The pathway intermediate 2-keto-3-deoxy-L-galactonate mediates the induction of genes involved in D-galacturonic acid utilization in Aspergillus niger

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Pectins are heterogeneous plant cell wall polysaccharides rich in D-galacturonic acid (GA). They represent a natural carbon source for many saprotrophic fungi including Aspergillus niger [1,2]. The A. niger genome contains 58 genes encoding pectin-degrading enzymes [2,3]. GA, the most abundant uronic acid in pectin, is transported by A. niger into the cell via the transporter GatA [4] and then catabolized into pyruvate and glycerol by consecutive action of four enzymes: GaaA, D-galacturonate reductase; GaaB, L-galactonate dehydratase; GaaC, 2-keto-3-deoxy-L-galactonate aldolase; and GaaD, L-glyceraldehyde reductase [5–8] (Fig. 1A). This four-step GA catabolic pathway is evolutionarily conserved in Pezizomycotina fungi [5], and has been studied in detail in Botrytis cinerea [9] and Trichoderma reesei [10–13]. In B. cinerea, the first enzymatic step is catalyzed by two functionally redundant enzymes, BcGar1 and the A. niger GaaA ortholog BcGar2 [9]. In T. reesei, GA is converted into L-galactonate by TrGar1 [10]. In addition, GaaA and GaaD (LarA) of A. niger have been shown to be involved in D-glucuronate and L-arabinose catabolism, respectively [14,15].

Degradation of plant cell wall polysaccharides and subsequent transport and catabolism of released sugars are tightly controlled [16]. Genes required for pectin degradation, GA transport, and GA catabolism are subject to carbon catabolite repression via CreA [17,18]. They are specifically induced in the presence of GA [5,17] and are regulated by the GaaR/GaaX activator-repressor module [19,20]. The conserved Zn(II) 2Cys6 transcription factor GaaR is required for

Keywords: D-galacturonic acid catabolism; gene regulation; pectinase

Abbreviations
AP, apple pectin; CM, complete medium; GA, D-galacturonic acid; MM, minimal medium; NMR, Nuclear Magnetic Resonance Spectroscopy; PGA, polygalacturonic acid; RG-I, rhamnogalacturonan I; a-IPM, α-isopropylmalate.
growth on GA and for the activation of the GA-responsive genes in both *B. cinerea* and *A. niger* [19,21].

The mechanism of activation of transcription factors can be diverse, and possibly requires so-called inducer molecules. These inducer molecules are often metabolites related to the substrate [22]. Only a few examples of activation of a transcription factor via an inducer have been elucidated in fungi. Probably the best studied example is the Zn(II)2Cys6 transcription factor Gal4p that regulates galactose utilization in *Sacccharomyces cerevisiae*. Gal4p is repressed under noninducing conditions because the transcriptional activation domain of Gal4p is bound to the corepressor Gal80p. In the presence of galactose and ATP (inducing conditions), the sensor protein Gal3p binds to the Gal4p/Gal80p complex leading to dissociation of Gal4p and subsequent Gal4p-dependent transcription [23–27]. In the regulation of leucine biosynthesis, the Zn(II)2Cys6 transcription factor Leu3p interacts directly with a metabolic intermediate. The middle domain of the Leu3p protein masks the C-terminal activation domain by an intramolecular interaction in the absence of α-isopropylmalate (α-IPM), a metabolic intermediate of the leucine biosynthesis pathway. In the presence of α-IPM, which accumulates during leucine starvation, this self-masking is prevented, resulting in active Leu3p and activation of leucine biosynthesis genes [28–30]. The Gal4p and Leu3p transcription factors localize to the nucleus regardless of the presence or absence of inducer molecules [31,32]. On the other hand, the transcriptional activator AmyR, involved in starch degradation in *Aspergillus nidulans* and *Aspergillus oryzae*, is translocated from the cytoplasm to the nucleus only in the presence of its inducer isomaltose [33–35].

Fig. 1. (A) The evolutionarily conserved GA catabolic pathway in filamentous fungi as proposed by Martens-Uzunova and Schaap [5]. GA is converted in pyruvate and glycerol by consecutive action of GaaA, GaaB, GaaC, and GaaD enzymes. Growth profile of the reference strain (MA249.1) and GA catabolic pathway deletion mutants ΔgaaA, ΔgaaB, ΔgaaC, and ΔgaaD (B) on solid MM without any carbon source, or with 50 mM monomeric or 1% polymeric carbon sources after 7 days at 30 °C, and (C) in microtiter plate in liquid medium with 50 mM GA at 30 °C. Error bars represent standard deviation of six biological replicates.
In *A. niger*, GA or a derivative of GA was suggested to act as an inducer required for the activation of GA-responsive genes [17]. In *B. cinerea*, BcGaaR was shown to translocate from the cytoplasm to the nucleus in response to such an inducer [21]. Previous studies of *A. niger* and *B. cinerea* mutants disrupted in GA catabolic pathway did not unambiguously identify a specific inducer [6–9]. In this study, we constructed GA catabolic pathway deletion mutants (ΔgaaA, ΔgaaB, ΔgaaC, and ΔgaaD) to gain insight into regulation of GA-responsive genes in *A. niger*. Comparative analysis of these mutants indicates that 2-keto-3-deoxy-L-galactonate acts as the physiological inducer of the GA-responsive genes.

**Materials and methods**

**Strains, media and growth conditions**

All strains used in this study are listed in Table S1. MA249.1 was obtained by transformation of N593.20 (cspA1, pyrG, kuxA::umdS) [19] with a 3.8-kb *XhoI* fragment containing the *A. niger* pyrG gene, resulting in the full restoration of the pyrG locus.

Media were prepared as described previously [36]. Radial growth phenotype analyses were performed with minimal medium (MM) (pH 5.8) containing 1.5% (w/v) agar (Scharlau, Barcelona, Spain) and various carbon sources: 50 mM glucose (VWR International, Amsterdam, the Netherlands), α-fructose (Sigma-Aldrich, Zwijndrecht, the Netherlands), GA (Chemodex, St Gallen, Switzerland), L-rhamnose (Fluka, Zwijndrecht, the Netherlands), L-arabinose (Sigma-Aldrich) or glycerol (Glycerol 87%; BioChemica AppliChem, Darmstadt, Germany), or 1% (w/v) polygalacturonase acid (PGA) (Sigma), apple pectin (AP) (Sigma-Aldrich), or galactan (Acros Organics, Geel, Belgium). Filter sterilized α-fructose or GA solution was added after autoclaving MM with agar. Other carbon sources were autoclaved together with the medium. The plates were inoculated with 5 µL 0.9% NaCl containing 10^6 freshly harvested spores and cultivated at 30 °C for 7 days. For microtiter plate growth phenotype analysis, wells in a 96-well, flat bottom plate ( Sarstedt AG & Co., Nürnberg, Germany) were filled with 180 µL MM (pH 5.8) containing 55 mM GA as the sole carbon source, and 20 µL freshly harvested spores (7.5 x 10^5 spores·mL−1). The plate was incubated with lids in EnSpire Multimode Plate Reader (PerkinElmer, Waltham, MA, USA) at 30 °C. Lid temperature was set to 32 °C to prevent condensation on the lid. Optical density at 600 nm was measured every hour. The average OD from the GA-containing control wells was subtracted from the OD of the test wells and negative values were corrected as zero.

For gene expression and metabolic analyses, 10^8 freshly harvested spores were inoculated and grown in 100 mL complete medium (CM) (pH 5.8) with 2% (w/v) α-fructose for 16 h, and mycelia were harvested by filtration through sterile mycelloid. For northern blot and metabolic analyses, pregrown mycelia were washed twice with MM with no carbon source (pH 4.5) and 1.5 g (wet weight) mycelia were transferred and incubated in 50 mL MM (pH 4.5) with 50 mM α-fructose or 50 mM GA for 2 h. For metabolic analysis, 1.5 g (wet weight) mycelia were transferred and incubated in 50 mL MM (pH 4.5) with 50 mM GA for 55 h. Additionally, 30 g (wet weight) mycelia of SDP20.6 (AgaA) were transferred and incubated in 1 L MM (pH 4.5) with 50 mM GA for 55 h. For RNA-seq analysis, pregrown mycelia were washed with MM with no carbon source (pH 6) and 2.5 g (wet weight) were transferred to 50 mL MM (pH 6) with 25 mM GA and grown for 2 h. All incubations were carried out in a rotary shaker at 30 °C and 250 r.p.m.

**Construction of gene deletion strains**

Protoplast-mediated transformation of *A. niger*, purification of the transformants and genomic DNA extraction were performed as described [36].

To construct the deletion cassettes, 5' and 3' flanks of the *gaaA, gaaB, gaaC*, and *gaaD* genes were PCR-amplified using the primer pairs listed in Table S2 with N402 genomic DNA as template. For all cloning experiments *Escherichia coli* strain DH5α was used. To create SDP22.1 (AgaA), SDP21.5 (AgaB), and SDP20.6 (AgaC), gene deletion cassettes were made using MultiSite Gateway Three-fragment Vector Construction Kit (Invitrogen, Carlsbad, CA, USA) according to the supplier’s instructions. *Aspergillus oryzae pyrG* gene flanked by AttB1 and AttB2 sites was amplified by PCR using the primer pairs listed in Table S2 and plasmid pMA172 [37] as template. *gaaA, gaaB*, and *gaaC* deletion cassettes containing 5' and 3' flanks of the target genes with *A. oryzae pyrG* gene in between were obtained by restriction digestion. To create EA1.1 (AgaD), 5' flank of *gaaD* was ligated into pJET1.2/blunt cloning vector (Thermo Fisher Scientific, Carlsbad, CA, USA) and amplified in *E. coli*. Following plasmid isolation, the 5' flank was excised using restriction enzymes *KpnI* and *XhoI*, ligated into *KpnI*-*XhoI* opened pBluescript II SK(+) (Agilent Technologies, La Jolla, CA, USA) and amplified in *E. coli*. *Aspergillus oryzae pyrG* gene was obtained from plasmid pMA172 [37] by restriction digestion with *HindIII* and *XhoI*. Isolated pBluescript II SK(+) plasmid containing the 5' flank was opened with restriction enzymes *XhoI* and *NotI*, and the *A. oryzae pyrG* gene as *XhoI-NotI* fragment and *HindIII-NotI* fragment of the *gaaD* 3' flank were ligated into the plasmid. Ligation product was amplified in *E. coli* and the linear deletion cassette was obtained by PCR amplification from the plasmid using...
primers gaaDP1-KpnI and gaaDP4-NotI. Deletion cassettes were introduced into the pyrG strain N593.20. Gene deletions were confirmed via southern blot analysis.

Gene expression analysis

Northern blot and RNA-seq analyses were performed as described [19] with minor modifications: For northern blot analysis, total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Probes were PCR-amplified using the N402 genomic DNA and the primer pairs listed in Table S2.

Chemical analysis

One milliliter culture samples were taken 7, 24, 31, 48, and 55 h after the transfer of mycelia to MM with GA. About 250 µL of each culture sample was centrifuged at 16 000 g for 30 min and the supernatant was transferred to a new microfuge tube. After adding 1 x volume of cold methanol (−20 °C), the sample was incubated on ice for 15 min and centrifuged at 16 000 g for 30 min. The supernatant was collected in a new microfuge tube and 1 x volume of 0.1% formic acid was added. Metabolites in the extracellular culture fluids were analyzed by high pressure liquid chromatography–high-resolution mass spectrometry. Aliquots were loaded, using a Series 200 micropump (PerkinElmer), onto a reversed-phase Eclipse C18 2.1 x 150 mm column (Agilent, Santa Clara, CA, USA) connected in-line to a 7 Tesla LTQ-FT-ICR mass spectrometer (Thermo Electron Corporation, San Jose, CA, USA) and negative mode electrospray ionization spectra were acquired at a resolution of 100 000 at 200 m/z. Absolute GA concentration was calculated using a standard dilution calibration curve of commercially obtained GA (Chemodex). Standards for L-galactonate and 2-keto-3-deoxy-L-galactonate were not available, therefore, these metabolites were assigned based on accurate mass alone (matched within a 5 p.p.m. m/z window) and relative amounts in terms of extracted ion chromatograms peak areas were compared. One liter culture of SDP20.6 (AgaaC) was filtered through sterile myrcloth 55 h after the transfer of mycelia to MM with GA, and the filtrate was stored at −80 °C. After freeze-drying, dry materials from SDP20.6 (AgaaC) extracellular culture fluid were dissolved in D2O (Sigma Aldrich) for structural investigation by Nuclear Magnetic Resonance Spectroscopy (NMR). Spectra were recorded with a Varian VNMR-500 MHz at 25 °C. The presence of 2-keto-3-deoxy-L-galactonate was confirmed by 1H-NMR and 13C-NMR.

Bioinformatics

RNA-seq data were analyzed as described previously [19]. Differential expression was identified by Student’s t-test with a P-value cut-off of 0.05. RNA-seq data for FP-1132.1 (reference strain) and SDP20.6 (AgaaC) were submitted to Gene Expression Omnibus [38] with accession numbers GSE80227 [19] and GSE95776 (this study), respectively.

Results

Growth analysis of α-galacturonic acid catabolic pathway deletion mutants

Aspergillus niger GA catabolic pathway deletion mutants, ΔgaaA, ΔgaaB, ΔgaaC, and ΔgaaD, were constructed and were verified by southern blot analysis (Fig. S1). We compared the growth phenotype of the strains on monomeric and polymeric carbon sources (Fig. 1, Fig. S2). Disruption of gaaA and gaaD resulted in reduced growth and sporulation on plates containing GA or PGA as carbon source. However, both mutants showed better growth on plates containing MM with GA compared to plates containing MM with no carbon source, indicating that they can still metabolize GA. The ΔgaaB and ΔgaaC mutants exhibited a more drastically reduced growth on plates containing GA, PGA, or AP (Fig. 1B). The growth defects of the GA catabolic pathway deletion mutants on GA plates were confirmed in microtiter plate-based growth assays (Fig. 1C, Fig. S2A). None of the GA catabolic pathway deletion mutants exhibited defects in growth on other carbon sources tested, except that the deletion of gaaD, also known as the l-arabinose reductase gene larA, resulted in a poor growth on l-arabinose (Fig. S2B), confirming previous observations [15]. The inability of ΔgaaB or ΔgaaC to use GA as a carbon source suggests that there are no functionally redundant enzymes capable of replacing GaaB and GaaC.

ΔgaaB and ΔgaaC accumulate the α-galacturonic acid catabolic pathway intermediates L-galactonate and 2-keto-3-deoxy-L-galactonate, respectively

Since the roles of GaaB and GaaC in GA catabolism cannot be replaced by redundant enzymes, we expect the accumulation in the medium of the corresponding enzyme substrate in ΔgaaB and ΔgaaC, as shown previously [7,8]. The extracellular GA concentration and the extracellular metabolites were examined by FTICR mass spectrometry over time during growth in GA. This analysis revealed that the reference strain utilized all GA in the medium within 48 h of growth, whereas in the GA catabolic pathway deletion mutants GA was still present in the medium after 55 h of
growth (Fig. 2A). In ΔgaaA and ΔgaaD, the concentration of GA gradually decreased to approximately 45% of the initial GA concentration in the medium, which reflects the slow catabolism of GA in these mutants. ΔgaaB consumed about 35% of the initial GA in 55 h and secreted α-galactonate. The time course consumption of GA by ΔgaaB was proportional to its release of α-galactonate (Fig. 2A). The ΔgaaC mutant took up about 78% of the initial GA in 55 h, and extracellular 2-keto-3-deoxy-α-galactonate accumulated in the medium of the ΔgaaC mutant over time (Fig. 2A). The presence of 2-keto-3-deoxy-α-galactonate in the extracellular culture fluid of the ΔgaaC mutant was confirmed by structural resolution by 1H-NMR and 13C-NMR (Fig. S3).

Expression of α-galacturonic acid-responsive genes is increased in ΔgaaC

Genes involved in the degradation of the pectic substructures PGA (e.g., NRRL3_03144 exo-polygalacturonase and pgx28B) and rhamnogalacturonan I (RG-I) (e.g., NRRL3_10865 αrabinofuranosidase), GA transport (gatA), and GA catabolism (gaaA-D) have been shown to be induced in the presence of GA [5,18] and are part of the proposed GaaR/GaaX panregulon [19]. To test the effect of the GA catabolic pathway gene deletions on the induction of GA-responsive genes, northern blot analysis was performed. The reference and ΔgaaA, ΔgaaB, ΔgaaC and ΔgaaD strains were pregrown in α-fructose medium and transferred to either GA or α-fructose medium. Rapid induction of gatA, gaaA, gaaB, gaaC, gaaD, and NRRL3_10865 was observed in the reference strain upon transfer from α-fructose to GA as expected (Fig. 2B). Induction of these genes upon transfer to GA was also found in ΔgaaA, but at lower levels compared to the reference strain. The induction of GA-responsive genes was nearly absent in ΔgaaB. As shown in Fig. 2B, deletion of gaaC resulted in a hypperinduction of GA-responsive genes, especially pectinases (NRRL3_03144, pgx28B, and NRRL3_10865). Expression of gatA, gaaA, gaaB, gaaC, and the pectinases in ΔgaaD was similar to the expression in the reference strain (Fig. 2B).

Transcriptome analysis of ΔgaaC

In order to analyze the expression of a larger number of genes controlled by GaaR/GaaX activator-repressor module in ΔgaaC, a genome-wide gene expression analysis was performed using RNA-seq. The reference strain and the ΔgaaC mutant were pregrown in α-fructose medium and transferred to GA medium. Seventeen of the 53 GaaR/GaaX panregulon genes were significantly upregulated (FC ≥ 2 and P-value ≤ 0.05) in the ΔgaaC mutant cultured in GA as compared to the reference strain (Table 1, Table S3). These 17 genes include gaaA and 6 pectinases (NRRL3_03144, pgx28B, NRRL3_05252, NRRL3_04916, NRRL3_10559, and NRRL3_11738), as well as genes encoding four transporters and six genes for which the function has not yet been established. The expression of 24 of the remaining GaaR/GaaX panregulon genes was higher in ΔgaaC compared to the reference strain, but differences were relatively small and did not pass the stringent P-value of ≤ 0.05.

In addition to GaaR/GaaX-controlled genes, we also compared the expression of all 58 pectinases identified in the genome of A. niger [2] between the reference strain and the ΔgaaC mutant (Table S4, Fig. 2C). Apart from the six pectinases that depend on GaaR for induction [19], nine additional pectinases acting on the RG-I backbone and arabinan and arabinogalactan side chains were significantly upregulated (FC ≥ 2 and P-value ≤ 0.05) in the ΔgaaC mutant compared to the reference strain (Table 2). It has been reported that many of these genes are regulated by transcription factors RhaR (NRRL3_02832, NRRL3_07501, NRRL3_07501, and raeB), XlnR (NRRL3_05407 and lac35B), or AraR (lac35B), which are required for the utilization of α-rhamnose, xylan/α-xylene, and arabinan/α-arabinose, respectively [39–42]. To address the possibility that deletion of gaaC affected the expression of these genes via their specific transcription factors, the expression of rhaR, xlnR, and araR was analyzed in more detail. Expression of rhaR (FC = 5.84 and P-value = 4.76E-03) and xlnR (FC = 2.68 and P-value = 5.60E-03) was significantly higher in ΔgaaC, which might explain the upregulation observed in these genes. The araR gene was not significantly differentially regulated in the ΔgaaC mutant.

Discussion

In this study, we used GA catabolic pathway deletion mutants to investigate the induction mechanism of the GA-responsive genes in A. niger. We observed that the gaaA and the gaaD deletion mutants show reduced growth on GA or PGA compared to the reference strain, whereas growth of ΔgaaB and ΔgaaC is more severely reduced on GA, PGA, or AP (Fig. 1B,C). These results are in line with the previous reports showing the inability of ΔgaaB and ΔgaaC to grow on GA [7,8]. ΔgaaA was reported to be unable to grow on GA in a previous study [6], where the tenuous
Fig. 2. Metabolic and gene expression analyses of *Aspergillus niger* GA catabolic pathway deletion mutants ΔgaaA, ΔgaaB, ΔgaaC, and ΔgaaD (A) Extracellular GA, L-galactonate, and 2-keto-3-deoxy-L-galactonate concentration in cultures of the reference strain (FP-1132.1) and GA catabolic pathway deletion mutants. GA concentration is given in mM and L-galactonate and 2-keto-3-deoxy-L-galactonate amounts are presented as ion chromatogram peak areas relative to ΔgaaB 55 h and ΔgaaC 55 h samples, respectively. (B) Northern blot analysis of selected GA-responsive genes in the reference strain (MA249.1) and GA catabolic pathway deletion mutants. Actin (NRRL3_03617) was used as a control. (C) RNA-seq analysis of pectinase genes in the reference strain (FP-1132.1) and ΔgaaC in GA (FPKM). Expression in ΔgaaR in GA (FPKM) [19] and in the reference strain [MA234.1] and ΔgaaX in α-fructose (TPM) [20] was shown for comparison. Pectinase genes that belong to the GaaR/GaaX panregulon [20] are indicated with an asterisk. Strains were pregrown in CM with 2% D-fructose. For metabolic analysis, mycelia were transferred to and grown in MM containing 50 mM GA. For northern blot analysis, mycelia were transferred to and grown in MM containing 25 mM GA for 2 h. For RNA-seq analysis, mycelia were transferred to and grown in MM containing 25 mM GA for 2 h.
### Table 1. RNA-seq analysis of 53 genes of the GaaR-GaaX panregulon [20] in ΔgaaC in GA. 27 genes belonging to GaaR-GaaX core regulon [20] are written in bold. Expression values (FPKM) are averages of duplicates. Significantly upregulated genes (FC ≥ 2 and P-value ≤ 0.05) are highlighted.

| Gene ID | CBS513.88 | Description | Gene name | Ref | ΔgaaC | FC ΔgaaC/Ref | P-value |
|---------|------------|-------------|-----------|-----|-------|-------------|---------|
| NRRL3_00958 | An14g04280 | O-galacturonic acid transporter GatA | gatA | 888.35 | 1062.68 | 1.20 | 6.95E-02 |
| NRRL3_03144 | An12g07500 | Exo-polygalacturonase | | 698.90 | 3384.63 | 4.84 | 1.34E-02 |
| NRRL3_05260 | An02g12450 | Exo-polygalacturonase Pgx28C | pgx28C | 99.93 | 192.85 | 1.93 | 9.11E-02 |
| NRRL3_05649 | An02g07720 | 2-Keto-3-deoxy-D-galactonate aldolase GaaC | gaaC | 5658.32 | 14.60 | 0.00 | 2.88E-04 |
| NRRL3_05650 | An02g077710 | O-Galacturonic acid reductase GaaA | gaaA | 2599.98 | 6710.72 | 2.58 | 1.04E-02 |
| NRRL3_06053 | An02g02540 | Carbohydrate esterase family 16 protein | | 522.81 | 1301.08 | 2.49 | 8.01E-02 |
| NRRL3_06890 | An16g05390 | L-Galactonate dehydratase GaaB | gaaB | 11309.00 | 13990.90 | 1.24 | 1.91E-01 |
| NRRL3_07470 | An04g00790 | Repressor of D-galacturonic acid utilization | gaaX | 381.34 | 529.21 | 1.39 | 1.97E-01 |
| NRRL3_08325 | An03g06310 | Pectin methylesterase Pme8A | pme8A | 6.54 | 6.74 | 1.03 | 8.79E-01 |
| NRRL3_07094 | An16g02730 | Endo-1,5-alpha-arabinanase | | 4.57 | 3.48 | 0.76 | 2.43E-01 |
| NRRL3_08194 | An05g02440 | Carbohydrate esterase family 16 protein | | 35.48 | 19.78 | 0.56 | 3.56E-02 |
| NRRL3_09811 | An15g07160 | Pectin lyase | | 35.48 | 19.78 | 0.56 | 3.56E-02 |
| NRRL3_10559 | An18g04810 | Glycoside hydrolase family 28 protein | | 20.00 | 97.18 | 4.86 | 1.19E-02 |
| NRRL3_00965 | An14g04370 | Pectin lyase Pel1A | pel1A | 56.54 | 113.40 | 2.01 | 3.58E-01 |
| NRRL3_04281 | An07g00780 | MFS-type transporter | | 106.09 | 227.29 | 2.14 | 1.71E-01 |
| NRRL3_02571 | An01g11520 | Endo-polygalacturonase Pga28I | pga28I | 56.38 | 59.67 | 1.06 | 5.83E-01 |
| NRRL3_08910 | An12g03550 | MFS-type transporter | | 137.63 | 170.01 | 1.24 | 5.21E-01 |
| NRRL3_03342 | An12g04990 | Short-chain dehydrogenase/reductase | | 261.62 | 440.98 | 2.19 | 1.92E-01 |
| NRRL3_00502 | An09g06200 | Hypothetical protein | | 28.91 | 319.96 | 11.07 | 4.60E-02 |
| NRRL3_00957 | An14g01130 | Rhamnogalacturonan lyase | | 7.87 | 13.23 | 2.29 | 2.61E-01 |
| NRRL3_02571 | An01g11520 | Endo-polygalacturonase Pga28I | pga28I | 56.38 | 59.67 | 1.06 | 5.83E-01 |
| NRRL3_08325 | An03g06310 | Pectin methylesterase Pme8A | pme8A | 6.54 | 6.74 | 1.03 | 8.79E-01 |
| NRRL3_02881 | An03g06740 | Exo-polygalacturonase Pga28B | pgx28B | 888.35 | 1062.68 | 1.20 | 6.95E-02 |
| NRRL3_03525 | An02g077710 | O-Galacturonic acid reductase GaaA | gaaA | 2599.98 | 6710.72 | 2.58 | 1.04E-02 |
| NRRL3_05252 | An02g12450 | Exo-polygalacturonase Pgx28C | pgx28C | 99.93 | 192.85 | 1.93 | 9.11E-02 |
| NRRL3_00684 | An14g04280 | O-galacturonic acid transporter GatA | gatA | 888.35 | 1062.68 | 1.20 | 6.95E-02 |
| NRRL3_00958 | An14g04280 | O-galacturonic acid transporter GatA | gatA | 888.35 | 1062.68 | 1.20 | 6.95E-02 |
| NRRL3_00958 | An14g04280 | O-galacturonic acid transporter GatA | gatA | 888.35 | 1062.68 | 1.20 | 6.95E-02 |
growth of $\Delta gaaA$ could have been interpreted as no growth. GA catabolic pathway deletion mutants derived from N593.20 in this study and from ATCC1015 in previous studies [6-8] showed the same growth defects on GA (unpublished results), excluding the possibility of a phenotypic difference caused by strain background.

Deletion of $gaaB$ and $gaaC$ severely impaired growth on MM containing GA (Fig. 1B,C), indicating that there are no alternative enzymes replacing GaaB and GaaC. The residual growth of $\Delta gaaA$ and $\Delta gaaD$ on GA indicates that GA is catabolized in these reductase deletion mutants via partially redundant enzymes.

In B. cinerea, there are two nonhomologous d-galacturonate reductases, BcGar1, and BcGar2. While single gene deletion mutants ($\Delta Bcgar1$ or $\Delta Bcgar2$) could still grow on GA, the double gene deletion mutant $\Delta Bcgar1\Delta Bcgar2$ showed a complete loss of growth [9]. Aspergillus niger also contains a BcGar1 ortholog, NRRL3_06930, which shows no protein homology to GaaA. As in B. cinerea, NRRL3_06930 might enable the residual growth of $\Delta gaaA$ on GA. However, the expression of NRRL3_06930 is considerably lower than the expression of $gaaA$ in GA, and unlike the expression of $gaaA$, does not depend on GaaR or GaaX [19,20]. It is also possible that the two dehydrogenases belonging to the GaaR-GaaX panregulon, NRRL3_03342, and NRRL3_09863, partially replace GaaA or GaaD.

The recently proposed model related to the regulation of GA-responsive gene expression [20] postulates that under noninducing conditions the repressor GaaX inhibits the transcriptional activity of GaaR. The repressing activity of GaaX is suggested to be lost in the presence of an inducer and subsequent activation of GaaR, resulting in the induction of GA-responsive genes in A. niger [20]. The results of metabolic and northern blot analyses indicate that accumulation of 2-keto-3-deoxy-L-galactonate produced is enough for the induction of GA-responsive genes. However, this induction is lower compared to the reference strain (Fig. 2B). This result is supported by a previous finding that
gaaB and gaaC were expressed at lower levels in ΔgaaA compared to the reference strain [6]. In contrast, ΔgaaB possibly does not produce 2-keto-3-deoxy-L-galactonate from L-galactonate, since the growth phenotype of the ΔgaaB mutant suggests that there are no functionally redundant enzymes replacing GaaB. As a result, expression of GA-responsive genes is not induced in ΔgaaB (Fig. 2B). Reduced expression of gatA, gaaA, and gaaC in the ΔgaaB mutant was also observed previously [7].

RNA-seq analysis of ΔgaaC revealed significant upregulation of several genes from the GaaR/GaaX panregulon involved in pectin breakdown and GA utilization, as well as genes with currently unknown link to GA utilization, such as transporters that might facilitate the faster GA transport in ΔgaaC compared to other GA catabolic pathway deletion mutants observed both in this study (Fig. 2A) and previous studies [6–8]. Deletion of gaaC also induced the expression of several pectinases acting on RG-I that do not belong to GaaR/GaaX panregulon (Table 2). A possible explanation is that starvation in ΔgaaC results in the induction of these genes. Several pectinases acting on side chains of RG-I, including NRRL3_05407, lac35B and NRRL3_07501, were previously reported to be induced upon starvation [43]. Another explanation is that the increased transcript levels of rhaR and xlnR results in an increase in the expression of these genes that were suggested to be under control of RhaR and XlnR (see above).

Although both ΔgaaB and ΔgaaC cannot utilize GA, residual growth of ΔgaaC was observed on AP, whereas the growth of ΔgaaB on AP was more impaired (Fig. 1B). This could be explained by the high capacity of ΔgaaC to secrete pectinases acting on RG-I and release monosaccharides (L-arabinose, L-rhamnose, D-galactose) other than GA to support growth, and the less efficient pectinase production in ΔgaaB.

Previously, we identified 53 genes as the GaaR/GaaX panregulon downregulated in ΔgaaR under inducing condition and/or upregulated in ΔgaaX under non-inducing condition. However, only a core set of 27 genes was significantly differentially regulated under both conditions [19,20], and only 17 of 53 panregulon genes, 10 of which belong to the core regulon, were hyperinduced in response to deletion of gaaC (Table 1), demonstrating the complex regulation of GA-responsive gene expression. A dynamic equilibrium is suggested to exist between the free and DNA-bound states of a transcription factor, and the binding of a transcription factor to the promoters of its target genes depends on its concentration, as well as its cooperative/competitive interactions with other proteins and the chromatin accessibility [44,45]. Deletion of gaaR would result in the lack of GaaR in the cell, whereas deletion of gaaX or intracellular accumulation of 2-keto-3-deoxy-L-galactonate in ΔgaaC would, possibly to different degrees, increase the concentration of active GaaR by elimination or reducing the repressing activity of GaaX. GaaR concentration might also be regulated transcriptionally: gaaX is highly upregulated in GA [5], whereas gaaR expression is significantly increased in the ΔgaaC mutant (FC = 5.10 and P-value = 7.88E-03). Moreover, different levels of CreA mediated repression on different GA-responsive genes [18] and accessibility of the promoter regions of these genes under different conditions might play a role in the observed differences in gene regulation. Condition specific cross-regulation between transcription factors and coregulation of target genes might add additional complexity to GA-responsive gene expression, as discussed above.

To conclude, in this study we identified the GA catabolic pathway intermediate 2-keto-3-deoxy-L-galactonate as the probable inducer of the GA-responsive genes in A. niger. Considering that both the GA catabolic pathway enzymes and the GaaR/GaaX activator–repressor module is evolutionarily conserved in the Pezizomycotina subdivision of Ascomycetes [5,20], it is highly probable that the mechanism by which 2-keto-3-deoxy-L-galactonate acts as an inducer and interacts with the activator–repressor module is also conserved.

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**Author contributions**

EA, CK, TGH, SdP, MA, MDF, TTMP performed experiments. EA, MDF, MP, MVAP performed bioinformatics analysis. EA, JV, AT, RPdV, and AFJR wrote the manuscript with input of all authors.

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Supporting information

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

*Fig. S1.* Verification of the GA catabolic pathway deletion strains (A) Δ*gaaA* (SDP22.1), (B) Δ*gaaB* (SDP21.5), (C) Δ*gaaC* (SDP20.6), and (D) Δ*gaaD* (EA1.1) via southern blot analysis of genomic DNA.

*Fig. S2.* Growth profile of the *Aspergillus niger* reference strain (MA249.1) and GA catabolic pathway deletion mutants Δ*gaaA*, Δ*gaaB*, Δ*gaaC*, and Δ*gaaD*.

*Fig. S3.* (A) Predominant form (pyranose) of 2-keto-3-deoxy-L-galactonate in the extracellular culture fluid of *Aspergillus niger*.

*Fig. S4.* RNA-seq analysis of pectinases in Δ*gaaA* and Δ*gaaR* in GA and in Δ*gaaX* in D-fructose.

Table S1. Strains used in this study.

Table S2. Primers used in this study.

Table S3. RNA-seq analysis of 53 genes of the GaaR-GaaX panregulon [20] in Δ*gaaC* and Δ*gaaR* in GA and in Δ*gaaX* in D-fructose.

Table S4. RNA-seq analysis of pectinases in Δ*gaaA* and Δ*gaaR* in GA and in Δ*gaaX* in D-fructose.