]	10.65	0.34	31.32	9.10E-03	- strand -594
NRRL3_08281	An03g06740	*pgxB* *[1]	200.31	12.39	16.17	2.62E-02	- strand -298 and -823
NRRL3_05260	An02g12450	*pgxC* *[1]	99.93	4.10	24.40	6.24E-04	+ strand -268 and - strand -642
NRRL3_06053	An02g02540	*paeA* (putative) *[1]	522.81	22.99	22.75	4.57E-03	+ strand -1238
NRRL3_04916	An07g08940	*paeB* (putative) *[3]	13.41	10.57	1.27	7.42E-01	
NRRL3_08325	An03g06310	*pmeA*	6.54	0.42	15.75	1.18E-02	+ strand -983 and - strand -308
NRRL3_07470	An04g09690	*pmeB* (putative)	30.16	4.67	6.46	1.41E-02	+ strand -389
NRRL3_05252	An02g12505	*pmeC* (putative) *[2]	558.37	24.68	22.62	4.20E-03	+ strand -275, -246 and -35
NRRL3_02571	An01g11520	*pgaI*	56.38	6.56	8.59	6.96E-04	+ strand -221
NRRL3_05859	An02g04900	*pgaB* *[3]	15.10	3.11	4.86	6.74E-02	- strand -753 and -934
NRRL3_08805	An05g02440	*pgaC*	5.26	0.59	8.99	3.65E-02	+ strand -374, -196 and -865
NRRL3_02835	An01g14670	*pgaE* *[3]	4.26	2.40	1.78	4.12E-01	
NRRL3_00965	An14g04370	*pelA* *[1]	56.54	9.74	5.80	2.12E-04	
NRRL3_09811	An11g04030	*pelC*	0.51	0.00	NA	4.77E-03	
NRRL3_01237	An19g00270	*pelD*	18.95	0.34	55.74	6.03E-04	- strand -409 and -465
NRRL3_04153	An15g07160	*pelF* *[3]	35.48	37.02	0.96	8.73E-01	- strand -644
NRRL3_10559	An18g04810	*rgxC* (putative)	20.00	0.90	22.22	1.26E-02	+ strand -880 and -852 and - strand -250

NRRL3_00684	An14g01130	*rglA*	5.77	1.03	5.63	9.23E-03	- strand -188
NRRL3_01606	An01g00330	*abfA* *[3]	87.96	59.62	1.48	5.81E-01	
NRRL3_10865	An08g01710	*abfC* (putative) *[1]	201.62	67.16	3.00	4.04E-02	
NRRL3_07094	An16g02730	*abnD* (putative)	4.57	1.53	2.99	2.66E-02	+ strand -246
NRRL3_02479	An01g10350	*lacB* (putative)	137.63	41.24	3.34	1.36E-02	
NRRL3_11738	An06g00290	*lacC* (putative)	28.91	9.08	3.19	3.55E-02	+ strand -267
NRRL3_10643	An18g05940	*galA*	105.64	29.24	3.61	3.53E-02	+ strand -307

*[1] Genes identified as the GA-regulon by Martens-Uzunova and Schaap [7]

*[2] *pmeC* not present on the Affymetrix microarray

*[3] Genes not significantly differentially expressed on GA, but differentially expressed on SBP (see Table 2)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gaaB
gaaC
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Ref SBP 2 h
Ref SBP 8 h
Ref SBP 24 h
ΔgaaR SBP 2 h
ΔgaaR SBP 8 h
ΔgaaR SBP 24 h
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mRNA Expression
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500
0
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pgaA
pgaB
pgaC
pgaA
pmeA
pmeB
pmeC
C
mRNA Expression
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160
140
120
100
80
60
40
20
0
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pgaD
pgaC
pgaB
pgaI
D
mRNA Expression
900
800
100
90
80
70
60
50
40
30
20
10
0
pelA
pelB
pelC
pelD
pelE
xghA
E
mRNA Expression
8000
7000
6000
5000
4000
3000
2000
1000
0
abfA
abfB
abnA
abnB
abnC
faeA
faeB
faeD
F
mRNA Expression
4000
3500
3000
2500
2000
1500
1000
500
0
lacA
lacB
lacC
lacD
gdgA
galA
G
mRNA Expression
1200
1000
800
600
400
200
0
rgx3
rhg1
rhg2
rhaA
rhaR
H
mRNA Expression
1800
1600
1400
1200
1000
800
600
400
200
0
lacA
xlnA
xlnB
xlnD
xlnR

Figure 2 Transcript levels of pectin utilization genes in *A. niger* reference and *ΔgaaR* on GA or SBP. **A)** GA transporters and GA catabolic pathway enzymes, **B)** exo-polygalacturonases and pectin acetyl- and methylesterases, **C)** endo-polygalacturonases, **D)** pectin lyases and endo-xylogalacturonan hydrolase, **E)** α-L-arabinofuranosidases, arabinan endo-1,5-α-L-arabinofuranosidase, endo-arabinanases, ferulic acid esterases and feruloyl esterase D, **F)** β-galactosidases, galactan 1.3-β-galactosidase and β-1.4-ensogalactanase, **G)** the L-rhamnose regulator *rhaR* and L-rhamnose catabolic pathway enzymes and **H)** the L-arabinose and D-xylose catabolic pathway enzymes. Mycelia of the reference strain (FP-1132.1) and *ΔgaaR* (FP-1126.1) were pre-grown in CM with 2% D-fructose, washed and transferred to MM with 25mM GA or 1% SBP in and incubated for 2, 8 or 24h.

GaaR is required for the induction of genes related to polygalacturonic acid degradation and D-galacturonic acid utilization on complex pectin

Both the strongly reduced growth phenotype on GA and PGA and the expression analysis in *ΔgaaR* suggest that that GaaR is required for GA utilization in *A. niger*. Growth and sporulation of *ΔgaaR* on complex pectins such as SBP was also reduced, but not as severe as on GA and PGA (Figure. 1A). This could be explained by two (not mutually exclusive) hypotheses. The first explanation could be that *A. niger* has alternative mechanisms (independent of GaaR) to induce genes involved in GA utilization. The second possibility is that additional sugars such as L-arabinose, D-galactose, D-xylose or L-rhamnose that are present in SBP are metabolized and used for growth. To gain insight in the expression of pectinase genes in *ΔgaaR* on complex pectin, the reference strain and *ΔgaaR* were transferred from D-fructose to SBP and grown for 2, 8 and 24h before harvesting mycelia and extraction of RNA.

Expression profiles of pectinase genes in the reference strain and *ΔgaaR* were pairwise compared for identical time points (Table 2 and Figure 2, Figure S3). Most of the genes in the GA-regulon, including those required for GA transport and catabolism, are dependent on GaaR for induction on SBP (Figure 2A-D). This observation strongly suggests that *ΔgaaR* is not utilizing GA from SBP. FThe expression of *gaaD*/*larA* can be explained by the dual activity of the enzyme encoded by this gene as both an L-glyceraldehyde reductase and an L-arabinose reductase (Mojzita *et al.* 2010a) and the utilization of L-arabinose from SBP in *ΔgaaR* (see below). The expression profile of exo-polygalacturonases, pectin acetyl- and methylesterases, endo-polygalacturonases and pectin lyases (Table 2 and Figure 2B-D) all acting on the PGA backbone support the conclusion that the GaaR target genes are not induced during growth on SBP in *ΔgaaR*.

Table 2 RNA-seq analysis on SBP of the genes that depend on GaaR for induction. Expression values (FPKM) are averages of duplicates. Fold changes ≥2 and p-values ≤0.05 are highlighted. Genes identified as the GA-regulon by Martens-Uzunova and Schaap [7] are marked with an asterisk (*[1])

NRRL3 protein ID	CBS 513.88 gene ID	Gene name	Ref SBP 2h	*ΔgaaR* SBP 2h	Fold change Ref/*ΔgaaR* SBP 2h	p-value	Ref SBP 8h	*ΔgaaR* SBP 8h	Fold change Ref/*ΔgaaR* SBP 8h	p-value	Ref SBP 24h	*ΔgaaR* SBP 24h	Fold change Ref/*ΔgaaR* SBP 24h	p-value
NRRL3_00958	An14g04280	*gatA**[1]	849.85	12.60	67.45	6.18E-05	1077.70	21.92	49.17	2.84E-03	57.92	1.82	31.91	5.06E-02
NRRL3_08663	An03g01620	GA transporter (putative)*[1]	2647.36	642.68	4.12	1.01E-04	387.81	273.28	1.42	2.41E-01	2.69	3.20	0.84	7.16E-01
NRRL3_04281	An07g00780	GA transporter (putative)*[1]	33.99	7.43	4.58	1.89E-01	14.34	6.24	2.30	5.60E-02	34.49	2.63	13.11	8.63E-03
NRRL3_05650	An02g07710	*gaaA* *[1]	4649.59	70.19	66.24	2.16E-03	2785.77	78.98	35.27	1.73E-03	215.02	24.06	8.94	9.85E-02
NRRL3_06890	An16g05390	*gaaB* *[1]	11722.91	113.85	102.97	2.38E-03	9634.93	229.60	41.96	2.72E-04	208.56	65.62	3.18	8.85E-02
NRRL3_05649	An02g07720	*gaaC* *[1]	7306.08	92.88	78.66	2.95E-05	6041.04	78.17	77.29	2.63E-03	527.35	34.38	15.34	1.02E-01
NRRL3_10050	An11g01120	*gaaD* *[1]	11412.45	3807.08	3.00	5.06E-03	7573.39	1434.68	5.28	8.61E-04	621.40	409.01	1.52	4.46E-01
NRRL3_03144	An12g07500	*pgaX* *[1]	948.28	5.71	166.22	1.16E-02	1154.06	21.72	53.13	4.37E-03	133.41	4.97	26.87	1.59E-03
NRRL3_09810	An11g04040	*pgxA* *[1]	19.61	0.11	186.71	1.72E-02	72.87	0.60	121.44	2.09E-03	3.23	0.00	NA	NA
NRRL3_08281	An03g06740	*pgxB* *[1]	483.28	3.15	153.66	1.81E-02	784.73	13.84	56.72	6.23E-02	364.35	3.40	107.32	4.37E-02
NRRL3_05260	An02g12450	*pgxC* *[1]	206.37	3.08	67.11	8.92E-03	191.37	4.23	45.29	2.90E-04	7.90	8.21	0.96	7.98E-01
NRRL3_06053	An02g02540	*paeA* (putative) *[1]	585.00	14.43	40.55	1.48E-02	1836.37	30.62	59.97	1.75E-02	300.70	11.83	25.43	1.46E-02
NRRL3_04916	An07g08940	*paeB* (putative) *[3]	166.46	7.51	22.16	1.47E-02	427.08	62.09	6.88	9.60E-02	151.00	10.65	14.18	9.63E-04
NRRL3_08325	An03g06310	*pmeA*	15.92	1.30	12.24	3.81E-02	27.43	0.99	27.70	3.18E-03	0.90	0.12	7.50	2.43E-01
NRRL3_07470	An04g09690	*pmeB* (putative)	41.34	4.84	8.55	3.34E-02	130.79	52.94	2.47	1.77E-02	33.31	2.06	16.17	2.20E-01
NRRL3_05252	An02g12505	*pmeC* (putative) *[2]	957.92	7.59	126.29	1.59E-02	1917.18	26.77	71.63	1.09E-02	249.81	0.40	624.53	1.67E-02

NRRL3_02571	An01g11520	*pgaI*	18.75	4.38	4.29	1.44E-02	106.37	6.59	16.14	8.39E-03	9.88	4.83	2.04	4.97E-01
NRRL3_05859	An02g04900	*pgaB* *[3]	7.84	11.01	0.71	2.52E-01	31.37	17.51	1.79	3.24E-02	4.29	2.07	2.07	3.62E-01
NRRL3_08805	An05g02440	*pgaC*	2.97	0.65	4.60	3.05E-01	58.43	6.22	9.40	2.60E-02	17.67	0.41	43.09	2.87E-01
NRRL3_02835	An01g14670	*pgaE* *[3]	3.16	1.35	2.34	8.30E-02	177.86	19.69	9.03	3.46E-03	28.57	15.13	1.89	1.99E-01
NRRL3_00965	An14g04370	*pelA* *[1]	40.64	11.09	3.67	1.14E-02	861.91	9.02	95.55	2.19E-02	25.43	5.41	4.70	1.82E-01
NRRL3_09811	An11g04030	*pelC*	2.39	4.58	0.52	4.27E-01	0.00	0.00	NA	NA	0.00	0.00	NA	NA
NRRL3_01237	An19g00270	*pelD*	11.72	0.61	19.37	1.10E-02	17.97	0.58	31.24	1.26E-03	1.28	0.42	3.05	8.54E-03
NRRL3_04153	An15g07160	*pelF* *[3]	18.03	21.02	0.86	4.67E-01	44.85	14.09	3.18	2.56E-02	64.95	25.60	2.54	5.26E-04
NRRL3_10559	An18g04810	*rgxC* (putative)	86.41	0.94	92.41	6.26E-02	207.96	3.76	55.38	4.39E-02	116.98	1.11	105.39	1.02E-01
NRRL3_00684	An14g01130	*rglA*	2.46	0.00	NA	NA	6.60	2.32	2.84	2.96E-02	3.98	2.30	1.73	6.60E-01
NRRL3_01606	An01g00330	*abfA* *[3]	3864.76	705.67	5.48	6.85E-03	133.72	176.93	0.76	3.59E-01	1.56	4.72	0.33	1.76E-01
NRRL3_10865	An08g01710	*abfC* (putative) *[1]	2406.40	527.90	4.56	2.46E-02	441.88	483.86	0.91	2.48E-01	97.31	36.96	2.63	2.77E-02
NRRL3_07094	An16g02730	*abnD* (putative)	3.92	2.82	1.39	1.06E-01	14.38	6.20	2.32	4.33E-02	55.45	12.53	4.43	2.40E-01
NRRL3_02479	An01g10350	*lacB* (putative)	487.09	48.89	9.96	1.33E-03	115.20	24.31	4.74	8.71E-03	13.85	8.31	1.67	2.24E-01
NRRL3_11738	An06g00290	*lacC* (putative)	297.53	12.04	24.71	6.23E-03	314.50	8.56	36.74	3.61E-02	113.92	2.81	40.61	3.74E-02
NRRL3_10643	An18g05940	*galA*	154.02	30.49	5.05	1.82E-02	398.55	26.21	15.21	2.85E-04	19.05	20.72	0.92	5.82E-01

*[1] Genes identified as the GA-regulon by Martens-Uzunova and Schaap [7]

*[2] *pmeC* not present on the Affymetrix microarray

*[3] Genes not significantly differentially expressed in on GA, but differentially expressed on SBP

The results described above indicate that the residual growth of *ΔgaaR* on SBP is due to the utilization of other monosaccharides released from SBP. Analysis of the monosaccharide composition of the SBP used in this study was performed as described previously (Santander *et al.* 2013) and showed that it contains 55 mol% GA, as well as 17 mol% L-arabinose, 16 mol% D-galactose and 10 mol% L-rhamnose. Analysis of the expression of the genes involved in the degradation of RG-I such as exo-rhamnogalacturonases (*rgx*), rhamnogalacturonases (*rhg*), rhamnogalacturonan acetyl esterases (*rgae*), rhamnogalacturonyl hydrolases (*urhg*), arabinofuranosidases (*abf*), endo-arabinanases (*abn*), ferulic acid esterases (*fae*) and β-galactosidases (*lac*), and the genes responsible for catabolism of L-rhamnose, L-arabinose and D-xylose showed that these genes were still expressed in *ΔgaaR* (Figure. 2E-H, Figure S3), indicating that the degradation and metabolism of RG-I support the growth of *ΔgaaR* on SBP.

A clustering analysis of the expression of genes encoding the (putative) GA transporters, GA catabolic pathway genes and pectinases provided further insight in the groups of co-regulated genes (Figure 3). Clusters E and G consist of genes that are members of the GA-regulon (Table 1) and represent genes involved in the release and utilization of GA. Cluster F also consists mostly of genes that are part of the GA-regulon (Table 1, 2). Genes in Cluster F, like genes in Clusters E and G, are expressed in the reference strain on GA and SBP at 2 and 8h, but unlike genes in Clusters E and G also expressed in the *ΔgaaR* strain on SBP at 2 and 8h. Cluster F mainly includes pectinases acting on RG-I side-chains. Their expression profile indicates that they are regulated by GaaR as well as other TFs involved in pectin degradation. Genes in Clusters A, B, C and D are generally expressed in a GaaR independent fashion and represent pectinases acting on RG-I and XGA. Pectinase genes of Cluster D are predominantly expressed in the *ΔgaaR* strain on SBP at 2 and 8h. Genes in Clusters A, B and C are expressed predominantly in the reference strain and *ΔgaaR* on SBP at 24h or in *ΔgaaR* on GA, suggesting that these genes are likely induced on starvation or derepressed conditions.

In conclusion, in this paper we showed that the conserved Zn_2Cys_6 TF GaaR of *A. niger* is required for the utilization of GA and PGA. We also showed that GaaR is essential for GA utilization from complex pectic substrates and that residual growth of *ΔgaaR* on complex pectins is likely due to induction of pectinases releasing L-rhamnose from the RG-I backbone and L-arabinose and D-galactose from the RG-I “hairy regions”. These monosaccharides are metabolized independently of *gaaR*. With the identification of the GaaR in *A. niger*, we identified the missing link to further understand the interplay between several TFs involved in

plant cell wall degradation. Insight in the regulation of pectin degradation and GA utilization in *A. niger* can help in exploiting *A. niger* for more efficient pectinase production.

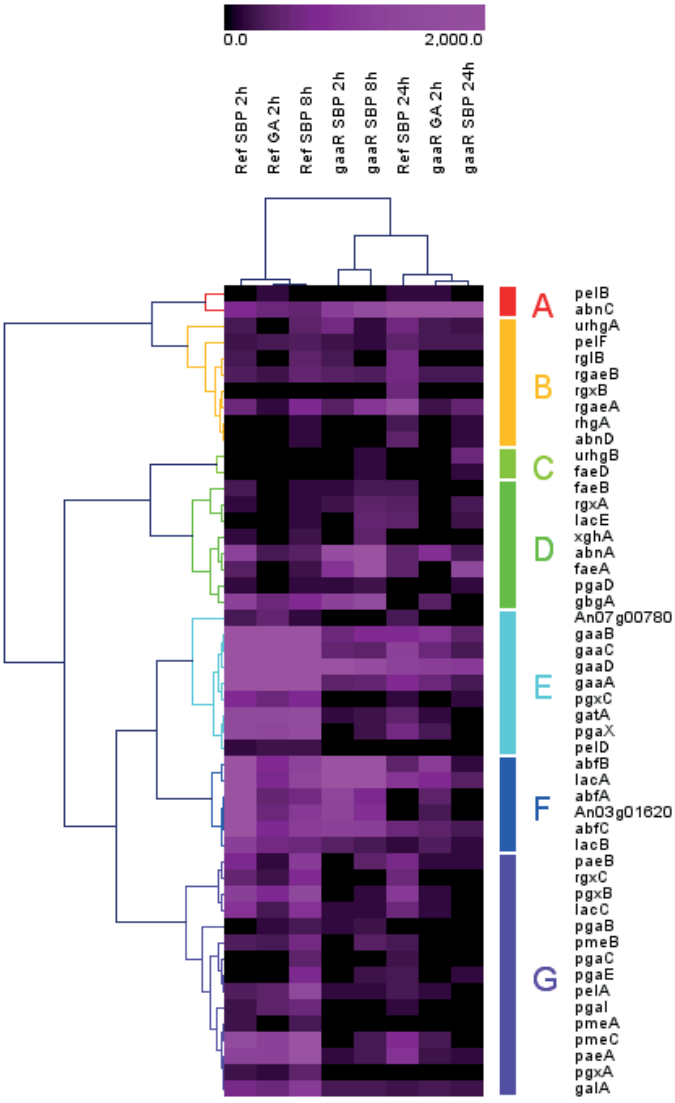

Figure 3 Hierarchical clustering of pectin utilization genes according to their expression in the reference strain (FP-1132.1) and *ΔgaaR* (FP-1126.1) on GA and SBP. The color code displayed represents the transcript levels of the genes. Clusters E and G include genes that are members of the GA-regulon.

Acknowledgements

EA and JN were supported by a grant from BE-Basic (Flagship 10) and the China Scholarship Council, respectively. JEK and MVAP were supported by a grant of the Dutch Technology Foundation STW, Applied Science division of NWO and the Technology Program of the Ministry of Economic Affairs 016.130.609 to RPdV. We thank Patricia vanKuyk and Mark Arentshorst for constructing the *kusA* deletion strains. The pectin composition analysis was conducted at Complex Carbohydrate Research Centre and supported by the Chemical Sciences, Geosciences and Biosciences Division, Office of Basic Energy Sciences, U.S. Department of Energy grant (DE-FG02-93ER20097) to Parastoo Azadi.

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Supporting information

The supplementary material of this chapter are available via

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5111758/ and comprises the following:

Table S1 Strains used in this study

Table S2 Primers used in this study. Overlapping sequences for fusion PCR are written in bold.

Table S3A RNA-seq analysis of pectinases on GA and SBP. Expression values (FPKM) are averages of duplicates. Fold changes ≥2 and p-values ≤0.05 are highlighted.

Table S3B RNA-seq analysis of pectinases on GA and SBP. Expression values (FPKM) are averages of duplicates. Fold changes ≥2 and p-values ≤0.05 are highlighted.

Table S3C RNA-seq analysis of pectinases on GA and SBP. Expression values (FPKM) are averages of duplicates. Fold changes ≥2 and p-values ≤0.05 are highlighted.

Table S3D RNA-seq analysis of pectinases on GA and SBP. Expression values (FPKM) are averages of duplicates. Fold changes ≥2 and p-values ≤0.05 are highlighted.

Figure S1 Alignment of AnGaaR and BcGaaR using EMBOSS Needle with standard settings (http://www.ebi.ac.uk/Tools/psa/emboss_needle/)

Figure S2 Verification of the gaaR deletion strain in the MA234.1 background. A) Strategy and primer design for disruption the gaaR gene using the split marker method [27]. Primers P1 till P8 correspond to primers gaaRP1f, gaaRP2r, gaaR3Pf, gaaRP4r, hygP6f, hygP9r, hygP8f and hygP7r in Table S2. B) Schematic representation of the gaaR locus in the reference strain and after gaaR deletion. Predicted sizes of the DNA fragment hybridizing with the indicated probes are shown. C) Southern blot analysis of genomic DNA of MA234.1 (lane 1), JN35.1 (lane 2), JN35.2 (lane 3), JN36.1 (lane 4), JN37.4 (lane 5) and JN37.5 (lane 6). Left panel: agarose gel stained with ethidium bromide. Marker size (M, in kb) is indicated. Right panel: Southern blot after hybridization. D) Growth analysis of the reference strain MA234.1, gaaR deletion strains and complemented strains on MM with 50 mM or 1% different carbon sources.

Figure S3 Transcript levels of pectinases acting on RG-I backbone in A. niger reference and ΔgaaR on GA or SBP. A) exo-rhamnogalacturonases and rhamnogalacturonase A B) rhamnogalacturonan lyases, rhamnogalacturonan acetyl esterases and rhamnogalacturonyl hydrolases. Mycelia of the reference strain (FP-1132.1) and ΔgaaR (FP-1126.1) were pre-grown in CM with 2% D-fructose, washed and transferred to MM with 25mM GA or 1% SBP in and incubated for 2, 8 or 24h.