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

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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 the expression of pectinases acting on rhamnogalacturonan and subsequent metabolism of the monosaccharides other than GA.

Keywords: gene regulation; pectinase; polygalacturonic acid; transcriptomics; Zn_2Cys_6 transcription factor

Pectins are complex heterogeneous polysaccharides found in plant cell walls. Four substructures 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) [1]. 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 [1]. 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 [1,2]. 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 [1].

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* [3–5]. 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 [6]. GA is then catabolized into pyruvate and glycerol [7], through a pathway consisting of four enzymes: GaaA, D-galacturonate reductase; GaaB, L-galactonate

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.

dehydratase; GaaC, 2-keto-3-deoxy-L-galactonate aldolase; and GaaD, L-glyceraldehyde reductase [7]. Deletion of *gaaA*, *gaaB*, or *gaaC* abolished growth on GA as the sole carbon source [8–10]. *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 [11].

The production of extra- and intracellular enzymes in *A. niger* is regulated by a network of transcription factors (TFs) [12]. 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 [13,14]. 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 [9,10,13].

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 coregulated genes [12,15,16]. 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* [17–21]. 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 [17,18,20], suggesting that the utilization of GA, the main component of this substrate, is not affected.

Martens-Uzunova and Schaap [7] 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*). 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 [7]. Deletion and mutational analysis of GARE showed that the element is required for GA-induced gene expression in both *A. niger* and *Botrytis cinerea* [14,22]. 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 [22]. 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 [23] with a deletion construct (*kusA::amdS*) [24] 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*$^-$) [25] 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 counterselection as described [26] 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 [26]. 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, Amsterdam, the Netherlands), GA (Chemodex, St Gallen, Switzerland), L-rhamnose (Fluka, Zwijndrecht, the Netherlands), L-arabinose (Sigma-Aldrich, Zwijndrecht, the Netherlands), or D-xylose (Merck, Amsterdam, the Netherlands), and 1% (w/v) PGA (Sigma, Zwijndrecht, the Netherlands), SBP (Pectin Betapec RU301 Herbstreith & Fox KG, Neuenbürg, Germany), citrus pectin (CP) (Acros Organics, Geel, Belgium), 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 0.9 % (w/v) NaCl solution 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^{-1} in 100-mL complete medium (CM) (pH 5.8) with 2% (w/v) D-fructose (Sigma-Aldrich) and were pregrown for 16 h. 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 6 h. For

RNA-seq analysis, 2.5 g of pregrown mycelia were transferred to 50 mL MM (pH 4.5) with 25 mM GA and incubated for 2 h or to 50 mL MM with 1% SBP and incubated for 2, 8, or 24 h. All incubations were performed in rotary shaker at 30 °C and 250 r.p.m.

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 [26]. 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 [27] 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, Leiden, the Netherlands) 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, Landsmeer, the Netherlands), amplified in the *E. coli* strain DH5α 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 (*ΔgaaR*) were pregrown in CM with D-fructose. At the time of transfer ($t = 0$) and 2, 4, and 6 h 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, Düren, Germany) following the protocol provided by the supplier, including the rDNase treatment. Total RNA samples were stored at −80 °C. Quantitation and purity assessment of total RNA was done by spectrophotometric method (NanoDrop 2000; Thermo Scientific, Breda, the Netherlands). Standard molecular techniques were applied as described [28]. About 3.5 μg RNA was loaded per sample and hybridized with [α-32P]-dCTP labeled 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 (*ΔgaaR*) was ground in Tissue Lyser II (Qiagen, Venlo, the Netherlands) and RNA was extracted using TRIzol reagent (Invitrogen, Breda, the Netherlands) 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™ 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, China). 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 [29] and BWA software [30]. 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) [31] using RSEM tool [32]. 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 cutoff 0.05. The RNA-seq data have been submitted to Gene Expression Omnibus (GEO) [33] with accession number: GSE80227. Homology searches were performed using the BLASTP algorithm from NCBI against the nonredundant database and proteins with an *E*-value ≤ 1E-50 were defined as homologous [34]. Hierarchical clusters using the average expression values of genes were made via GENESIS 1.7.7 [35] 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) [22]. 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 (Fig. 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 (Fig. S2 and data not shown). The growth phenotype of the *ΔgaaR* strains was analyzed on different monomeric and polymeric carbon sources (Fig. 1A). Deletion of *gaaR* in the AB4.1 background (MA234.1, Fig. S2) and N593 background (N593.20, Fig. 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

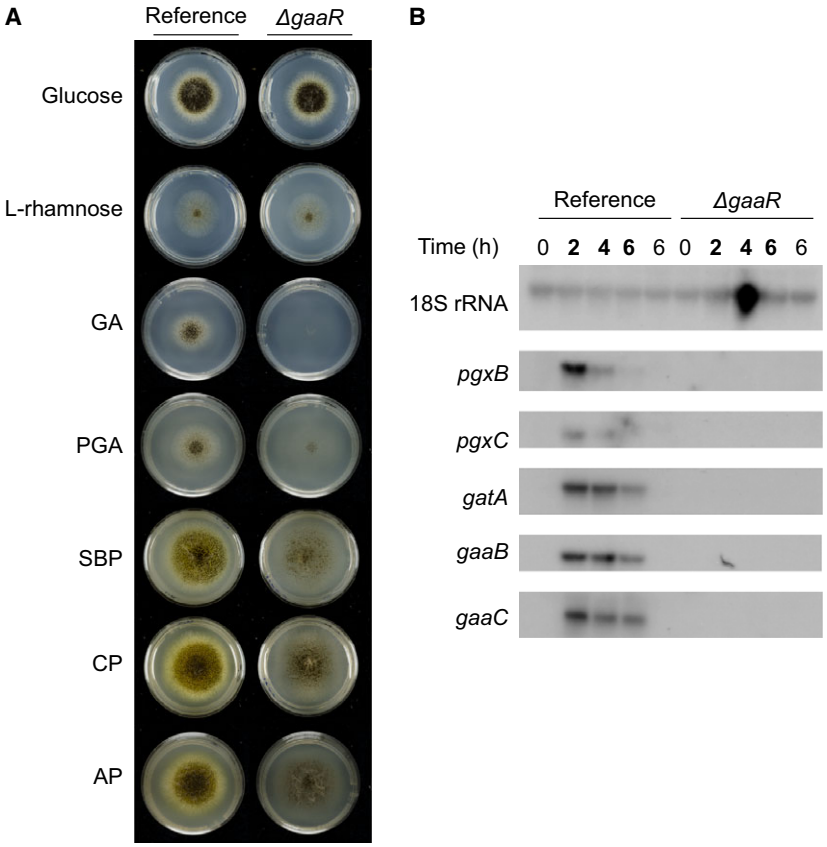


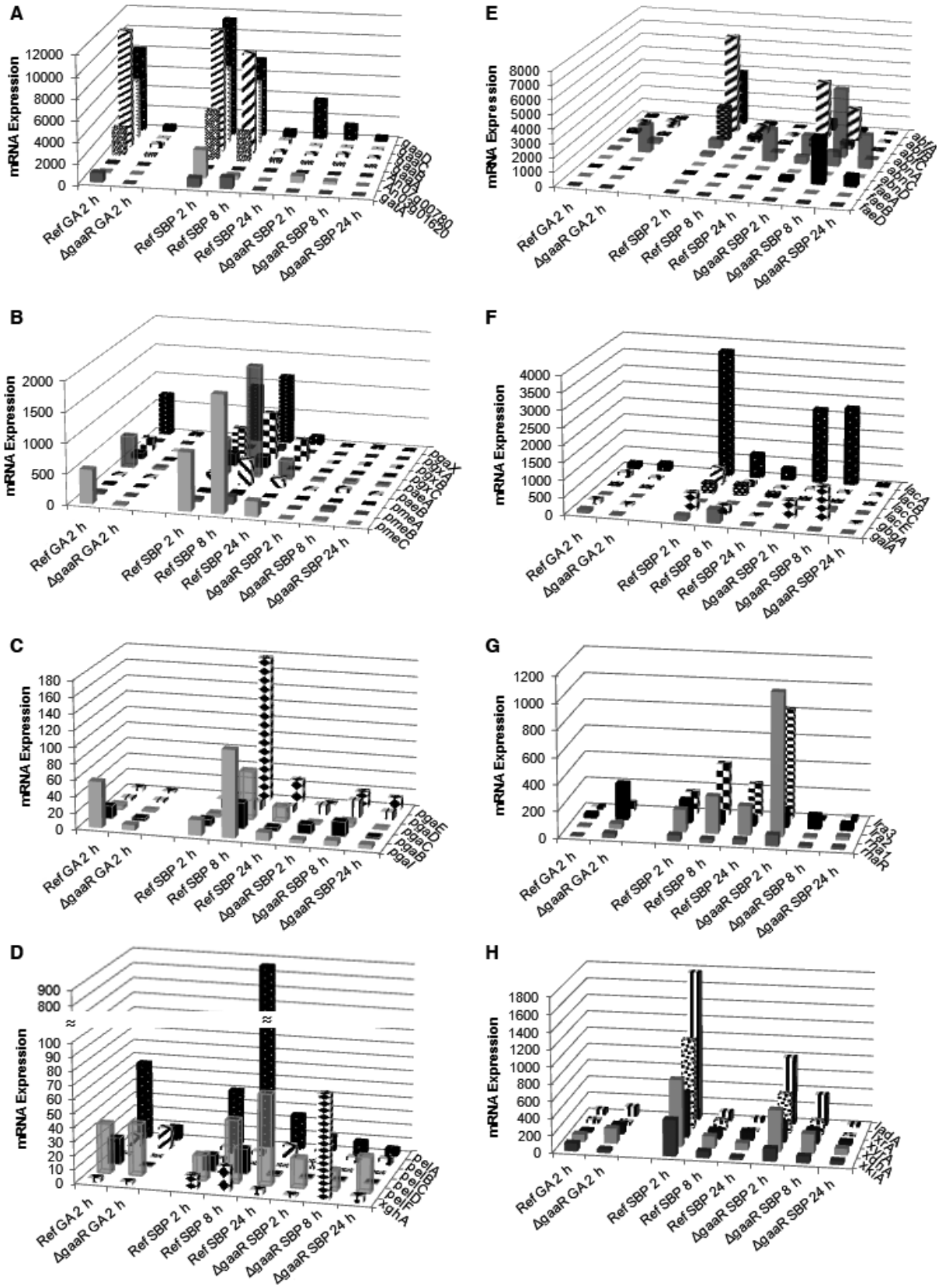
Fig. 1. Phenotypic and gene expression analyses of *A. niger ΔgaaR* (A) Growth profile of the reference strain (FP-1132.1) and *ΔgaaR* (FP-1126.1) on MM with 25 mM monomeric and 1% polymeric carbon sources. Strains were grown for 4 days at 30 °C. (B) Northern blot analysis of selected GA-induced genes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

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.

NRRL3 protein ID	CBS 513.88 gene ID	Gene name	Ref GA 2 h	*ΔgaaR* GA 2 h	Fold change Ref/*ΔgaaR* GA 2 h	*P*-value	GARE (CCNCCAA) position
NRRL3_00958	An14g04280	*gatA*[a]	888.35	13.32	66.69	1.54E-03	+ strand −360
NRRL3_08663	An03g01620	GA transporter (putative)[a]	106.09	30.34	3.50	1.25E-01	+ strand −673
NRRL3_04281	An07g00780	GA transporter (putative)[a]	90.41	1.86	48.74	7.77E-03	− strand −42 and −994
NRRL3_05650	An02g07710	*gaaA* [a]	2599.98	117.53	22.12	1.69E-04	+ strand −414 and −100
NRRL3_06890	An16g05390	*gaaB* [a]	11309.00	344.03	32.87	1.88E-03	+ strand −326
NRRL3_05649	An02g07720	*gaaC*[a]	5658.32	106.21	53.27	2.98E-04	− strand −292 and −606
NRRL3_10050	An11g01120	*gaaD*[a]	8104.43	506.79	15.99	7.01E-03	− strand −538, −583, −801 and −813
NRRL3_03144	An12g07500	*pgaX*[a]	698.90	24.27	28.80	1.19E-02	+ strand −388
NRRL3_09810	An11g04040	*pgxA*[a]	10.65	0.34	31.32	9.10E-03	− strand −594
NRRL3_08281	An03g06740	*pgxB*[a]	200.31	12.39	16.17	2.62E-02	− strand −298 and −823
NRRL3_05260	An02g12450	*pgxC*[a]	99.93	4.10	24.40	6.24E-04	+ strand −268 and − strand −642
NRRL3_06053	An02g02540	*paeA* (putative)[a]	522.81	22.99	22.75	4.57E-03	+ strand −1238
NRRL3_04916	An07g08940	*paeB* (putative)[c]	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)[b]	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*[c]	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*[c]	4.26	2.40	1.78	4.12E-01	
NRRL3_00965	An14g04370	*pelA*[a]	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*[c]	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*[c]	87.96	59.62	1.48	5.81E-01	
NRRL3_10865	An08g01710	*abfC* (putative)[a]	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

[a]Genes identified as the GA-regulon by Martens-Uzunova and Schaap [7]. [b]*pmeC* not present on the Affymetrix microarray. [c]Genes not significantly differentially expressed on GA, but differentially expressed on SBP (Table 2).

Fig. 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-endogalactanase; (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 pregrown in CM with 2% D-fructose, washed and transferred to MM with 25 mM GA or 1% SBP and incubated for 2, 8 or 24 h.


A
mRNA Expression
12000
10000
8000
6000
4000
2000
0
Ref GA 2 h
ΔgaaR GA 2 h
Ref SBP 2 h
Ref SBP 8 h
Ref SBP 24 h
ΔgaaR SBP 2 h
ΔgaaR SBP 8 h
ΔgaaR SBP 24 h
gaaD
gaaC
gaaB
gaaA
An02g00780
An01g01620
galX
B
mRNA Expression
2000
1500
1000
500
0
pgaX
pgxA
pgxB
pgxC
paeA
paeB
pmeA
pmeB
pmeC
C
mRNA Expression
180
160
140
120
100
80
60
40
20
0
pgaE
pgaD
pgaC
pgaB
pgaI
D
mRNA Expression
900
800
100
90
80
70
60
50
40
30
20
10
0
pelA
pelB
pelC
pelE
pelD
xghA
E
mRNA Expression
8000
7000
6000
5000
4000
3000
2000
1000
0
abfA
abfB
abfC
abnA
abnC
abnD
faeA
faeB
faeD
F
mRNA Expression
4000
3500
3000
2500
2000
1500
1000
500
0
lacA
lacB
lacC
lacE
gtaA
galA
G
mRNA Expression
1200
1000
800
600
400
200
0
rra3
rra2
rra1
rhaR
H
mRNA Expression
1800
1600
1400
1200
1000
800
600
400
200
0
xdaA
xlrA
xlnA
xgtA
xkiA

other carbon sources tested (Fig. 1A, Fig. S2). The strongly reduced growth of *ΔgaaR* on GA and PGA was fully complemented by reintroducing the *gaaR* gene ectopically (Fig. 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*) [7,14]. 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* that made the AB4.1 background were pregrown 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* (Fig. 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 pregrown 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 [7] is dependent on GaaR (Table 1 and Fig. 2). The only exception is a putative GA transporter (An03g01620) that is expressed more than threefold 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 [7] 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.

To identify additional pectinase genes controlled directly or indirectly by GaaR, the expression of all 58 pectinolytic genes [3] was examined (Table S3). An overview of the gene abbreviations and their (putative) function is given in Martens-Uzunova and Schaap [3]. This analysis resulted in the identification of several additional pectinase genes for which the expression on GA is dependent of GaaR (Table 1 and Fig. 2, Fig. 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 [7]. 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.

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 (Fig. 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 24 h 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 Fig. 2, Fig. 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 (Fig. 2A–D). This observation strongly suggests that *ΔgaaR* is not utilizing GA from SBP. The 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 [11] 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 Fig. 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*.

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

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.

NRRL3 protein ID	CBS 513.88 gene ID	Gene name	Ref SBP 2 h	*ΔgaaR* SBP 2 h	Fold change Ref/*ΔgaaR* SBP 2 h	*P*-value	Ref SBP 8 h	*ΔgaaR* SBP 8 h	Fold change Ref/*ΔgaaR* SBP 8 h	*P*-value	Ref SBP 24 h	*ΔgaaR* SBP 24 h	Fold change Ref/*ΔgaaR* SBP 24 h	*P*-value
NRRL3_00958	An14g04280	*gatA*[a]	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)[a]	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)[a]	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*[a]	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*[a]	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*[a]	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*[a]	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*[a]	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*[a]	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*[a]	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*[a]	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)[a]	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)[c]	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)[b]	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*[c]	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*[c]	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*[a]	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*[c]	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*[c]	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

Table 2. (Continued).

NRRL3 protein ID	CBS 513.88 gene ID	Gene name	Ref SBP 2 h	*ΔgaaR* SBP 2 h	Fold change Ref/*ΔgaaR* SBP 2 h	*P*-value	Ref SBP 8 h	*ΔgaaR* SBP 8 h	Fold change Ref/*ΔgaaR* SBP 8 h	*P*-value	Ref SBP 24 h	*ΔgaaR* SBP 24 h	Fold change Ref/*ΔgaaR* SBP 24 h	*P*-value
NRRL3_10865	An08g01710	*abfC* (putative)[a]	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

[a]Genes identified as the GA-regulon by Martens-Uzunova and Schaap [7]. [b]*pmeC* not present on the Affymetrix microarray. [c]Genes not significantly differentially expressed on GA, but differentially expressed on SBP.

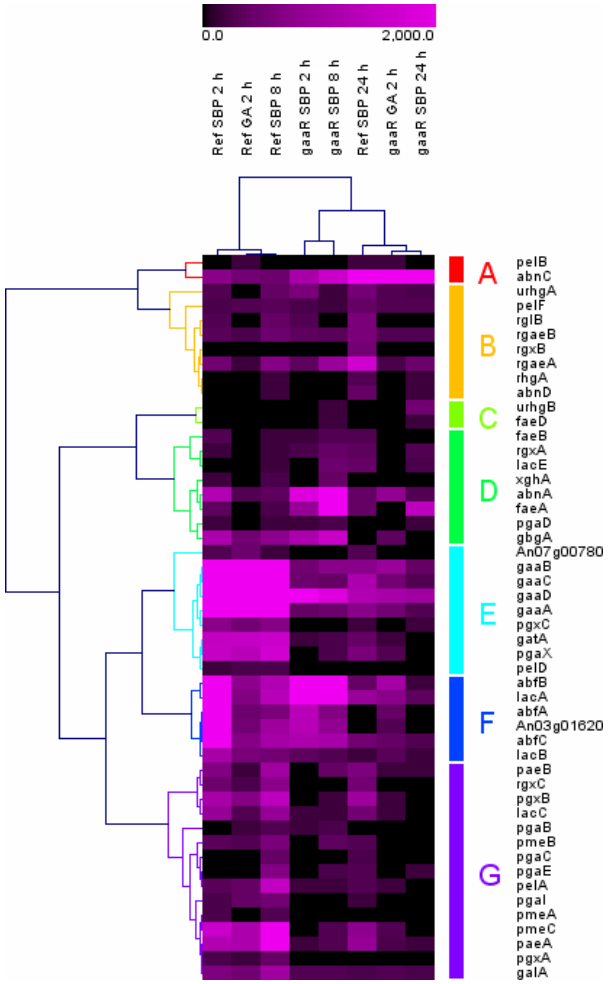


Fig. 3. Hierarchical clustering of pectin utilization genes according to their expression in the reference strain (FP-1132.1) and *ΔgaaR* (FP-1126.1) on GA and SBP. The color code displayed represents the transcript levels of the genes. Clusters E and G include genes that are members of the GA-regulon.

used in this study was performed as described previously [36] 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* (Fig. 2E–H, Fig. 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 coregulated genes (Fig. 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 (Tables 1 and 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 8 h, but unlike genes in Clusters E and G also expressed in the *ΔgaaR* strain on SBP at 2 and 8 h. 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 8 h. Genes in Clusters A, B, and C are expressed predominantly in the reference strain and *ΔgaaR* on SBP at 24 h 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.

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Author contributions

EA, JN and JEK performed experiments, MP, MVAP and EA performed bioinformatics analysis, all authors analyzed results, EA, JEK, JALK, JV, RPV and AFJR wrote the manuscript with input of all authors, RPV and AFJR designed experiments and supervised the study.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

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

Fig. S2. Verification of the *gaaR* deletion strain in the MA234.1 background.

Fig. S3. Transcript levels of pectinases acting on RG-I backbone in *A. niger* reference and *ΔgaaR* on GA or SBP.

Table S1. Strains used in this study.

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

Table S3. RNA-seq analysis of pectinases on GA and SBP.