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

Alazi, E.

CHAPTER 7

W361R mutation in GaaR, the regulator of D-galacturonic acid responsive genes, leads to constitutive expression of pectinases in *Aspergillus niger*

Alazi E, Niu J, Otto SB, Pham TTM, Tsang A, Ram AFJ

Submitted to *MicrobiologyOpen*

ABSTRACT

Polysaccharides present in plant biomass, such as pectin, are the main carbon source for filamentous fungi. *Aspergillus niger* naturally secretes pectinases to degrade pectin and utilize the released monomers, mainly D-galacturonic acid. The transcriptional activator GaaR, the repressor of D-galacturonic acid-utilization GaaX, and the physiological inducer 2-keto-3-deoxy-L-galactonate play important roles in the transcriptional regulation of D-galacturonic acid-responsive genes, which include the genes encoding pectinases. In this study, we described the mutations found in *gaaX* and *gaaR* that enabled constitutive (i.e. inducer-independent) expression of pectinases by *A. niger*. Via gene expression analyses using promoter-reporter strains (P*pgaX-amdS*) and PGA-plate assay, we showed that W361R mutation in GaaR results in constitutive expression of pectinase genes. Analysis of subcellular localization of C-terminally eGFP-tagged GaaR/GaaR^{W361R} revealed important differences in nuclear accumulation of N- versus C-terminal eGFP-tagged GaaR.

INTRODUCTION

Polysaccharides present in plant biomass, such as pectin, are the principle carbon source for filamentous fungi (Kowalczyk *et al.*, 2014). All of the four pectin substructures, i.e. polygalacturonic acid (PGA), rhamnogalacturonan I, rhamnogalacturonan II and xylogalacturonan, contain D-galacturonic acid (GA) in their backbones, with various monomers or polymers attached to their backbones. PGA is the major polysaccharide present in pectin, and consists of α-1,4-linked GA subunits (Caffall and Mohnen, 2009).

When *Aspergillus niger* encounters pectin in its environment, it secretes pectinases to degrade pectin, transports the released monosaccharides into the cell and catabolizes them to generate energy. The catabolic intermediate 2-keto-3-deoxy-L-galactonate produced from GA was shown to induce the production of pectinases by *A. niger* (Alazi *et al.*, 2017). The regulated production of pectinases, especially polygalacturonases, under inducing conditions requires the Zn_2Cys_6 type transcription factor GaaR (Alazi *et al.*, 2016), the action of which is suggested to be inhibited by the repressor protein GaaX (Niu *et al.*, 2017). Additionally, the expression of pectinase genes is subject to carbon catabolite repression via the repressor protein CreA in the presence of energetically favorable carbon sources, such as glucose or D-fructose (Niu *et al.*, 2015; Alazi *et al.*, 2018).

Pectinases produced by *A. niger* are used industrially (Kashyap *et al.,* 2001; Toushik *et al.*, 2017; Khan *et al.*, 2013; Edwards and Doran-Peterson, 2012), especially in food industry and in hydrolysis of plant biomass for the subsequent production of biofuel and high-value biopolymers. Inducer-independent production of pectinases by *A. niger* would enable the use of waste and residues from agriculture, forestry and food industries as a cheap and sustainable feedstock for fungal cultivations. In this study, we describe mutations in *gaaX* and *gaaR* that result in constitutive expression of pectinase genes by *A. niger*. A mutation in *gaaR* causing an amino acid change from tryptophan to arginine at position 361 (W361R) is shown to lead to a constitutively active form of the GaaR transcription factor, leading to inducer-independent production of polygalacturonases.

MATERIALS AND METHODS

Strains, media and growth conditions

All strains used in this study are listed in Table S1. Media were prepared as described in Arentshorst *et al.* (2012). Radial growth phenotype of the strains were analysed on minimal medium (MM) (pH 5.8) containing 1.5% (w/v) agar (Scharlau, Barcelona, Spain) and various carbon sources: 50 mM D-glucose (VWR International, Amsterdam, The Netherlands), D-fructose (Sigma-Aldrich, Zwijndrecht, The Netherlands) or GA (Chemodex, St Gallen, Switzerland); or 1% (w/v) PGA (Sigma, Zwijndrecht, The Netherlands) or apple pectin (AP) (Sigma-Aldrich, Zwijndrecht, The Netherlands). Media of the uridine auxotrophic strains were supplemented with 10 mM uridine. Plates were inoculated with 5 µL 0.9% NaCl containing $1x10^4$ or $5x10^4$ freshly harvested spores, and cultivated at 30 °C for 7 or 5 days, respectively. MM (pH 5.8) containing 1.5% (w/v) agar, 10 mM acetamide (Sigma-Aldrich, Steinheim, Germany) as the sole nitrogen source, and glucose, D-fructose, GA or 2-keto-3-deoxy-L-galactonate as the carbon source was prepared as described previously (Arentshorst *et al.*, 2012). Plates were inoculated with 5 µL 0.9% NaCl

containing 10^4 freshly harvested spores and cultivated at 30 °C for 7 or 10 days. Filter sterilized carbon source solutions were added after autoclaving MM containing agar. PGA and AP were autoclaved together with the medium. All growth experiments were performed in duplicate.

For enzymatic analysis, 300 mL shake flasks that include 100 mL MM (pH 5.8) containing 50 mM D-fructose and 0.003% yeast extract were inoculated with $7.5x10^7$ freshly harvested spores and cultivated for 36h in a rotary shaker at 30 °C and 250 rpm. Experiments were performed in duplicate.

For microscopic analysis of the localization of the eGFP-tagged GaaR or GaaR^{W361R} proteins, $4x10^5$ freshly harvested spores were inoculated on cover slips in Petri dishes that include 4 mL MM containing 0.003% yeast extract and 10 mM D-fructose or 2-keto-3-deoxy-L-galactonate, and grown at 30 °C for approximately 21 h. For each condition, two biological replicates were performed.

Sequencing *gaaX* and *gaaR* genes in the mutants with constitutive expression of genes involved in PGA utilization

Sixty five *trans*-acting mutants, spontaneous or obtained after mild UV mutagenesis with constitutive expression of genes involved in PGA utilization were obtained as previously described (Niu *et al*., 2017) on solid MM containing 50 mM glucose as the carbon source and 10 mM acetamide as the sole nitrogen source. Genomic DNA of 11 spontaneous and 53 UV mutants was extracted as described by Arentshorst *et al.* (2012), and *gaaX* and/or *gaaR* genes were PCR-amplified using the primers gaaX_P5f and gaaX_GSP9r, or gaaRP7f and gaaRP8r, respectively (Table S2). The PCR fragments were sequenced in both directions using *gaaX* or *gaaR* sequencing primers, respectively (Table S2).

Construction of the promoter-reporter strains expressing *gaaR^{W361R}*

Protoplast-mediated transformation of *A. niger* and purification of the transformants were performed as described by Arentshorst *et al.* (2012).

The *gaaR^{W361R}* gene together with its 964-bp promoter and 992-bp terminator regions was amplified by PCR using the primers gaaRP5f and gaaRP6r (Table S2) with JN103.1 genomic DNA as template. The PCR product was transformed into strains JC1.5 and JN29.2, yielding in the strains JN130.4 and JN129.1, respectively. Transformants were selected on transformation plates (Arentshorst *et al.*, 2012) containing 10 mM acetamide as the sole nitrogen source. Correct gene replacements in strains JN130.4 and JN129.1 were verified by Southern blot and sequencing analyses. For sequence analysis, the *gaaR* locus was amplified using the primers gaaRP7f and gaaRP8r (Table S2) and JN130.4 and JN129.1 genomic DNA as template, and ligated into pJET1.2/blunt cloning vector (Thermo Fisher Scientific, Carlsbad, California). The resulting plasmids were amplified in *Escherichia coli* and sequenced using *gaaR* sequencing primers. Integration of the *gaaR^{W361R}* into the endogenous *gaaR* locus was confirmed via Southern blot analysis. Genomic DNA was restricted overnight with *Nco*I or *Hin*dIII restriction enzymes. A 501-bp fragment containing the *gaaR* gene was PCR-amplified using the primer pairs listed in Table S2 with

N402 genomic DNA as template, and was used as a probe.

Construction of strains expressing *gaaR-eGFP or gaaR-(GA)$_4$-eGFP*

The plasmid pEA9 containing the P*gaaR-gaaR-eGFP*-T*gaaR* construct was created as follows: The *eGFP* gene and the 715-bp terminator of the *gaaR* gene were amplified by PCR using the primer pairs listed in Table S2 with the plasmid pFG029 (unpublished vector, containing P*gpdA-eGFP*-T*trpC*) and N402 genomic DNA as template, respectively. *eGFP* and T*gaaR* were combined by fusion PCR using primers GaaR_GFP1F and GaaR_GFP3R. The *gaaR* gene without the stop codon and together with the 731-bp promoter region was PCR amplified using the primer pairs listed in Table S2. P*gaaR-gaaR* and *eGFP*-T*gaaR* were combined by fusion PCR using primers eGFP-gaaR-For and eGFP-gaaR-Rev, ligated into pJET1.2/blunt cloning vector (Thermo Fisher Scientific, Carlsbad, California) and amplified in *E. coli*. Sequencing of pEA9 showed that a PCR error (T to C) has occurred in T*gaaR* at a distance of 519 bp downstream of the stop codon of the *eGFP* gene.

The plasmid pEA10 containing the P*gaaR-gaaR-(GA)$_4$-eGFP*-T*gaaR* construct was created in a similar way to pEA9, except that P*gaaR-gaaR-(GA)$_4$* was amplified using the primer GaaR_GFP6R adding a linker containing four repeats of glycine and alanine residues between GaaR and eGFP. Sequencing of pEA10 showed that a PCR error (C to T) has occurred in T*gaaR* at a distance of 430 bp downstream of the stop codon of the *eGFP* gene.

To create the strain EA29.14, pEA9 was co-transformed into strain JN36.1 (*ΔgaaR*) together with the plasmid pMA357 containing the *A. nidulans amdS* gene behind the *A. nidulans gpdA* promoter (Alazi *et al.*, 2016). EA30.6 was created by co-transformation of pEA10 into strain JN36.1 (*ΔgaaR*) together with the plasmid pMA357. Transformants were selected on transformation plates containing acetamide as the sole nitrogen source.

Construction of the *gaaR::AOpyrG* deletion strain

SO1.1 (*ΔgaaR::AOpyrG*) was created using the split marker approach (Arentshorst *et al.*, 2015). 5' and 3' flanks of *gaaR* gene were PCR-amplified using the primer pairs listed in Table S2 with N402 genomic DNA as template. The *A. oryzae pyrG* gene was PCR-amplified as two fragments using the primer pairs listed in Table S2 and the plasmid pAO4-13 (de Ruiter-Jacobs *et al.*, 1989) as template. Split marker fragments with the *AOpyrG* selection marker were created by fusion PCR and used to transform the strain MA169.4, resulting in the strain SO1.1. Proper deletion of *gaaR* was confirmed by diagnostic PCR (data not shown) and via Southern blot analysis.

Construction of strains expressing *gaaR^{W361R}, gaaR^{W361R}-eGFP or gaaR-eGFP* from the endogenous *gaaR* locus

To construct plasmid pSO1.2, the P*gaaR-gaaR^{W361R}*-T*gaaR* allele was amplified by PCR using the primers gaaRP5f and gaaRP6r (Table S2) with JN103.1 genomic DNA as template and ligated into pJET1.2/blunt cloning vector (Thermo Fisher Scientific, Carlsbad, California). The resulting plasmid pSO1.2 was amplified in *E. coli*. pSO2.1 (containing the

P*gaaR-gaaR*^{W361R}*-eGFP*-T*gaaR* construct) was created by digesting the plasmids pSO1.2 and pEA9 (containing the P*gaaR-gaaR-eGFP*-T*gaaR* construct) with the restriction enzymes *Bcu*I and *Bam*HI, and by ligating the 2408-bp *Bcu*I-*Bam*HI fragment from pSO1.2 (containing the *gaaR*^{W361R} mutation) with the *Bcu*I and *Bam*HI cut opened pEA9. pSO1.2 and pSO2.1 were sequenced to ensure no PCR errors have occurred and proper ligation and orientation of the fragments.

Repair fragments for CRISPR-Cas9 mediated targeted integration (see below) were obtained as follows: The P*gaaR-gaaR*^{W361R}-T*gaaR* construct containing the 964-bp P*gaaR* and 992-bp T*gaaR* was excised from pSO1.2 using the restriction enzymes *Not*I and *Xba*I. The P*gaaR-gaaR-eGFP*-T*gaaR* and P*gaaR-gaaR*^{W361R}*-eGFP*-T*gaaR* constructs containing the 731-bp P*gaaR* and 419-bp T*gaaR* were excised from pEA9 and pSO2.1, respectively, using the restriction enzyme *Bgl*II.

The strains SO2.1 (*gaaR*^{W361R}), EA31.1 (*gaaR-eGFP*) and EA32.1 (*gaaR*^{W361R}*-eGFP*) were created using the CRISPR-Cas9 technique (Nødvig *et al.*, 2015; Song *et al.*, manuscript in preparation). A guide RNA protospacer sequence GTGGATACGTACTCCTTTTA targeting the *AOpyrG* gene was designed using E-CRISP (Heigwer *et al.*, 2014).

To create SO2.1, the DNA template for the *in vitro* synthesis of the guide RNA via the T7 promoter was amplified by PCR using the primers sgRNAP1 and OTL19 (Table S2), and the plasmid p426-SNR52p-gRNA.CAN1.Y-SUP4t (Addgene, USA) (DiCarlo *et al.*, 2013) as template. The PCR consisted of the following reactions: Initial denaturation at 98 °C for 30 sec; 30 cycles of denaturation at 98 °C 5 sec, annealing at 77 °C 5 sec, extension at 72 °C 2 sec; and final extension at 72 °C 30 sec. The amplified DNA template was transcribed *in vitro* using the MEGAscript T7 Kit (Thermo Fisher Scientific, Carlsbad, California). P*gaaR-gaaR*^{W361R}-T*gaaR* repair fragment was co-transformed into strain SO1.1 (*ΔgaaR::AOpyrG*) together with the *in vitro* transcribed guide RNA and the plasmid pFC332 containing the *cas9* gene and the hygromycin selection marker (Nødvig *et al.*, 2015), yielding to SO2.1. Transformants were selected on transformation plates containing hygromycin and uridine, and purified twice on MM containing GA, 5'-fluoroorotic acid, uridine and hygromycin. pFC332 was cured by further purifying transformants strains twice without hygromycin selection on MM containing glucose and uridine. Transformants were not able to grow on MM containing glucose, uridine and hygromycin after the first round of purification, indicating that pFC332 was successfully cured.

The DNA template for the *in vivo* synthesis of the guide RNA via the RNA polymerase III promoter was amplified in two parts by PCR using the primers Fw_LIC2 and Rev_P1, and Fw_P1 and Rev_LIC2 (Table S2) using the plasmid ANEp8_Cas9-gRNAalbA (Song *et al.*, manuscript in preparation) as template. The two PCR products were combined by fusion PCR using the primers Fw_LIC2 and Rev_LIC2. The fusion PCR product was amplified by PCR using the primers for_pTE1 and rev_pTE1 to introduce *Pac*I restriction sites at both ends, digested with *Pac*I, and ligated into *Pac*I cut opened pFC332, yielding to plasmid pTE1. pTE1 was cotransformed together with P*gaaR-gaaR-eGFP*-T*gaaR* or P*gaaR-gaaR*^{W361R}*-eGFP*-T*gaaR* repair fragments into the strain SO1.1 (*ΔgaaR::AOpyrG*) to create EA31.1 or EA32.1, respectively. Transformants were selected on transformation plates containing hygromycin and uridine, and purified twice on MM containing GA, 5'-fluoroorotic acid and uridine. Transformants were not able to grow on MM containing GA,

5'-fluoroorotic acid, uridine and hygromycin after the first round of purification, indicating that pTE1 was successfully cured.

The *gaaR* locus in SO2.1 and EA31.1 was amplified using the primers gaaRP7f and gaaRP8r, and the one in EA32.1 using the primers PgaaR_seq_P2 and TgaaR_seq_P2, and ligated into pJET1.2/blunt cloning vector (Thermo Fisher Scientific, Carlsbad, California). The resulting plasmids were amplified in *E. coli* and sequenced using *gaaR* sequencing primers to confirm the presence of the mutated *gaaR* allele in SO2.1 and EA32.1, and the wild type *gaaR* allele in EA31.1. Integration of the repair fragments into the endogenous *gaaR* locus in SO2.1, EA31.1 and EA32.1 was confirmed via Southern blot analysis (Figure S3). SO2.1, EA31.1 and EA32.1 were made uridine prototroph by transforming with the plasmid pAB4.1 (van Hartingsveldt *et al.*, 1987), yielding to strains EA34.1, EA36.1 and EA39.1, respectively.

Enzymatic analysis

Supernatants from shake flask cultures were obtained by filtration through glass microfiber filters (Whatman, Buckinghamshire, UK), and the filtrate was stored at -80 °C. PGA plate assays were performed as described by Niu *et al.* (2017). Twenty-five microlitres of supernatant from each culture was spotted on plates containing 0.2% PGA, and plates were incubated at 37 °C for 24 h before staining with 0.1% Congo Red (Sigma-Aldrich, Zwijndrecht, The Netherlands) solution.

Microscopy

The cover slips with adherent germlings were placed upside down on glass slides. The GFP fluorescence was excited using 488 nm laser line in a Zeiss Observer confocal laser scanning microscope (Zeiss, Jena, Germany). The images were analysed with the ImageJ software (Abramoff *et al.*, 2004). On each image, the exact same brightness and contrast adjustments were applied.

RESULTS

Genetic characterization of *A. niger* mutants showing constitutive expression of pectinases

Mutants showing constitutive expression of pectinases were previously obtained via a forward genetic screen using the promoter-reporter strain P*pgaX-amdS ΔcreA* (JN29.2) (Niu *et al.*, 2017). This strain contains the P*pgaX-amdS* reporter construct and is defective in carbon catabolite repression due to disruption of the *creA* gene (*ΔcreA*). P*pgaX-amdS ΔcreA* (JN29.2) is able to grow on acetamide as the sole nitrogen source only when the GA-responsive promoter of the *pgaX* (NRRL3_03144) gene gets activated to express the *amdS* gene encoding the acetamidase enzyme (Niu *et al.*, 2015). In total, 65 mutants showing constitutive expression of pectinases were isolated on plates containing MM with glucose as the carbon source and acetamide as the sole nitrogen source. Eleven of these mutants were analysed for mutations in the GA-responsive transcription factor *gaaR*

(NRRL3_08195) but no mutations were found. The genomes of five mutants were sequenced and it was found that they are all mutated in a common gene, which was named the repressor of D-galacturonic acid utilization, *gaaX* (NRRL3_08194) (Niu *et al*., 2017). It was also shown that deletion of *gaaX* results in constitutive expression of pectinases (Niu *et al*., 2017).

We sequenced the *gaaX* gene in the other 59 mutants showing constitutive expression of pectinases (Table S3, Figure S1). In total, 28 and nine mutants were found to carry nonsense and frameshift mutations, respectively. The nonsense or frameshift mutation closest to the C-terminus was the frameshift mutation V653G, indicating the importance of the last 45 aa residues for a functional GaaX. Twenty-three mutants were found to have missense mutations in the *gaaX* gene. These mutations were found throughout *gaaX*, indicating the importance of the different domains of GaaX for proper functioning. Four mutants did not carry any mutations in *gaaX*. Sequencing the *gaaR* gene in these mutants resulted in the identification of a mutation in *gaaR* in one of the mutants.

The UV mutant P*pgaX-amdS ΔcreA gaaR*$^{W361R\text{-UV}}$ (JN103.1) carries a missense mutation (W 361 to R) caused by codon change from TGG to CGG at a distance of 1285 bp from the start codon of *gaaR*. The tryptophan361 residue lies in the fungal-specific transcription factor domain that spans the residues 139-518 in the 740 aa long GaaR (Figure 1A), and is 100 % conserved in the homologous GaaR sequences present in *Aspergillus* species (Figure 1B) and in other Ascomycetes analysed belonging to the Pezizomycotina subdivision (Figure 1C). Growth of the P*pgaX-amdS ΔcreA gaaR*$^{W361R\text{-UV}}$ (JN103.1) strain was similar to the growth of the *ΔcreA* (XY1.1) strain on MM containing glucose, D-fructose, GA, PGA or AP (Figure S2), both displaying a smaller radial growth compared to the reference (MA234.1) strain due to *creA* deletion.

Analyses of gene expression in *gaaR*^{W361R}

The UV mutant P*pgaX-amdS ΔcreA gaaR*$^{W361R\text{-UV}}$ (JN103.1) possibly carries multiple mutations in its genome. To create a strain that carries only the W361R mutation in *gaaR*, the *gaaR*^{W361R} gene was transformed into a *ΔgaaR* (SO1.1) strain. Southern blot analysis indicated that the P*gaaR-gaaR*^{W361R}-T*gaaR* construct was successfully integrated into the endogenous *gaaR* locus in the genome of the *gaaR*^{W361R} (SO2.1) strain (Figure S3). Growth of *gaaR*^{W361R} (SO2.1) was analysed on different monomeric and polymeric carbon sources (Figure S2). *ΔgaaR* (SO1.1) displayed a strongly impaired growth on GA, PGA and AP, as it was shown previously (Alazi *et al.*, 2016). Introduction of *gaaR*^{W361R} gene resulted in a full complementation of growth on GA containing carbon sources, indicating the presence of a functional GaaR. Growth of *gaaR*^{W361R} (SO2.1) was similar to the growth of the reference (MA234.1) strain on all carbon sourcestested.

The pectinase production capacity of *gaaR*^{W361R} (EA34.1), a uridine prototroph derived from *gaaR*^{W361R} (SO2.1), was assessed via PGA plate assay (Figure 2A). Strains were grown in liquid medium containing D-fructose, a carbon source that does not induce the expression of GA-responsive genes, and the culture supernatants were spotted on PGA plates. Comparison of hydrolysis zones on PGA plates showed that *gaaR*^{W361R} (EA34.1) constitutively produces pectinases involved in PGA degradation, as the UV mutant P*pgaX-*

amdS ΔcreA gaaR$^{W361R\text{-UV}}$ (JN103.1) does (Figure 2A). This indicates that the W361R mutation in *gaaR*, and no other additional mutation(s) induced by UV mutagenesis in P*pgaX-amdS ΔcreA gaaR*$^{W361R\text{-UV}}$ (JN103.1), is responsible for the constitutive expression of the genes encoding pectinases. The reference (MA234.1) strain did not display any polygalacturonase activity in its supernatant under non-inducing conditions, while the P*pgaX-amdS ΔgaaX* (JN123.1) strain did, as it was shown previously (Niu *et al.*, 2017).

Figure 1. Schematic representation of the domains present in GaaR **(A)** and conservation of GaaR^{W361} in *Aspergillus* species **(B)** and other Ascomycetes **(C)**. Domains in GaaR (NRRL3_08195) were identified using NCBI's CDD (Marchler-Bauer *et al.*, 2015). Protein sequences homologous to the *A. niger* GaaR were retrieved using the blastp algorithm from NCBI (Altschul *et al.*, 1990) against the nonredundant protein sequences database, and were aligned using Clustal Omega (Sievers *et al.*, 2011).

The effect of the W361R mutation in *gaaR* on the expression of pectinase genes was also analysed using the promoter-reporter strains expressing the *amdS* gene via the *pgaX*

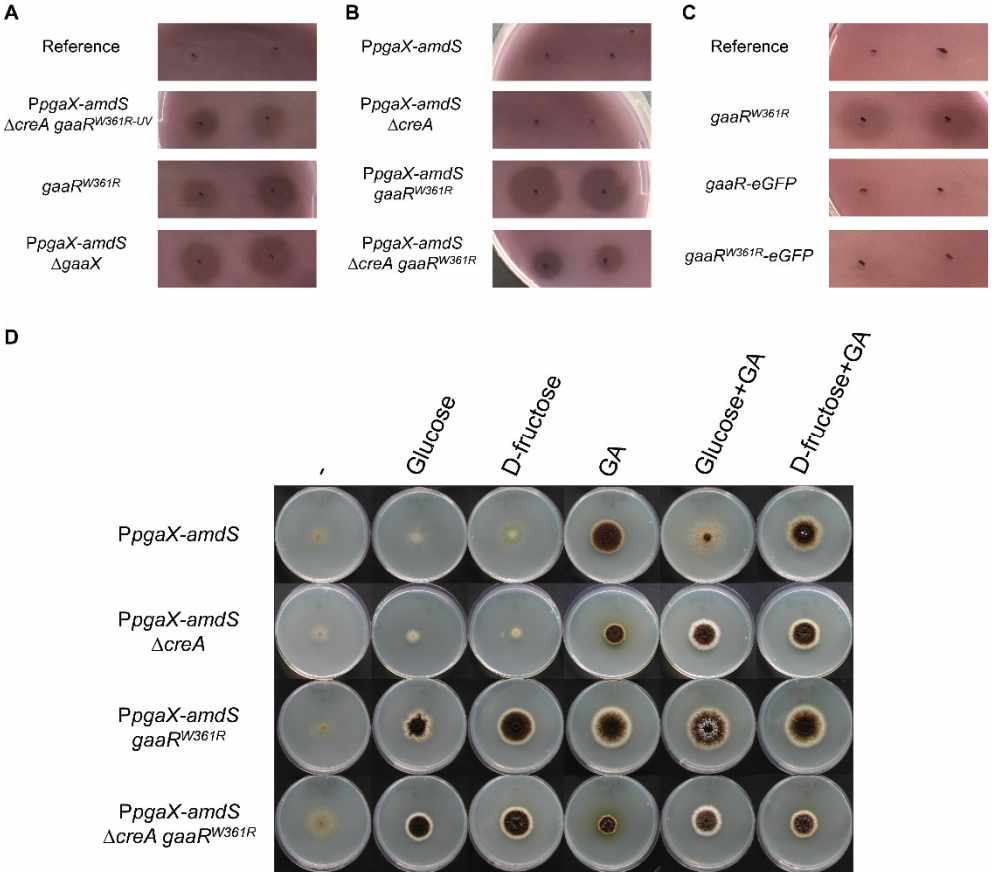

Figure 2. Effect of the W361R mutation in *gaaR* on the expression of pectinase genes. **A-C** PGA plate assay. Strains were grown in MM containing 50 mM D-fructose for 36 h, and culture supernatants were spotted PGA plates. Enzymatic activities in the supernatants from duplicate cultures are shown: **(A)** the reference (MA234.1), P*pgaX-amdS ΔcreA gaaR*$^{W361R\text{-}UV}$ (JN103.1), *gaaR*^{W361R} (EA34.1), P*pgaX-amdS ΔgaaX* (JN123.1); **(B)** P*pgaX-amdS* (JC1.5), P*pgaX-amdS ΔcreA* (JN29.2), P*pgaX-amdS gaaR*^{W361R} (JN130.4), P*pgaX-amdS ΔcreA gaaR*^{W361R} (JN129.1); and **(C)** reference (MA234.1), *gaaR*^{W361R} (EA34.1), *gaaR-eGFP* (EA36.1) and *gaaR*^{W361R}*-eGFP* (EA39.1). **D** Growth phenotype of the P*pgaX-amdS* (JC1.5), P*pgaX-amdS ΔcreA* (JN29.2), P*pgaX-amdS gaaR*^{W361R} (JN130.4) and P*pgaX-amdS ΔcreA gaaR*^{W361R} (JN129.1) strains after 7 days at 30 °C on solid MM containing no carbon source (-); 50 mM glucose, D-fructose or GA as the sole carbon source; or 50 mM glucose or D-fructose together with 50 mM GA. All plates contain 10 mM acetamide as the sole nitrogen source.

promoter. The *gaaR*^{W361R} gene was transformed into the P*pgaX-amdS* (JC1.5) and P*pgaX-amdS ΔcreA* (JN29.2) strains. Transformants were selected on medium containing a non-inducing carbon source and acetamide as the nitrogen source, and only transformants in which the *gaaR*^{W361R} was integrated were expected grow on the transformation plate because of constitutive activation of the P*pgaX-amdS* reporter. Southern blot analysis and sequencing of the *gaaR* locus showed that the endogenous *gaaR* gene was replaced with *gaaR*^{W361R} in the resulting strains P*pgaX-amdS gaaR*^{W361R} (JN130.4) and P*pgaX-amdS ΔcreA gaaR*^{W361R} (JN129.1), respectively (Figure S3). Growth of P*pgaX-amdS gaaR*^{W361R} (JN130.4)

and P*pgaX-amdS ΔcreA gaaR*^{W361R} (JN129.1) was similar to the growth of their parental strains on MM containing glucose, D-fructose, GA, PGA or AP (Figure S2).

The PGA plate assay showed that the parental strains P*pgaX-amdS* (JC1.5) and P*pgaX-amdS ΔcreA* (JN29.2) did not produce any polygalacturonases in D-fructose, while P*pgaX-amdS gaaR*^{W361R} (JN130.4) and P*pgaX-amdS ΔcreA gaaR*^{W361R} (JN129.1) showed polygalacturonase activity in their culture supernatants (Figure 2B). The expression of *pgaX* was previously shown to be induced only in the presence of an inducing carbon source, such as GA (Niu *et al.*, 2015). Radial growth assays on plates containing acetamide showed that P*pgaX-amdS gaaR*^{W361R} (JN130.4) and P*pgaX-amdS ΔcreA gaaR*^{W361R} (JN129.1) can grow on glucose or D-fructose, while their parental strains cannot (Figure 2D). This indicates that GaaR^{W361R} can activate the expression of *amdS* via the *pgaX* promoter in an inducer-independent way, confirming the results obtained by PGA plate assays.

The expression of *pgaX* is known to be repressed strongly on glucose and mildly on D-fructose via CreA (Niu *et al.*, 2015; Alazi *et al.*, 2018). As shown in Figure 2D, growth of the promoter-reporter strains on plates containing acetamide and GA decreased when glucose or D-fructose was added to the growth media, and this growth reduction was restored when *creA* is deleted. Although P*pgaX-amdS gaaR*^{W361R} (JN130.4) could grow both on glucose and D-fructose as the sole carbon source, its growth on glucose was reduced compared to on D-fructose (Figure 2D). This indicates that CreA-mediated repression on *pgaX* plays an important role even in the presence of the constitutively active GaaR^{W361R}.

Subcellular localization of GaaR-eGFP and GaaR^{W361R}-eGFP

We have previously shown that *eGFP-gaaR* (EA19.2), a strain expressing the N-terminally eGFP-tagged *gaaR*, shows a slightly reduced growth on GA compared to the reference strain (Alazi *et al.*, 2018), indicating that N-terminal GFP-tagging might result in a slight decrease in GaaR activity. To check whether C-terminal GFP-tagging affects GaaR activity, strains expressing C-terminally eGFP-tagged *gaaR*, with or without a linker between GaaR and eGFP, were created in a *ΔgaaR* background. While *ΔgaaR* (JN35.1) could not grow on GA, growth on GA was fully complemented in both *gaaR-eGFP* (EA29.14) and *gaaR-(GA)*$_4$*-eGFP* (EA30.6) (Figure S4). This indicates that C-terminal eGFP-tagging does not affect the transcriptional activity of GaaR, independently of the presence of a linker between GaaR and eGFP.

To analyse the subcellular localization of GaaR-eGFP and GaaR^{W361}-eGFP, strains that express C-terminally eGFP-tagged *gaaR* or *gaaR*^{W361R} from the endogenous *gaaR* locus were created, and integration of the P*gaaR-gaaR-eGFP*-T*gaaR* or P*gaaR-gaaR*^{W361R}*-eGFP*-T*gaaR* constructs into the endogenous *gaaR* locus was verified by Southern blot analysis (Figure S3). Growth of *gaaR-eGFP* (EA31.1) and *gaaR*^{W361R}*-eGFP* (EA32.1) was fully complemented on GA and GA-containing carbon sources (Figure S2), confirming previous results that GaaR-eGFP is fully functional under inducing conditions.

The ability of *gaaR-eGFP* (EA36.1) and *gaaR*^{W361R}*-eGFP* (EA39.1), uridine prototrophs derived from *gaaR-eGFP* (EA31.1) and *gaaR*^{W361R}*-eGFP* (EA32.1), respectively, to constitutively express pectinases was assessed via a PGA plate assay and compared to that

of *gaaR*^{W361R} (EA34.1) (Figure 2C). No polygalacturonase activity was observed in the culture supernatant of the reference (MA234.1) strain or *gaaR-eGFP* (EA36.1) after growth in D-fructose, indicating that C-terminal eGFP-tagging does not affect the inactive state of GaaR under non-inducing conditions, similar to N-terminal eGFP-tagging (Alazi *et al.*, 2018). While the *gaaR*^{W361R} (EA34.1) strain expressing the constitutively active *gaaR*^{W361R} produced pectinases in D-fructose, the *gaaR*^{W361R}*-eGFP* (EA39.1) strain expressing *gaaR*^{W361R}*-eGFP* did not (Figure 2C). This shows that, unlike GaaR^{W361R}, GaaR^{W361R}-eGFP did not activate the expression of pectinases under non-inducing conditions.

The subcellular localization of the C-terminally eGFP-tagged GaaR and GaaR^{W361R} in *gaaR-eGFP* (EA36.1) and *gaaR*^{W361R}*-eGFP* (EA39.1) grown under inducing (i.e. on 2-keto-3-deoxy-L-galactonate) or non-inducing (i.e. on D-fructose) conditions was analysed using confocal laser scanning microscopy (Figure 3). Beforehand, the potency of 2-keto-3-deoxy-L-galactonate, the physiological inducer (Alazi *et al.*, 2017), to induce the expression of the GA-responsive genes was tested using the promoter-reporter strain P*pgaX-amdS ΔcreA* (JN29.2) and was compared to that of GA (Figure S5). P*pgaX-amdS ΔcreA* (JN29.2) showed an impaired growth on plates containing acetamide and 50 mM glucose. Addition of 100 nM 2-keto-3-deoxy-L-galactonate to the growth media resulted in a major increase in growth, whereas addition of a 100 nM GA was not sufficient to induce the growth of P*pgaX-amdS ΔcreA* (JN29.2), indicating that 2-keto-3-deoxy-L-galactonate is more potent than GA in inducing the expression of pectinases.

We previously showed that the partially functional N-terminally eGFP-tagged GaaR in *eGFP-gaaR* (EA19.2) is localized mainly in the nucleus under both inducing (i.e. on GA) and non-inducing (i.e. on D-fructose) conditions (Alazi *et al.*, 2018). Here, we show again that eGFP-GaaR is mainly localized in the nucleus after growth in 2-keto-3-deoxy-L-galactonate or D-fructose (Figure 3). C-terminally eGFP-tagged GaaR and GaaR^{W361R} were also localized mainly in the nucleus after growth in 2-keto-3-deoxy-L-galactonate, while apparent nuclear fluorescence was not observed after growth in D-fructose. This result indicates that on D-fructose (under non-inducing and mildly repressing conditions) nuclear accumulation of C-terminally eGFP-tagged GaaR is restrained, suggesting that the inability of GaaR^{W361R}-eGFP to activate the expression of pectinases under non-inducing conditions might be caused by improper subcellular localization to execute its function.

DISCUSSION

We previously isolated via a forward genetic screen *A. niger* strains displaying constitutive expression of genes encoding pectinases (Niu *et al*., 2017). The majority of the mutant collection (60 strains) was found to contain a loss-of-function mutation in *gaaX*, the repressor of GA utilization. One mutant with no mutations in *gaaX* was found to possess the missense mutation W361R in *gaaR*. Introducing the W361R mutation in the wild type genetic background and subsequent gene expression analyses allowed us to conclude that this mutation results in constitutive activation of GaaR and therefore constitutive expression of pectinase genes.

To our knowledge, XlnR was the only transcription factor involved in plant biomass degradation in which missense mutations leading to constitutive expression of plant

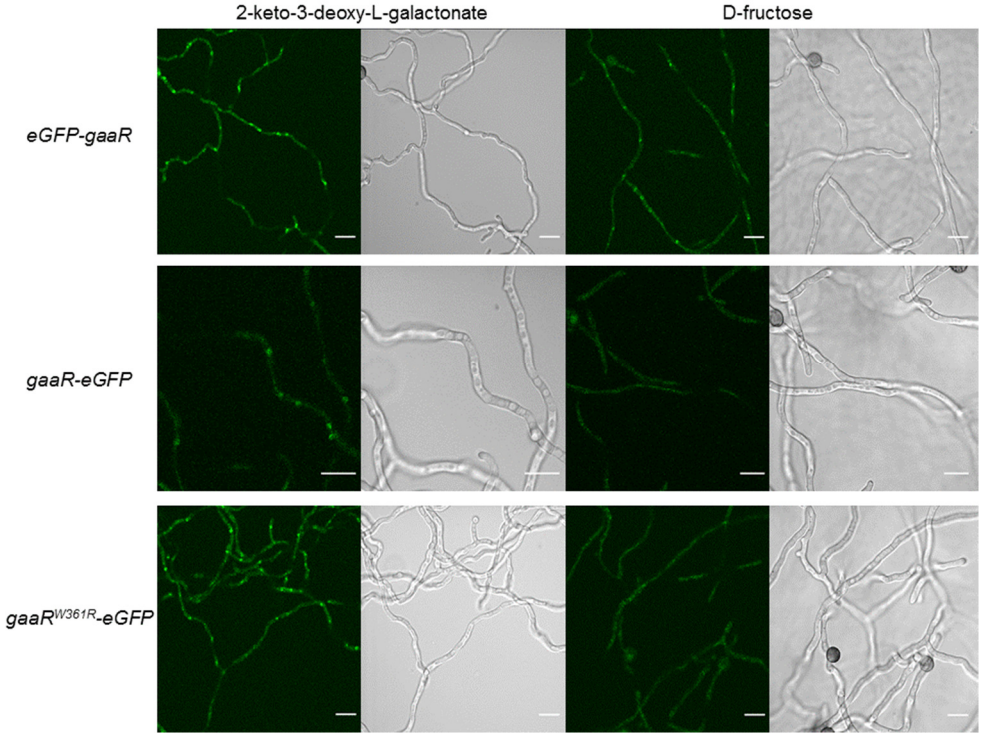

Figure 3. Nuclear and cytoplasmic fluorescence intensities of the eGFP-tagged GaaR and GaaR^{W361R} proteins. The *eGFP-gaaR* (EA19.2), *gaaR-eGFP* (EA36.1) and *gaaR^{W361R}-eGFP* (EA39.1) strains were grown in MM containing 10 mM D-fructose or 2-keto-3-deoxy-L-galactonate for 21 h. Example micrographs representing each condition are shown. Scale bar: 10 µm.

biomass degrading enzymes were reported, such as V756F in *A. niger*, or A824V in *Trichoderma reesei* and orthologous mutations in *Neurospora crassa* and *Penicillium oxalicum* (Hasper, 2004; Hasper *et al.*, 2004; Derntl *et al.*, 2013; Craig *et al.*, 2015; Gao *et al.*, 2017). Identification of the constitutively active GaaR^{W361R} would benefit the industrial production of pectinases in an inducer-independent way.

The activity of GaaR is proposed to be inhibited by GaaX under non-inducing conditions via protein-protein interaction (Niu et al., 2017). Pectinase genes are expressed under inducing conditions (i.e. in the presence of inducer), or under non-inducing conditions (i.e. in the absence of inducer) when *gaaX* is deleted or *gaaR* is overexpressed (Martens-Uzunova and Schaap, 2008; Niu *et al.*, 2017; Alazi *et al.*, 2018). These findings indicate that GA-responsive gene expression only requires the presence of active GaaR relieved/escaped from GaaX inhibition, and does not require the presence of the inducer. This suggests that under inducing conditions, the inducer binds to GaaX resulting in a GaaX-unbound and active form of GaaR. Therefore, we propose that W361R mutation in GaaR disrupts the interaction between GaaX and GaaR under non-inducing conditions. Loss-of-function mutations were found throughout all three domains of *gaaX* (shikimate kinase, 3-dehydroquinate dehydratase and shikimate dehydrogenase) (Figure S1), making it difficult to predict the domain that possibly interacts with GaaR.

N-terminally eGFP-tagged GaaR was previously shown to mainly localize in the nucleus under both inducing and non-inducing conditions, but was unable to fully complement the growth of *ΔgaaR* when grown on inducing carbon sources (Alazi *et al.*, 2018). C-terminally eGFP-tagged GaaR/GaaR^{W361R} accumulated in the nucleus under inducing conditions and complemented the growth of *ΔgaaR* when grown on inducing carbon sources. Based on that we conclude that C-terminally eGFP-tagged GaaR/GaaR^{W361R} is fully functional under inducing conditions. However, unlike the N-terminally eGFP-tagged GaaR, strains expressing the C-terminally eGFP-tagged GaaR/GaaR^{W361R} did not show higher nuclear fluorescence intensity compared to cytoplasmic fluorescence intensity under non-inducing conditions. This result indicates that C-terminal eGFP-tagging restrained nuclear accumulation of GaaR/GaaR^{W361R}, confirming the inability of GaaR^{W361R}-eGFP to constitutively activate the expression of pectinases, as GaaR^{W361R} can.

Although the probability of identifying gain-of-function mutations leading to constitutive activation of GaaR was very low compared to that of loss-of-function of GaaX, constitutively active forms of GaaR can be utilized in protein/metabolite interaction studies and provide insight in regulation of GA-responsive gene expression. The nature of mutations in three UV mutants with no mutations in *gaaX* or *gaaR* remains to be discovered.

ACKNOWLEDGEMENTS

EA and JN were supported by grants from BE-Basic (Flagship 10) and the China Scholarship Council, respectively. This work was in part supported by the Natural Sciences and Engineering Research Council of Canada Strategic Industrial Biocatalysis Network.

REFERENCES

Alazi E, Khosravi C, Homan TG, du Pré S, Arentshorst M, Di Falco M, Pham TTM, Peng M, Aguilar-Pontes MV, Visser J, Tsang A, de Vries RP, Ram AFJ (2017) The pathway intermediate 2-keto-3-deoxy-L-galactonate mediates the induction of genes involved in D-galacturonic acid utilization in *Aspergillus niger*. *FEBS Letters* 591:1408-1418

Alazi E, Knetsch T, Di Falco M, Reid ID, Arentshorst M, Visser J, Tsang A, Ram AFJ (2018) Inducer-independent production of pectinases in *Aspergillus niger* by overexpression of the D-galacturonic acid responsive transcription factor *gaaR*. *Applied Microbiology and Biotechnology* 102:2723-2736

Alazi E, Niu J, Kowalczyk JE, Peng M, Aguilar Pontes MV, van Kan JAL, Visser J, de Vries RP, Ram AFJ (2016) The transcriptional activator GaaR of *Aspergillus niger* is required for release and utilization of D-galacturonic acid from pectin. *FEBS Letters* 590:1804-1815

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *Journal of Molecular Biology* 215:403-410

Arentshorst M, Niu J, Ram AFJ (2015) Efficient Generation of *Aspergillus niger* Knock Out Strains by Combining NHEJ Mutants and a Split Marker Approach. In van den Berg MA, Maruthachalam K (ed), Genetic Transformation Systems in Fungi, Vol. 1, Springer International Publishing, pp 263-272 Arentshorst M, Ram AFJ, Meyer V (2012) Using Non-homologous End-Joining-Deficient Strains for Functional Gene Analyses in Filamentous Fungi. *Plant Fungal Pathogens Methods in Molecular*

Biology 835:133-150

Caffall KH, Mohnen D (2009) The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. *Carbohydrate Research* 344(14)

Carvalho NDSP, Arentshorst M, Kwon MJ, Meyer V, Ram AFJ (2010) Expanding the *ku70* toolbox for filamentous fungi: establishment of complementation vectors and recipient strains for advanced gene analyses. *Applied Microbiology and Biotechnology* 87:1463-1473

Craig JP, Coradetti ST, Starr TL, Glass NL (2015) Direct target network of the *Neurospora crassa* plant cell wall deconstruction regulators CLR-1, CLR-2, and XLR-1. *mBio* 6(5):1–11

de Ruiter-Jacobs YMJT, Broekhuijsen M, Unkles SE, Campbell EI, Kinghorn JR, Contreras R, Pouwels PH, van den Hondel CAMJJ (1989) A gene transfer system based on the homologous *pyrG* gene and efficient expression of bacterial genes in *Aspergillus oryzae*. *Current Genetics* 16:159-163

Derntl C, Gudynaite-Savitch L, Calixte S, White T, Mach RL, Mach-Aigner AR (2013) Mutation of the Xylanase regulator 1 causes a glucose blind hydrolase expressing phenotype in industrially used *Trichoderma* strains. Biotechnol. *Biofuels* 6:1–11

DiCarlo JE, Norville JE, Mali P, Rios X, Aach J, Church GM (2013) Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. *Nucleic Acids Research* 41(7):4336-4343

Edwards MC, Doran-Peterson J (2012) Pectin-rich biomass as feedstock for fuel ethanol production. *Applied Microbiology and Biotechnology* 95:565–575

Gao L, Li Z, Xia C, Qu Y, Liu M, Yang P et al. (2017) Combining manipulation of transcription factors and overexpression of the target genes to enhance lignocellulolytic enzyme production in *Penicillium oxalicum*. Biotechnol. *Biofuels* 10:1-16

Hasper AA (2004) Function and mode of regulation of the transcriptional activator XlnR from *Aspergillus*. [dissertation]. [Wageningen (The Netherlands)]: Wageningen University

Hasper AA, Trindade LM, van der Veen D, van Ooyen AJJ, de Graaff LH (2004) Functional analysis of the transcriptional activator XlnR from *Aspergillus niger*. *Microbiology* 150:1367-1375

Heigwer F, Kerr G, Boutros M (2014) E-CRISP: fast CRISPR target site identification. *Nature Methods* 11:122-123

Kashyap D, Vohra P, Chopra S, Tewari R (2001) Applications of pectinases in the commercial sector: a review. *Bioresource Technology* 77:215–227

Khan M, Nakkeeran E, Umesh-Kumar S (2013) Potential application of pectinase in developing functional foods. *Annual Review of Food Science and Technology* 4:21–34

Kowalczyk JE, Benoit I, de Vries RP (2014) Regulation of plant biomass utilization in *Aspergillus*. *Advances in Applied Microbiology* 88: 31-56

Marchler-Bauer A, Derbyshire MK, Gonzales NR, Lu S, Chitsaz F, Geer LY, Geer RC, He J, Gwadz M, Hurwitz DI, Lanczycki CJ, Lu F, Marchler GH, Song JS, Thanki N, Wang Z, Yamashita RA, Zhang D, Zheng C, Bryant SH (2015) CDD: NCBI's conserved domain database. *Nucleic Acids Research* 43:D222-226 Martens-Uzunova ES, Schaap PJ (2008) An evolutionary conserved D-galacturonic acid metabolic pathway operates across filamentous fungi capable of pectin degradation. *Fungal Genetics and Biology* 45(11):1449-1457

Niu J, Alazi E, Reid ID, Arentshorst M, Punt PJ, Visser J, Tsang A, Ram AFJ (2017) An Evolutionarily Conserved Transcriptional Activator-Repressor Module Controls Expression of Genes for D-Galacturonic Acid Utilization in *Aspergillus niger*. *Genetics* 205:169-183

Niu J, Homan TG, Arentshorst M, de Vries RP, Visser J, Ram AF (2015) The interaction of induction and repression mechanisms in the regulation of galacturonic acid-induced genes in *Aspergillus niger*. *Fungal Genetics and Biology* 82:32-42

Nødvig CS, Nielsen JB, Kogle ME, Mortensen UH (2015) A CRISPR-Cas9 System for Genetic Engineering of Filamentous Fungi. *PLoS One* 10(7):e0133085

Papadopoulos JS, Agarwala R (2007) COBALT: constraint-based alignment tool for multiple protein sequences. *Bioinformatics* 23:1073-1079

Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J, Thompson JD, Higgins DG (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology* 7:539

Toushik SH, Lee K, Lee J, Kim K (2017) Functional applications of lignocellulolytic enzymes in the fruit and vegetable processing industries. *Journal of Food Science* 88(3):583–595

van Hartingsveldt W, Mattern IE, van Zeijl CM, Pouwels PH, van den Hondel CA (1987) Development of a homologous transformation system for *Aspergillus niger* based on the *pyrG* gene. *Molecular and General Genetics* 206(1):71-5

Yuan XL, Goosen C, Kools H, van den Maarel MJEC, van den Hondel CAMJJ, Dijkhuizen L, Ram AFJ (2006) Database mining and transcriptional analysis of genes encoding inulin-modifying enzymes of *Aspergillus niger*. *Microbiology* 152:3061-3073

SUPPORTING INFORMATION

Table S1. Strains used in this study.

Strain	Accession Number	Genotype	Description	Reference
MA169.4	FGSC A1279	*cspA1, pyrG^{378}, kusA::DR-amdS-DR*	Transiently silenced *kusA* in AB4.1	Carvalho *et al.*, 2010
MA234.1	CBS 141255	*cspA1, kusA::DR-amdS-DR*	Restored *pyrG* in MA169.4	Alazi *et al.*, 2016
XY1.1	CBS 143276	*cspA1, pyrG^{378}, creA::pyrG*	*ΔcreA* in AB4.1	Yuan *et al.*, 2006
JC1.5	CBS 143271	*cspA1, Δku70,* P*pgaX-amdS* integrated to the *pyrG* locus	P*pgaX-amdS* in MA299.2	Niu *et al.*, 2015
JN29.2	CBS 143272	*cspA1, Δku70,* P*pgaX-amdS* integrated to the *pyrG* locus*, creA::hygB*	*ΔcreA* in JC1.5	Niu *et al.*, 2015
JN123.1	CBS 143275	*cspA1, Δku70,* P*pgaX-amdS* integrated to the *pyrG* locus*, gaaX::hygB*	*ΔgaaX* in JC1.5	Niu *et al.*, 2017
JN130.4		*cspA1, Δku70,* P*pgaX-amdS* integrated to the *pyrG* locus, *gaaR^{W361R}* integrated to the *gaaR* locus	*gaaR^{W361R}* in JC1.5	This study
JN103.1		*cspA1, Δku70,* P*pgaX-amdS* integrated to the *pyrG* locus*, creA::hygB, UV mutagenized yielding to gaaR^{W361R}*	*gaaR$^{W361R\text{-}UV}$* in JN29.2	This study
JN129.1		*cspA1, Δku70,* P*pgaX-amdS* integrated to the *pyrG* locus*, creA::hygB,* *gaaR^{W361R}* integrated to the *gaaR* locus	*gaaR^{W361R}* in JN29.2	This study
SO1.1		*cspA1, pyrG^{378}, kusA::DR-amdS-DR, gaaR::AOpyrG*	*ΔgaaR* in MA169.4	This study
SO2.1		*cspA1, pyrG^{378}, kusA::DR-amdS-DR, gaaR^{W361R}* integrated to the *gaaR* locus	*gaaR^{W361R}* in SO1.1	This study
EA34.1		*cspA1, pyrG^{378}, kusA::DR-amdS-DR, gaaR^{W361R}* integrated to the *gaaR* locus, ectopically integrated pAB4.1	Restored *pyrG* in SO2.1	This study
EA31.1		*cspA1, pyrG^{378}, kusA::DR-amdS-DR, gaaR-eGFP* integrated to the *gaaR* locus	*gaaR-eGFP* in SO1.1	This study
EA36.1		*cspA1, pyrG^{378}, kusA::DR-amdS-DR, gaaR-eGFP* integrated to the *gaaR* locus, ectopically integrated pAB4.1	Restored *pyrG* in EA31.1	This study
EA32.1		*cspA1, pyrG^{378}, kusA::DR-amdS-DR, gaaR^{W361R}-eGFP* integrated to the *gaaR* locus	*gaaR^{W361R}-eGFP* in SO1.1	This study
EA39.1		*cspA1, pyrG^{378}, kusA::DR-amdS-DR, gaaR^{W361R}-eGFP* integrated to the *gaaR* locus, ectopically integrated pAB4.1	Restored *pyrG* in EA32.1	This study
JN35.1	CBS 141256	*cspA1, kusA::DR-amdS-DR, gaaR::hygB*	*ΔgaaR* in MA234.1	Alazi *et al.*, 2016
JN36.1	CBS 143259	*cspA1, gaaR::hygB*	*amdS* loopout in JN35.1	Alazi *et al.*, 2016

Table S1. Strains used in this study. (Continued)

Strain	Accession Number	Genotype	Description	Reference
EA19.2	CBS 143267	*cspA1, gaaR::hygB, amdS⁺, ectopically integrated* P*gaaR-eGFP-gaaR*	*eGFP-gaaR* in JN36.1	Alazi *et al.*, 2018
EA29.14		*cspA1, gaaR::hygB, amdS⁺, ectopically integrated* P*gaaR-gaaR-eGFP*	*gaaR-eGFP* in JN36.1	This study
EA30.6		*cspA1, gaaR::hygB, amdS⁺, ectopically integrated* P*gaaR-gaaR-*$(GA)_4$*-eGFP*	*gaaR-*$(GA)_4$*-eGFP* in JN36.1	This study

Table S2. Primers used in this study. Fusion PCR overlapping regions are written in bold.

Primer name	Sequence (5' to 3')	Used for
gaaX_P5f	CGTTGGTTCGATGTAAATGGG	Amplification and sequencing of *gaaX*
gaaX_GSP10r	TGTATGTGGCATTGATGGAACC	Sequencing of *gaaX*
gaaX_GSP11f	GGGTTGTTTGTCGGTGTACTCG	Sequencing of *gaaX*
gaaX_GSP12r	CGGTTCTTGGTCTTCAGGTCCT	Sequencing of *gaaX*
gaaX_GSP13f	CCTGTACTCTTTGCGCAGTCCT	Sequencing of *gaaX*
gaaX_GSP14r	GCTGTCCCATGATGTGGAGG	Sequencing of *gaaX*
gaaX_GSP15f	TTTCGCCCATCACGCATC	Sequencing of *gaaX*
gaaX_GSP16r	CCATGCATCGACATCTTCAATC	Sequencing of *gaaX*
gaaX_GSP17f	AGTGTACCTGGACTTGACGC	Sequencing of *gaaX*
gaaX_GSP9r	CAAACGTGGAGAAGCCGGT	Amplification and sequencing of *gaaX*
gaaRP7f	TCCTCTTCGGCTTCTGCTTC	Amplification and sequencing of *gaaR*
gaaRP9r	GCATACTCCAGGCTCCCTTG	Sequencing *gaaR*
gaaRP10f	CCTACATGGGAAGCCCTATTGG	Sequencing *gaaR*
gaaRP11r	CGATAGAGCAGACAGTGCACGA	Sequencing *gaaR*
gaaRP12f	CCCTGTTGGTCGACATTACGC	Sequencing *gaaR*
gaaRP13r	AAATCGGGTCGGTCCAGTG	Sequencing *gaaR*
gaaRP14f	AGGACTTCCTGGACCGGTAT	Sequencing *gaaR*
gaaRP8r	CATGTGATCATTTCGTGGCCT	Amplification and sequencing of *gaaR*
gaaRP5f	TGGACGGCGTATGGGATT	Amplification of P*gaaR-gaaR*-T*gaaR*
gaaRP6r	CCCAAAGACGGGTGGTAGAGT	Amplification of P*gaaR-gaaR*-T*gaaR*
gaaR SBfor	CCTCGACGCCATTCCAGTT	Amplification of Southern blot probe

Table S2. Primers used in this study. Fusion PCR overlapping regions are written in bold. (Continued)

Primer name	Sequence (5' to 3')	Used for
gaaR SBrev	GGTCATGGACACCGCATTG	Amplification of Southern blot probe
GaaR_GFP4F	**GCATGGACGAGCTGTACAAG**TAAGAAGCCGAATATACGAGCCAT	Amplification of T*gaaR*
GaaR_GFP3R	GCGTGCATCAAGGCGATT	Amplification of T*gaaR*
GaaR_GFP1F	**ATGGTGAGCAAGGGCGAG**	Amplification of *eGFP*
GaaR_GFP1R	**CTTGTACAGCTCGTCCATG**	Amplification of *eGFP*
GaaR_GFP2F	TGGTGTGATGAAGGCAGTGTG	Amplification of P*gaaR-gaaR(-(GA)$_4$)*
GaaR_GFP5R	**TCCTCGCCCTTGCTCACCA**TAGGATTCTCCACCTCCACC	Amplification of P*gaaR-gaaR*
eGFP-gaaR-For	TGGTGTGATGAAGGCAGTGTG	Fusion PCR to amplify P*gaaR-gaaR(-(GA)$_4$)-eGFP*-T*gaaR*
eGFP-gaaR-Rev	TGGGTGTTTGGTGGGTTGA	Fusion PCR to amplify P*gaaR-gaaR(-(GA)$_4$)-eGFP*-T*gaaR*
GaaR_GFP6R	**TCCTCGCCCTTGCTCACCA**TAGCGCCAGCGCCAGCGCCAGCGCCAGGATTCT CCACCTCCACC	Amplification of P*gaaR-gaaR-(GA)$_4$*
gaaRP1f	GCTGCTGCTGCTGCTTTACA	Amplification of *gaaR* 5' flank to create SO1.1
gaaRP2r	**CAATTCCAGCAGCGGCTT**GGCATTGCCTGTGCATAGGA	Amplification of *gaaR* 5' flank to create SO1.1
gaaRP3f	**ACACGGCACAATTATCCATCG**GAAGCCGAATATACGAGCCA	Amplification of *gaaR* 3' flank to create SO1.1
gaaRP4r	GCGTGCATCAAGGCGATT	Amplification of *gaaR* 3' flank to create SO1.1
AOpyrGP12f	**AAGCCGCTGCTGGAATTG**	Amplification of *AOpyrG* 5' flank to create SO1.1
AOpyrGP15r	CCGGTAGCCAAAGATCCCTT	Amplification of *AOpyrG* 5' flank to create SO1.1
AOpyrGP13r	**CGATGGATAATTGTGCCGTGT**	Amplification of *AOpyrG* 3' flank to create SO1.1
AOpyrGP14f	ATTGACCTACAGCGCACGC	Amplification of *AOpyrG* 3' flank to create SO1.1
sgRNAP1	TAATACGACTCACTATAGGGTGGATACGTACTCCTTTTAGTTTTAGAGCTAG AAATAGCAA	Amplification of the DNA template for guide RNA
OTL19	GCACCACCGACTCGGTGC	Amplification of the DNA template for guide RNA
Fw_LIC2	CAACCTCCAATCCAATTTGACTCCGCCGAACGTACTG	Amplification of the DNA template for guide RNA
Rev_P1	**TAAAAGGAGTACGTATCCAC**GACGAGCTTACTCGTTTCG	Amplification of the DNA template for guide RNA
Fw_P1	**GTGGATACGTACTCCTTTTAGTTTTA**GAGCTAGAAATAGCAAG	Amplification of the DNA template for guide RNA
Rev_LIC2	ACTACTCTACCACTATTTGAAAAGCAAAAAAGGAAGGTACAAAAAAGC	Amplification of the DNA template for guide RNA
for_pTE1	CCTTAATTAAACTCCGCCGAACGTACTG	Introduction of *Pac*I site to the DNA template for guide RNA
rev_pTE1	CCTTAATTAAAAAAGCAAAAAAGGAAGGTACAAAAAAGC	Introduction of *Pac*I site to the DNA template for guide RNA
PgaaR_seq_P2	CATCCATCTTAGTCCACCGGC	Amplification of *gaaR*
TgaaR_seq_P2	TCTAGTATTCACTTGTCCCTAA	Amplification of *gaaR*

Table S3. Mutations in *gaaX* in the UV mutants showing constitutive expression of pectinases. aa and gDNA represents amino acid and genomic DNA, respectively.

Strain	Type of mutation	Mutation	Codon change	aa change	Predicted length of the mutated protein (aa)	Remarks
S1	Frameshift	Insertion of G	GTT-GGT	V653G	664	gDNA sequenced, no mutation found in *gaaR* (Niu *et al.*, 2017)
S3	Missense	T-C	CTG-CCG	L111P		
S5	Nonsense	C-T	CAA-TAA	Q369Stop	368	gDNA sequenced, no mutation found in *gaaR* (Niu *et al.*, 2017)
S6						No mutations found in *gaaX* or *gaaR*
S7	Missense	T-C	CTG-CCG	L676P		gDNA sequenced, no mutation found in *gaaR* (Niu *et al.*, 2017)
S8	Frameshift	Deletion of TG	GTG-GGG	V193-G	197	
S9	Nonsense	C-T	CGA-TGA	R425Stop	424	
S10	Frameshift	Deletion of G	GGA-GAG	G644E	702	
S11	Frameshift	Insertion G	GGAGCC-GGGAGC	GA469GS	664	
S12	Nonsense	C-T	CAA-TAA	Q369Stop	368	
S13	Nonsense	G-T	GAG-TAG	E129Stop	128	
UV 1	Nonsense	G-T	GAG-TAG	E458Stop	457	gDNA sequenced, no mutation found in *gaaR* (Niu *et al.*, 2017)
UV 3	Nonsense	T-A	TTA-TAA	L101Stop	100	No mutation found in *gaaR* (Niu *et al.*, 2017)
UV 4	Nonsense	C-T	CAA-TAA	Q369Stop	368	No mutation found in *gaaR* (Niu *et al.*, 2017)
UV 5	Nonsense	G-A	TGGGGA-TAGGAA	W270Stop	269	No mutation found in *gaaR* (Niu *et al.*, 2017)
UV 6	Missense	GT-AA	GTA-AAA	V684K		No mutation found in *gaaR* (Niu *et al.*, 2017)
UV 8	Missense	G-A	GGA-GAA	G526E		gDNA sequenced, no mutation found in *gaaR* (Niu *et al.*, 2017)
UV12	Missense	A-G	AAT-GAT	N438D		No mutation found in *gaaR* (Niu *et al.*, 2017)
UV13	Frameshift	Insertion of G	GCG-GGC	A308G	438	No mutation found in *gaaR* (Niu *et al.*, 2017)
UV14	Missense	A-G	AAT-GAT	N521D		
UV15	Missense	GT- AA	GTG-AAG	V193K		
UV16	Missense	GAC-ATT	GAC-ATT	D640I		

Table S3. Mutations in *gaaX* in the UV mutants showing constitutive expression of pectinases. aa and gDNA represents amino acid and genomic DNA, respectively. (Continued)

Strain	Type of mutation	Mutation	Codon change	aa change	Predicted length of the mutated protein (aa)	Remarks
UV17	Missense	T-A	GAT-GAA	D496E		
UV18	Nonsense	T-A	TTA-TAA	L548Stop	547	
UV19	Missense	T-A	ATT-AAT	I190N		
UV20	Missense	G-A	GGG-GAC	G194D		
UV21	Nonsense	C-T	CAG-TAG	Q106Stop	105	
UV22	Nonsense	G-A	TGG-TAG	W270Stop	269	
UV23	Nonsense	A-T	AAA-TAA	K435Stop	434	
UV24	Nonsense	G-T	GAG-TAG	E43Stop	42	
UV25	Nonsense	T-A	TTA-TAA	L38Stop	37	
UV26	Missense	A-T	AAT-ATT	N521I		
UV27	Nonsense	A-T	AAG-TAG	K415Stop	414	
UV28	Missense	A-T	AAC-TAC	N378Y		
UV29	Missense	G-C	GCG-CCG	A321P		
UV30	Missense and frameshift	CA-G	TCCACG-TCGCGC	ST426SR	445	
UV31	Missense and nonsense	AC-CT	TTACAA-TTCTAA	LQ101FStop	101	
UV32	Missense	A-C	TAC-TCC	Y638S		
UV33	Missense and nonsense	AC-CT	TTACAA-TTCTAA	LQ101FStop	101	
UV34	Frameshift	Deletion of T	TTT-TTG	F466L	516	
UV35	Missense	A-G	AAT-GAT	N438D		
UV36	Missense	GT-AA	GTC-AAC	V51N		
UV37	Missense and frameshift	CCC-TC	GGCCCG-GGTCGC	GP579GR	593	
UV38	Missense	T-G	CTG-CGG	L607R		
UV39	Missense	G-A	GGG-GAG	G194E		
UV40	Missense	A-G	AAT-GAA	N438E		
UV41	Missense or nonsense	A-T	ATG-TTG	M1L	- (if nonsense)	
UV42	Nonsense	C-A	TCG-TAG	S306Stop	305	
UV43	Nonsense	AC-CT	CAA-TAA	Q102Stop	101	

Table S3. Mutations in *gaaX* in the UV mutants showing constitutive expression of pectinases. aa and gDNA represents amino acid and genomic DNA, respectively. (Continued)

Strain	Type of mutation	Mutation	Codon change	aa change	Predicted length of the mutated protein (aa)	Remarks
UV44	Missense	T-A	CTG-CAG	L565Q		
UV45	Nonsense	C-T	CGA-TGA	R349Stop	348	
UV46	Nonsense	C-T	CAG-TAG	Q269Stop	268	
UV47	Nonsense	T-A	TTA-TAA	L101Stop	100	
UV48	Nonsense	A-T	AAA-TAA	K293Stop	292	
UV49	Frameshift	Deletion of C	CCG-CGC	P513R	516	
UV50	Nonsense	C-T	CAG-TAG	Q269Stop	268	
UV51						No mutations found in *gaaX* or *gaaR*
JN103.1						No mutation found in *gaaX*, mutation found in *gaaR*
UV53	Nonsense	AC-TT	CAA-TAA	Q102Stop	101	
UV54	Nonsense	T-G	TAT-TAG	Y535Stop	534	
UV55						No mutations found in *gaaX* or *gaaR*
UV56	Nonsense	C-T	CAG-TAG	Q269Stop	268	
UV57	Nonsense	C-A	TCG-TAG	S593Stop	592	
UV58	Missense	T-C	CTG-CCG	L345P		

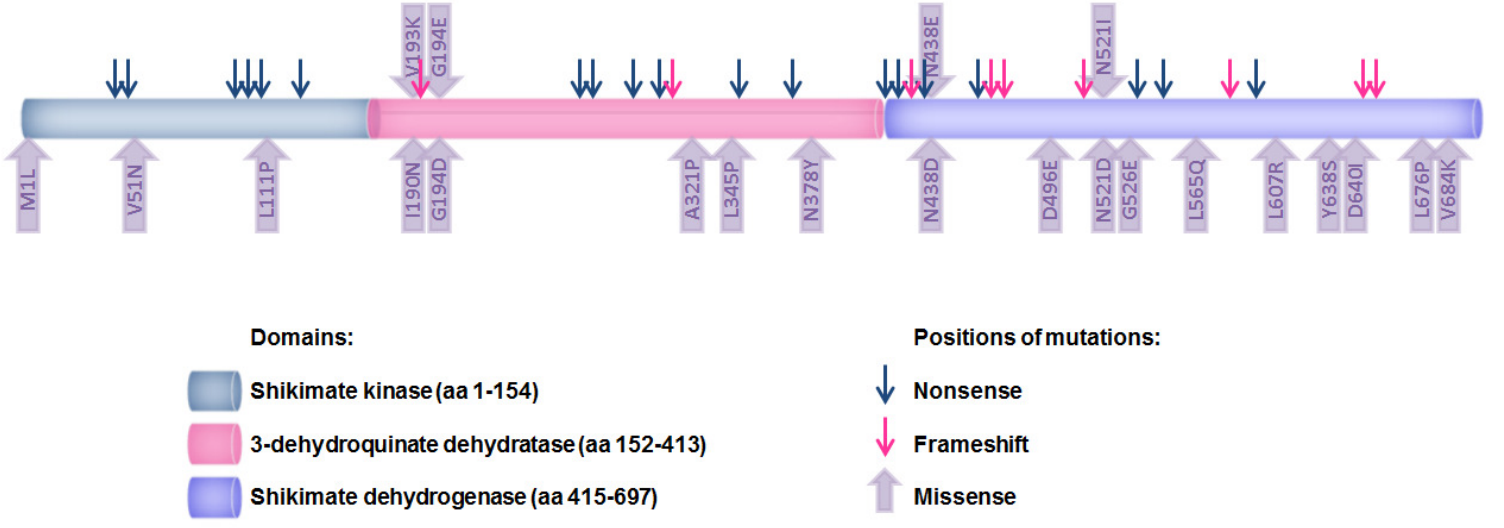

Figure S1. Schematic representation of the domains present in GaaX and mutations in the UV mutants showing constitutive expression of pectinases. Domains in GaaX were assigned based on multiple alignment of protein sequences of *A. niger* AROM (NRRL3_11273) C-terminal part (aa 863-1856) and GaaX (NRRL3_08194), and the *E. coli* shikimate kinase (AroL, NP_414922.1), 3-dehydroquinate dehydratase (AroD, NP_416208.1) and shikimate dehydrogenase (AroE, NP_417740.1), using COBALT (Papadopoulos and Agarwala, 2007).

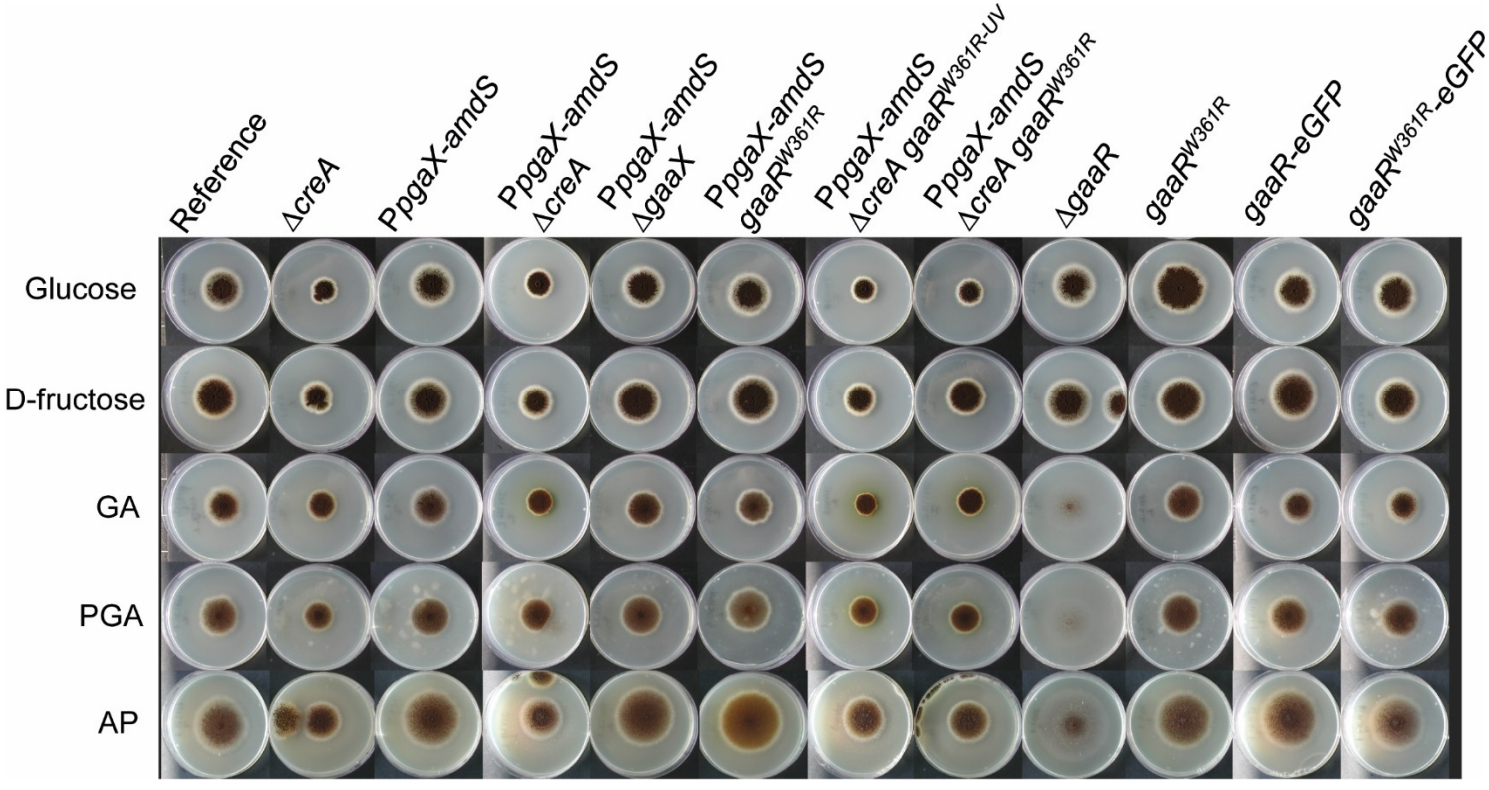

Figure S2. Radial growth assay of the reference (MA234.1), *ΔcreA* (XY1.1), P*pgaX-amdS* (JC1.5), P*pgaX-amdS ΔcreA* (JN29.2), P*pgaX-amdS ΔgaaX* (JN123.1), P*pgaX-amdS gaaR*^{W361R} (JN130.4), P*pgaX-amdS ΔcreA gaaR*$^{W361R\text{-}UV}$ (JN103.1), P*pgaX-amdS ΔcreA gaaR*^{W361R} (JN129.1), *ΔgaaR* (SO1.1), *gaaR*^{W361R} (SO2.1), *gaaR-eGFP* (EA31.1), *gaaR*^{W361R}*-eGFP* (EA32.1), and strains on solid MM containing 50 mM glucose, D-fructose or GA, or 1% PGA or pectin as the carbon source after 7 days at 30 °C.

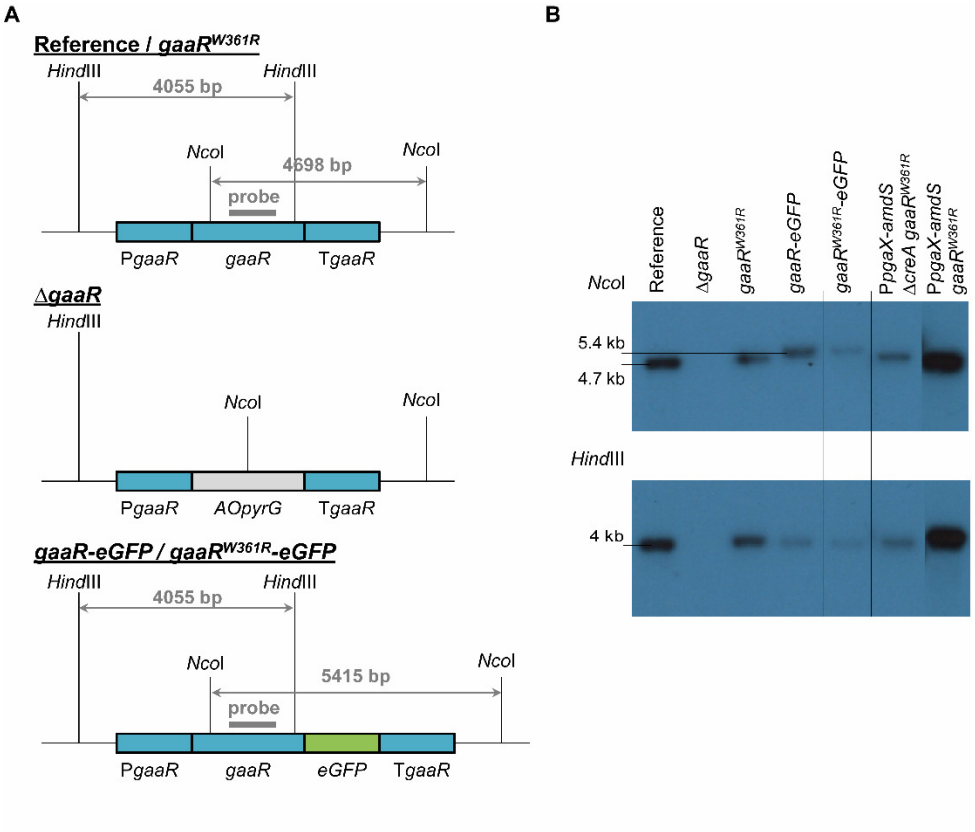

Figure S3. Southern blot analysis of genomic DNA to verify the integration of the *gaaR*^{W361R}, *gaaR-eGFP* and *gaaR*^{W361R}*-eGFP* constructs to the *gaaR* locus in the strains created in this study. **A** Schematic representation of the target gene locus in the reference (MA234.1), *ΔgaaR* (SO1.1), *gaaR*^{W361R} (SO2.1), *gaaR-eGFP* (EA31.1), *gaaR*^{W361R}*-eGFP* (EA32.1), P*pgaX-amdS ΔcreA gaaR*^{W361R} (JN129.1) and P*pgaX-amdS gaaR*^{W361R} (JN130.4) strains. The probe binds to *gaaR* downstream of the *Nco*I restriction site and upstream of the *Hind*III restriction site. Expected band sizes are indicated. **B** Southern blot after hybridization. The lines between genomic DNA samples indicate that the left and right parts of the same blot were combined after removing unnecessary lanes. 4698-bp and 4055-bp bands are visible when the genomic DNA of the reference strain is digested with *Nco*I and *Hind*III, respectively.

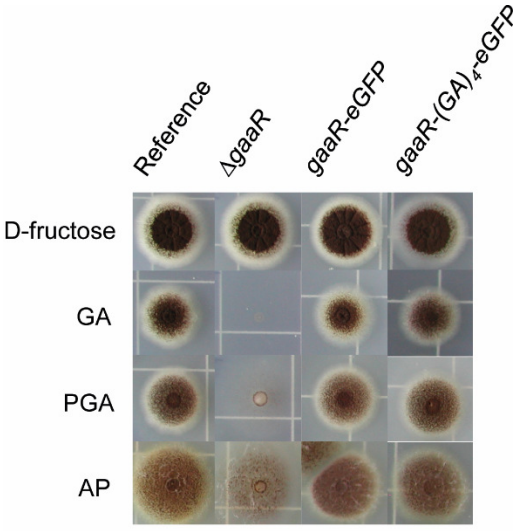

Figure S4. Radial growth assay of the reference (MA234.1), *ΔgaaR* (JN35.1), *gaaR-eGFP* (EA29.14) and *gaaR-(GA)$_4$-eGFP* (EA30.6) on solid MM containing 50 mM D-fructose or GA, or 1% PGA or pectin as the carbon source after 5 days at 30 °C.

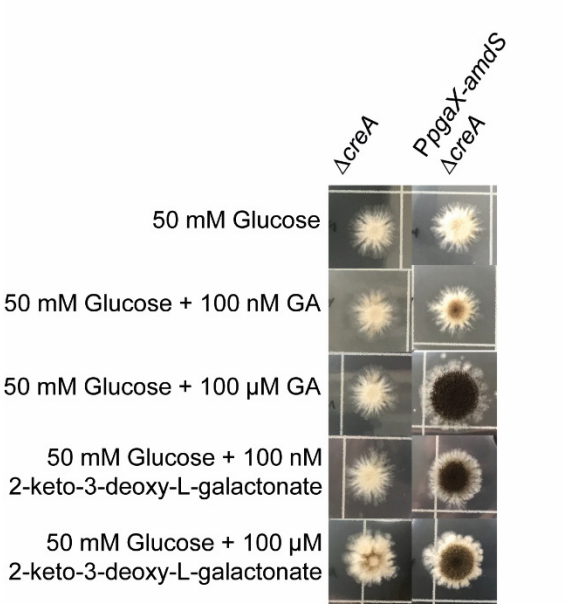

Figure S5. Growth phenotype of the *ΔcreA* (XY1.1) and P*pgaX-amdS ΔcreA* (JN29.2) strains after 10 days at 30 °C on solid MM containing 50 mM glucose or 50 mM glucose with 100 nM or 100 µM GA or 2-keto-3-deoxy-L-galactonate as the carbon source. All plates contain 10 mM acetamide as the sole nitrogen source.