INTRODUCTION

Polysaccharides present in plant biomass, such as pectin, are the principle carbon source for saprophytic filamentous fungi like Aspergillus niger and Neurospora crassa (Kowalczyk, Benoit, & de Vries, 2014; Znameroski & Glass, 2013). In plant pathogenic fungi, such as Botrytis cinerea, pectin degradation is considered an important virulence event as the fungus has to degrade the pectin that is part of the cell wall in order to invade the plant cell (Choquer et al., 2007; Zhang & van Kan, 2013). Different plants produce different kinds of pectins which have been categorized in four main substructures with increasing complexity. All four pectin substructures (i.e., polygalacturonic acid (PGA), xylogalacturonan, rhamnogalacturonan I, and rhamnogalacturonan II) 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 & Mohnen, 2009). When A. niger encounters pectin in its environment, it 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. Using promoter-reporter strains (PpgaX-amdS) and polygalacturonic acid plate assays, we showed that W361R mutation in GaaR results in constitutive production of pectinases. Analysis of subcellular localization of C-terminally eGFP-tagged GaaR/GaaRW361R revealed important differences in nuclear accumulation of N- versus C-terminally eGFP-tagged GaaR.

KEYWORDS
Aspergillus niger, constitutively active transcription factor, CRISPR-Cas9, missense mutation, pectinase, transcription factor localization
mutational analysis of this element (5'-CCNCCAA-3') showed its requirement for induction in response to the presence of GA (Niu et al., 2015). The same GARE element was also identified in the promoter regions of GA-induced genes in B. cinerea (Zhang et al., 2016), indicating a conserved transcriptional control mechanism. A yeast one-hybrid screen was conducted in B. cinerea which resulted in the identification of a fungal GA-responsive Zn2Cys6 type transcription factor named GaaR (Zhang et al., 2016). The A. niger GaaR orthologue was identified via its homology to the B. cinerea GaaR and was shown to be required for growth on GA and polygalacturonic acid (Alazi et al., 2016). GaaR was found to be essential for the GA-responsive expression of several pectinases, (putative) GA-transporters, and GA catabolic pathway enzymes (Alazi et al., 2016). The gene encoding GaaR is present in the genomes of Ascomycetes belonging to the Pezizomycotina subdivision which includes members of Eurotiomycetes (Aspergillus, Penicillium, and Talaromyces spp.), Leotiomycetes (Botrytis and Oidiodendron spp.), Sordariomycetes (Neurospora, Myceliophthora, Trichoderma, and Fusarium spp.), and Dothideomycetes (Zymoseptoria and Aureobasidium spp.). The function of GaaR has not been studied yet in species apart from A. niger and B. cinerea. In N. crassa, the PDR-1 transcription factor is important for growth on PGA and pectin (Thieme et al., 2017). Phylogenetically, PDR-1 is the ortholog of the A. niger rhamnose regulator RhaR. However, the ΔrhaR mutant of A. niger has no apparent growth defect on PGA or pectin (Ram et al., unpublished results; Gruben et al., 2014), indicating that expression of pectinases in A. niger and N. crassa is at least partially under control of different regulators.

Aspergillus niger mutants showing constitutive production of pectinases were previously obtained via a forward genetic screen using the promoter-reporter strain PpgaX-amdS ΔcreA (JN29.2) (Niu et al., 2017). PpgaX-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 is activated to express the amdS gene that encodes the acetamide catabolic enzyme acetamidase (Niu et al., 2015). In total, 64 mutants showing constitutive production of pectinases were isolated on plates containing minimal medium (MM) with glucose as the carbon source and acetamide as the sole nitrogen source. The genomes of five mutants were sequenced and it was found that they carry allelic mutations in a common gene, which was named the repressor of D-galacturonic acid utilization, gaaX (NRRL3_08194) (Niu et al., 2017). Transcriptome analysis revealed that deletion of gaaX results in the constitutive expression of pectinase genes (Niu et al., 2017). GaaX is suggested to inhibit GaaR function by stoichiometric binding to GaaR under non-inducing conditions (Alazi et al., 2018; Niu et al., 2017). Interestingly, the genes encoding the repressor protein (GaaX) and the transcriptional activator (GaaR) are located side-by-side on the chromosome of A. niger as well as in other Pezizomycotina species (Niu et al., 2017). The clustering of genes encoding the GA-specific transcriptional activator (GaaR) and the repressor protein (GaaX) is similar to that of the activator-repressor modules qaA-qaR and qa1F-qa1S involved in the utilization of the plant cell wall constituent quinic acid in Aspergillus nidulans and N. crassa, respectively. In both the quinic acid and the GA utilization systems, deletion of the repressor results in constitutive expression of target genes which requires the presence of the corresponding Zn2Cys6 type transcriptional activator (Giles et al., 1985; Grant, Roberts, Lamb, Stout, & Hawkins, 1988; Lamb et al., 1996; Niu et al., 2017). Moreover, A. nidulans strains expressing multiple copies of qaTA showed constitutive expression of the genes involved in quinic acid utilization (Lamb et al., 1996). Similarly, overexpression of gaaR in A. niger resulted in constitutive expression of the genes involved in the breakdown of pectin and utilization of GA (Alazi et al., 2018). These results indicate that the activity of GaaR is inhibited by GaaX under non-inducing conditions possibly via protein–protein interaction and that GA-responsive gene expression only requires the presence of active GaaR relieved/escaped from GaaX inhibition. This suggests that the inducer binds to GaaX resulting in a GaaX-unbound and active form of GaaR under inducing conditions. The catabolic intermediate 2-keto-3-deoxy-L-galactonate produced from GA was shown to induce the production of pectinases by A. niger which makes it reasonable to suggest that 2-keto-3-deoxy-L-galactonate binds to GaaX resulting in the dissociation of GaaX from GaaR, thereby releasing GaaR as an active transcription factor (Alazi et al., 2017). Because overexpression of the activators involved in GA or quinic acid utilization has similar effects, it is likely that activation mechanism of both QutA/QutR and GaaR/GaaX is conserved and comparable. Pectinases produced by A. niger are used industrially (Edwards & Doran-Peterson, 2012; Kashyap, Vohra, Chopra, & Tewari, 2001; Khan, Nakkeeran, & Umesh-Kumar, 2013; Toushiki, Lee, & Kim, 2017), 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 production of pectinases by A. niger. We show that a gain-of-function mutation in gaaR causing an amino acid change from tryptophan to arginine at position 361 (GaaR W361R) leads to a constitutively active form of the GaaR transcription factor, leading to inducer-independent production of pectinases.

2 | MATERIALS AND METHODS

2.1 | Strains, media, and growth conditions

All strains used in this study are listed in Table S1. Media were prepared as described in Arentshorst, Ram, and Meyer (2012). Radial growth phenotype of the strains was analyzed on minimal medium (pH 5.8) containing 1.5% (w/v) agar (Scharlab, 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-Aldrich) or apple pectin (AP)
(Sigma-Aldrich). Media of the uridine auxotrophic strains were supplemented with 10 mM uridine. Plates were inoculated with 5 μl 0.9% NaCl containing 1 × 10^4 or 5 × 10^5 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, Ram, and Meyer, 2012). Plates were inoculated with 5 μl 0.9% NaCl containing 10^5 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 7.5 × 10^7 freshly harvested spores and cultivated at 30°C and 250 rpm. Experiments were performed in duplicate.

For microscopic analysis of the localization of the eGFP-tagged GaaR or GaaR<sup>W361R</sup> proteins, 4 × 10^6 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 hr. For each condition, two biological replicates were performed.

### 2.2 Sequencing gaaX and gaaR genes from mutants displaying constitutive production of enzymes involved in PGA utilization

Sixty-four trans-acting mutants spontaneous or obtained after mild UV mutagenesis with constitutive production of enzymes 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, Ram, and Meyer (2012), and gaaX and/or gaaR genes were PCR-amplified using the primers gaaX<sub>P5f</sub> and gaaX<sub>GSP9r</sub> or gaaRP7f and gaaRP8r, respectively (Table S2). The PCR fragments were sequenced in both directions using gaaX or gaaR sequencing primers, respectively (Table S2).

### 2.3 Construction of the promoter-reporter strains expressing gaaR<sup>W361R</sup>

Protoplast-mediated transformation of A. niger and purification of the transformants were performed as described by Arentshorst, Ram, and Meyer (2012).

The gaaR<sup>W361R</sup> 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 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, CA). The resulting plasmids were amplified in *Escherichia coli* and sequenced using gaaR sequencing primers. Integration of the gaaR<sup>W361R</sup> into the endogenous gaaR locus was confirmed via Southern blot analysis. Genomic DNA was digested overnight with *NcoI* or *HindIII* 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.

### 2.4 Construction of strains expressing gaaR-eGFP or gaaR-(GA)<sub>4</sub>-eGFP

The plasmid pEA9 containing the PgaaR–gaaR-eGFP-TgaaR 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*pdA*-eGFP-∗TrpC*) and N402 genomic DNA as template, respectively. eGFP and TgaaR were combined by fusion PCR using primers GaaR<sub>GFP</sub>1F and GaaR<sub>GFP</sub>3R and GaaR<sub>GFP</sub>3R or GaaR<sub>GFP</sub>1F and GaaR<sub>GFP</sub>2R. 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. PgaaR–gaaR and eGFP–TgaaR were combined by fusion PCR using primers eGFP-gaaR-For and eGFP-gaaR-Rev, ligated into pJET1.2/blunt cloning vector (Thermo Fisher Scientific) and amplified in *E. coli*. Sequencing of pEA9 showed that a PCR error (T to C) has occurred in TgaaR at a distance of 519 bp downstream of the stop codon of the eGFP gene.

The plasmid pEA10 containing the PgaaR–gaaR-(GA)<sub>4</sub>-eGFP-TgaaR construct was created in a similar way to pEA9, except that PgaaR–gaaR-(GA)<sub>4</sub> was amplified using the primer GaaR<sub>GFP</sub>6R and GaaR<sub>GFP</sub>7R, and together with the 731-bp promoter region was PCR amplified using the primer pairs listed in Table S2. PgaaR–gaaR and eGFP–TgaaR were combined by fusion PCR using primers eGFP-gaaR-For and eGFP-gaaR-Rev, ligated into pJET1.2/blunt cloning vector (Thermo Fisher Scientific) and amplified in *E. coli*. Sequencing of pEA10 showed that a PCR error (C to T) has occurred in TgaaR 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 (∆gaaR) 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 (∆gaaR) together with the plasmid pMA357. Transformants were selected on transformation plates containing acetamide as the sole nitrogen source.

### 2.5 Construction of the ∆gaaR::AOpypG deletion strain

SO1.1 (∆gaaR::AOpypG) was created using the split marker approach (Arentshorst, Niu, & Ram, 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 Aspergillus oryzae pyrG gene was PCR-amplified as two fragments using the primer pairs listed in Table S2 and the plasmid pAO4-13 (de Ruijt-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 (Carvalho, Arentshorst, Kwon, Meyer, & Ram, 2010), resulting in the strain SO1.1. Proper deletion of gaaR was confirmed by diagnostic PCR (data not shown) and via Southern blot analysis.

2.6 Construction of strains expressing gaaRW361R, gaaRW361R-eGFP, and gaaR-eGFP at the endogenous gaaR locus

To construct plasmid pSO1.2, the PgaAR-gaaRW361R-TgaaR 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). The resulting plasmid pSO1.2 was amplified in E. coli. pSO2.1 (containing the PgaAR-gaaRW361R-eGFP-TgaaR construct) was created by digesting the plasmids pSO1.2 and pEA9 (containing the PgaAR-gaaR-eGFP-TgaaR construct) with the restriction enzymes BcU1 and BamHI, and by ligating the 2408-bp BcU1-BamHI fragment from pSO1.2 (containing the gaaRW361R mutation) with the BcU1 and BamHI cut open 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 PgaAR-gaaRW361R, TgaaR construct containing the 964-bp PgaAR and 992-bp TgaaR was excised from pSO1.2 using the restriction enzymes NotI and XbaI. The PgaAR-gaaR-eGFP-TgaaR and PgaAR-gaaRW361R-eGFP-TgaaR constructs containing the 731-bp PgaAR and 419-bp TgaaR were excised from pEA9 and pSO2.1, respectively, using the restriction enzyme BglII.

The strains SO2.1 (gaaRW361R), EA31.1 (gaaR-eGFP), and EA32.1 (gaaRW361R-eGFP) were created using the CRISPR-Cas9 technique (Nadvig, Nielsen, Kogle, & Mortensen, 2015; manuscript in preparation Song et al., 2018). A guide RNA protospacer sequence GTGGATACGTACTCCTTTTA targeting the SNR52p-gRNA.CAN1.Y- SUP4t (Addgene, USA) (DiCarlo et al., 2013) of the guide RNA via the T7 promoter was amplified by PCR using the primers gaaRP5f and gaaRP6r (Table S2) and the plasmid pAD4.1 genomic DNA as template. The two PCR products were combined by fusion PCR using the primers pAE1 and rev_pE1 to introduce PacI restriction sites at both ends, digested with PacI, and ligated into pAE1 cut open pFC332, yielding to plasmid pE1. pE1 was co-transformed together with PgaAR-gaaR-eGFP-TgaaR or PgaAR-gaaRW361R-eGFP-TgaaR repair fragments into the strain SO1.1 (gaaR::AOPyrG) to create EA31.1 or EA32.1, respectively. Transforms were selected on transformation plates containing hygromycin and uridine, and purified twice on MM containing GA, 5′-fluoroorotic acid, and uridine. Transforms were not able to grow on MM containing GA, 5′-fluoroorotic acid, uridine, and hygromycin after the first round of purification, indicating that pE1 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). 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, Mattern, van Zeijl, Pouwels, & van den Hondel, 1987), yielding to strains EA34.1, EA36.1, and EA39.1, respectively.

2.7 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 microliters of supernatant from each culture were spotted on plates containing 0.2% PGA, and plates were incubated at 37°C for 24 hr before staining with 0.1% Congo Red (Sigma-Aldrich, Zwijndrecht) solution.

2.8 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 analyzed with the ImageJ software (Abramoff, Magalhaes, & Ram, 2004). On each image, the exact same brightness and contrast adjustments were applied.

3 | RESULTS

3.1 | Genetic characterization of A. niger mutants showing constitutive production of pectinases

In a previous study, we isolated 64 mutants showing constitutive production of pectinases (Niu et al., 2017). Genome sequencing of five mutants showed that all five mutants harbor a mutation in a putative repressor protein that represses the expression of pectinases under non-inducing conditions (Niu et al., 2017). We sequenced the gaaX gene in the remaining 59 mutants showing constitutive production of pectinases (Table S3, Figure S1). In total, 28 and nine mutants were found to carry nonsense and frameshift mutations, respectively. Twenty-three mutants were found to have missense mutations in the gaaX gene. These missense mutations were found throughout gaaX, indicating the importance of the different domains of GaaX for proper functioning. The nonsense or frameshift mutation closest to the C-terminus was the frameshift mutation V653G, indicating the importance of the last 45 amino acid residues for a functional GaaX, where the missense mutations L676P and V684K were also found (Figure S1). Four mutants did not carry any mutations in gaaX. Sequencing the gaaR gene in these mutants did not reveal any mutations.

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 Aspergillus niger GaaR were retrieved using the blastp algorithm from NCBI (Altschul, Gish, Miller, Myers, & Lipman, 1990) against the nonredundant protein sequences database and were aligned using Clustal Omega (Sievers et al., 2011)
resulted in the identification of a mutation in gaaR in one of the mutant strains (JN103.1).

The UV mutant PpgaX-amdS ΔcreA gaaRW361R-UV (JN103.1) carries a missense mutation (W361 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 (cd12148) that spans the residues 139-518 in the 740 amino acid-long GaaR (Figure 1a), and is 100% conserved in the homologous GaaR sequences present in Aspergillus species (Figure 1b) and in other Ascomycetes analyzed belonging to the Pezizomycotina subdivision (Figure 1c).

### 3.2 Analyses of pectinase production in gaaRW361R

To show that the W361R mutation in GaaR is responsible for the constitutive production of pectinases, the GaaR gene containing the W361R mutation was introduced via a CRISPR/Cas9 approach into the endogenous gaaR locus in SO1.1, a strain carrying

![FIGURE 2](image-url)

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 hr, and culture supernatants were spotted PGA plates. Enzymatic activities in the supernatants from the duplicate cultures are shown: (a) the reference (MA234.1), PpgaX-amdS ΔcreA gaaRW361R-UV (JN103.1), gaaRW361R (EA34.1), PpgaX- amdS ΔgaaX (JN123.1); (b) PpgaX- amdS (JC1.5), PpgaX- amdS ΔcreA (JN29.2), PpgaX- amdS gaaRW361R (JN130.4), PpgaX- amdS ΔcreA gaaRW361R (JN129.1); and (c) reference (MA234.1), gaaRW361R (EA34.1), gaaR-eGFP (EA36.1), and gaaRW361R-eGFP (EA39.1). (d) Growth phenotype of the PpgaX- amdS (JC1.5), PpgaX- amdS ΔcreA (JN29.2), PpgaX- amdS gaaRW361R (JN130.4), and PpgaX- amdS ΔcreA gaaRW361R (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.
The expression of pgaX is known to be repressed strongly by glucose and mildly by D-fructose in a CreA-dependent way (Alazi et al., 2018; Niu et al., 2015). These previous results were confirmed as shown in Figure 2d, where growth of the promoter-reporter strains on plates containing acetamide and GA decreased when glucose was added to the growth media, and a short delay in growth and sporulation was observed when fructose was added as a repressing sugar. The repressing effect of glucose or fructose was lost upon creA deletion, indicating that CreA is required for the repressing effect of glucose and fructose. To examine whether the CreA-mediated carbon catabolite repression was dominant over constitutive activation of GaaR, the growth the PpgaX-amsD gaaRW361R reporter strain was analyzed under non-inducing/repressing conditions (Figure 2d). The PpgaX-amsD gaaRW361R (JN130.4) could grow both on glucose and D-fructose as the sole carbon source indicating constitutive expression even under carbon catabolite repressing conditions (Figure 2d). The colony growth on glucose was irregular and reduced compared to the growth on fructose, indicating that constitutive activation of GaaR was not fully dominant over CreA-mediated repression. Deletion of creA in the PpgaX-amsD gaaRW361R reporter strain alleviated glucose repression indicating that CreA-mediated repression on pgaX plays an important role even in the presence of the constitutively active GaaRW361R.

3.3 Subcellular localization of GaaR-eGFP and GaaRW361R-eGFP

We previously showed that eGFP-gaaR (EA19.2), a strain expressing the N-terminally eGFP-tagged gaaR, exhibits slightly reduced growth on GA compared to the reference strain (Alazi et al., 2018), indicating that N-terminal eGFP-tagging might affect the function of GaaR. To check whether C-terminal eGFP-tagging affects GaaR functionality, 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 restored 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 function of GaaR, independently of the presence of a linker between GaaR and eGFP.

To analyse the subcellular localization of GaaR-eGFP and GaaRW361R-eGFP, strains that express C-terminally eGFP-tagged gaaR or gaaRW361R from the endogenous gaaR locus were created, and integration of the PpgaR-gaaR-eGFP-Tgaar or PpgaR-gaaRW361R-eGFP-Tgaar constructs into the endogenous gaaR locus was verified by Southern blot analysis (Figure S3). Growth of gaaR-eGFP (EA31.1) and gaaRW361R-eGFP (EA32.1) was complemented on GA and GA-containing carbon sources (Figure S2), confirming previous results that GaaR-eGFP is functional under inducing conditions.

The ability of gaaR-eGFP (EA36.1) and gaaRW361R-eGFP (EA39.1) to constitutively produce pectinases was assessed via a PGA plate assay.
assay and compared to that of gaaRW361R (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 gaaRW361R (EA34.1) strain expressing the constitutively active gaaRW361R produced pectinases in D-fructose, the gaaRW361R-eGFP (EA39.1) strain expressing gaaRW361R-eGFP did not (Figure 2c). This indicates that GFP-tagging of the C-terminus of GaaRW361R interferes with constitutive function of the protein.

The subcellular localization of the C-terminally eGFP-tagged GaaR and GaaRW361R in gaaR-eGFP (EA36.1) and gaaRW361R-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 analyzed 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 PpgaX-amsS ΔcreA (JN29.2) and was compared to that of GA (Figure S5). 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 PpgaX-amsS ΔcreA (JN29.2), indicating that 2-keto-3-deoxy-L-galactonate is able to induce pgaX expression and that 2-keto-3-deoxy-L-galactonate is more potent than GA in inducing the expression.

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 repeated this experiment using 2-keto-3-deoxy-L-galactonate as an inducer instead of GA and confirmed a visual signal of eGFP-GaaR in the nucleus after induction by 2-keto-3-deoxy-L-galactonate or on the non-inducing carbon source D-fructose (Figure 3). C-terminally eGFP-tagged GaaR and GaaRW361R were also visualized mostly in the nucleus after growth in 2-keto-3-deoxy-L-galactonate. Surprisingly, this 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 and suggests that the C-terminal tagging of GaaR with eGFP affects is localization under non-inducing conditions. The inability of the GaaR-eGFP protein to localize in the nucleus under non-inducing conditions is most likely also the reason why the GaaRW361R-eGFP protein does not exert its constitutive activity.

4 | DISCUSSION

We previously isolated via a forward genetic screen A. niger strains displaying constitutive expression of pectinases (Niu et al., 2017). The majority of the mutants in the collection (60 out of 64 strains) was found to contain mutations in GaaX and are probably all loss-of-function mutations as deletion of gaaX leads to the same phenotype (Niu et al., 2017). From the four mutants that did not carry mutations in gaaX, one mutant was found to possess a missense mutation leading to an amino acid change from tryptophan to arginine at position 361 in GaaR. The nature of the mutations in the three UV mutants with no mutations in gaaX or gaaR remains to be discovered. Introducing the GaaRW361R mutation in the wild-type genetic background and subsequent phenotypic analyses allowed us to conclude that this mutation results in constitutive activation of GaaR and therefore constitutive production of pectinases under non-inducing conditions.

To our knowledge, XlnR/Xyr1 is the only transcription factor involved in plant biomass degradation in which missense mutations leading to constitutive expression of enzymes are described. The two mutations are the XlnRV756F mutation in A. niger, and the Xyr1A824V mutation in Trichoderma reesei and orthologous mutations in N. crassa and Penicillium oxalidum, resulting in constitutive production of xylanases (Craig, Coradetti, Starr, & Glass, 2015; Derntl et al., 2013; Gao et al., 2017; Hasper, Trindade, van der Veen, van Ooyen, & de Graaff, 2004). In protein sequence alignment, these two mutations are only three amino acids apart from each other (Figure S6). Whereas the GaaRW361R mutation is located roughly half way of the 740 amino acid-long GaaR protein, the XlnRV756F mutation is located closer to the C-terminal in the 875 amino acid-long XlnR protein. The GaaRW361R mutation is predicted to be in the fungal-specific transcription factor domain (cd12148) which comprises amino acids 139–518 in GaaR. The cd12148 domain comprises amino acids 341–653 in A. niger XlnR and 368–717 in T. reesei Xyr1 (NCBI’s CDD (Marchler-Bauer et al., 2015)), and the constitutive mutations in XlnR/Xyr1 are therefore outside of the predicted fungal-specific transcription factor domain in the C-terminal parts of XlnR and Xyr1, which has been suggested to be the activation domain. The XlnR/Xyr1 constitutive mutations are not located in a defined protein domain family, but the C-terminal parts of XlnR and Xyr1, including the amino acids V756 and the A824, respectively, have been suggested to be the activator domain (Derntl et al., 2013; Hasper et al., 2004). Alignment of protein sequences of XlnR and GaaR from A. niger, A. nidulans, Aspergillus fumigatus and A. oryzae and Xyr1 of T. reesei using Clustal omega (Sievers et al., 2011) showed low sequence similarity in the amino acid residues surrounding the GaaRW361 residue between GaaR and XlnR/Xyr1 (Figure S6). Whereas the W361 residue is fully conserved in GaaR in all four Aspergilli, only A. niger, A. oryzae, and T. reesei had a W residue positioned at the aligned protein sequence of XlnR/Xyr1 (Figure S6). Of note is that sequence similarity surrounding this region of XlnR/ Xyr1 is weak, making it possible that this conservation has no biological relevance. To test its biological relevance, it will be of interest to examine whether mutations in XlnR of A. niger at position W639 would also result in a constitutive phenotype. Protein sequence alignment revealed that the V756 and A824 residues are fully conserved in the XlnR/Xyr1 proteins of the four Aspergilli and T. reesei, but not strictly conserved between XlnR/Xyr1 and GaaR (Figure S6). Because of the low level of conservation between the regions and amino acids that result in constitutive activation of the respective transcription factors, it is reasonable to suggest that the mechanisms leading to the activation of GaaR and XlnR/Xyr1 are different. We identified in the A. niger genome four transcription factor/repressor modules (Niu et al., 2017). For two modules, we know that they are involved in either GA or quinic acid utilization (Niu et al., 2017; Ram et al., unpublished results). Analysis of the knockouts for the two remaining genes encoding the repressors, and subsequent phenotypic and transcriptomic analysis has shown that the two repressors are not involved in the expression of genes encoding xylanases (Alazi and Ram, unpublished results). Therefore, the activity of XlnR is likely not controlled by a GaaX-like repressor protein.

The activity of GaaR is proposed to be inhibited by GaaX under non-inducing conditions via protein–protein interactions (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 (Alazi et al., 2018; Martens-Uzunova & Schaap, 2008; Niu et al., 2017). These findings indicate that GA-responsive gene expression only requires the presence of active GaaR relieved/escaped from GaaX inhibition but does not require the presence of the inducer. This suggests that the inducer binds to GaaX under inducing conditions resulting in a GaaX-unbound and active form of GaaR. Therefore, we propose that W361R in GaaR disrupts the interaction between GaaX and GaaR under non-inducing conditions. Both PpgaX-amdS gaaR W361R and PpgaX-amdS ΔgaaX mutants were found to produce pectinases under non-inducing conditions (Figure 2a and b). The qualitative phenotypic similarity of these strains resulting from the W361R mutation in GaaR or the absence of GaaX, respectively, suggests that the GaaR-GaaX interaction is disturbed in the GaaRW361R mutant. In future experiments, the proposed protein–protein interaction of GaaR and GaaX and mutant variants thereof will be studied further in detail. Initial attempts to express GaaR and GaaX in E. coli to perform interaction studies have been unsuccessful and will be subject for further research. In this respect, it would also be of interest to isolate and identify additional mutations in GaaR that lead to constitutive expression of pectinases to identify regions in GaaR that are necessary for the proposed interaction with GaaX. GaaX of A. niger, QuR of A. nidulans, and qa-15 of N. crassa are all multidomain repressor proteins with sequence similarity to each
other and to the three C-terminal domains of AROM, a large penta-functional protein involved in the shikimate pathway. This suggests that these repressors share a common evolutionary origin. The last three domains of the AROM protein encode the shikimate kinase, 3-dehydroquinate dehydratase, and shikimate dehydrogenase enzymes (Lamb et al., 1996). The sequence similarity of the repressor domains to AROM is low (between 20% and 30% identity), suggesting that the enzymatic functions that are present in AROM are lost in the repressors. Loss-of-function mutations (nonsense and missense mutations) were found throughout all three domains of GaaX (shikimate kinase, 3-dehydroquinate dehydratase, and shikimate dehydrogenase domains) (Figure S1). The 22 missense mutations could especially be valuable for structure/function relationships to predict which domains exert different function as they might affect the proposed protein–protein interaction between GaaX and GaaR. It is however difficult to predict whether a missense mutation affects the overall secondary/tertiary structure of the protein or whether a mutation affects the interaction without affecting its structure. We identified a hot spot for mutations in the region 190–194 in which four mutants were found carrying mutations in three amino acid positions. This region could either be important for GaaR–GaaX interaction or could be important for the structure of GaaX. Alignment of protein sequences homologous to GaaX present in 18 Pezizomycotina species using Clustal omega (Sievers et al., 2011) revealed that these four mutations were found in three residues that are highly conserved (Figure S7). Several other missense mutations were found in conserved residues and are indicated in Figures S1 and S7. Relatively more missense mutations were found in the shikimate dehydrogenase domain (one mutation per 24 amino acids) (Figure S1) compared to the shikimate kinase (one mutation per 76 amino acids), or 3-dehydroquinate dehydratase domains (one mutation per 37 amino acids), indicating that the shikimate dehydrogenase domain might be more important in relation to binding to GaaR than the other domains.

In N. crassa, dehydroquinic acid is suggested to be the physiological inducer of the quinic acid catabolic pathway genes, as mutants that accumulate or cannot produce dehydroquinic acid showed induced or diminished expression of these genes, respectively (Giles, Partridge, Ahmed, & Case, 1967). On the other hand, Huiet and Giles identified two missense mutations in non-inducible N. crassa strains in the Qa-1S domain showing homology with the AROM shikimate dehydrogenase, indicating that this domain might interact with the inducer (Huiet & Giles, 1986). Hawkins, Lamb, Moore, Charles, and Roberts (1993) have proposed that the QutR domain homologous to the AROM 3-dehydroquinate dehydratase can recognize and bind to dehydroquinic acid produced from quinic acid, but does not react on it enzymatically in A. nidulans. For A. niger, accumulation of 2-keto-3-deoxy-L-galactonate in ΔgaoC results in hyper-induction of GA-responsive genes and the inducer 2-keto-3-deoxy-L-galactonate is likely to interact with the GaaX repressor protein (Alazi et al., 2017). The GA catabolic pathway enzyme GaaC is an aldolase and converts 2-keto-3-deoxy-L-galactonate to pyruvate and L-glyceraldehyde. Both the aldolase domain of GaaC and the 3-dehydroquinate dehydratase domain of GaaX belong to the same Aldolase Class I_superfamily (NCBI’s CDD Marchler-Bauer et al., 2015). These findings suggest that 2-keto-3-deoxy-L-galactonate could bind to the GaaX domain homologous to the AROM 3-dehydroquinate dehydratase. However, direct protein-metabolite interaction studies are required to determine which part (or parts) of the repressor protein interacts with the inducer.

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/GaaRW361R accumulated in the nucleus under inducing conditions and complemented the growth of ΔgaaR when grown on inducing carbon sources. Based on these observations, we conclude that C-terminally eGFP-tagged GaaR/GaaRW361R is functional under inducing conditions. However, prominent nuclear eGFP-fluorescence signal was not observed in the strains producing the C-terminally eGFP-tagged GaaR/GaaRW361R under non-inducing conditions. This result indicates that C-terminally eGFP-tagging restrained nuclear accumulation of GaaR/GaaRW361R under non-inducing conditions, explaining the inability of GaaRW361R-eGFP to constitutively activate the production of pectinases, as GaaRW361R can. In B. cinerea, expression of the C-terminally GFP-tagged BcGaaR via the strong o1C promoter resulted in higher nuclear GFP fluorescence intensity under both inducing and non-inducing conditions (Zhang et al., 2016). This indicates that nuclear accumulation of overexpressed and wild-type BcGaaR is not restrained by C-terminal GFP-tagging under non-inducing conditions, unlike the GaaR-eGFP/GaaRW361-eGFP expressed from its endogenous promoter.

Constitutive production of plant biomass degrading enzymes like pectinases is interesting for the industrial production of these enzymes because it allows the production independently of the feedstock. By understanding the transcriptional regulatory mechanism of pectinases, several approaches have been conducted leading to inducer independent and abundant production of pectinases, such as deletion of GaaX (Niu et al., 2017), overexpression of GaaR from a constitutive promoter (Alazi et al., 2018), or accumulation of the inducer (Alazi et al., 2017). Here, we show that a mutation in GaaR can also lead to constitutive production of pectinases. Combination of these approaches can be considered to further boost pectinase production in A. niger. The conservation of GaaR and GaaX in other fungi also allows improved pectinase production in other industrially important filamentous fungi.

**ACKNOWLEDGMENTS**

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

Abramoff, M. D., Magalhaes, P. J., & Ram, S. J. (2004). Image Processing with ImageJ. Biophotonics International, 11(7), 36–42.

Alazi, E., Khosravi, C., Homan, T. G., de Prê, S., Arentshorst, M., Di Falco, M., ... Ram, A. F. J. (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. https://doi.org/10.1002/1873-3468.12654

Alazi, E., Knetsch, T., Di Falco, M., Reid, I. D., Arentshorst, M., Visser, J., ... Ram, A. F. J. (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. https://doi.org/10.1007/s00253-018-8753-7

Alazi, E., Niu, J., Kowalczyk, J. E., Peng, M., Aguilar Pontes, M. V., van Kan, J. A. L., ... Ram, A. F. J. (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. https://doi.org/10.1002/1873-3468.12211

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. Journal of Molecular Biology, 215, 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Arentshorst, M., Niu, J., & Ram, A. F. J. (2015). Efficient generation of Aspergillus niger knock out strains by combining NHEJ mutants and a split marker approach. In M. A. van den Berg & K. Maruthachalam (Eds.), Genetic transformation systems in fungi (Vol. 1, pp. 263–272). Cham, Switzerland: Springer International Publishing.

Arentshorst, M., Ram, A. F. J., & 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. https://doi.org/10.1007/978-1-61779-501-5

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

Carvalho, N. D. S. P., Arentshorst, M., Kwon, M. J., Meyer, V., & Ram, A. F. J. (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. https://doi.org/10.1007/s00253-010-2588-1

Choquer, M., Fournier, E., Kunz, C., Levis, C., Pradier, J. M., Simon, A., & Viaud, M. (2007). Botrytis cinerea virulence factors: New insights into a necrotrophic and polyphagous pathogen. FEMS Microbiology Letters, 277, 1–10. https://doi.org/10.1111/j.1574-6968.2007.00930.x

Craig, J. P., Coradetti, S. T., Starr, T. L., & Glass, N. L. (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.

Derrnt, C., Gudynaike-Savitch, L., Calixte, S., White, T., Mach, R. L., & Mach-Aigner, A. R. (2013). Mutation of the Xylanase regulator 1 causes a glucose blind hydrolase expressing phenotype in industrially used Trichoderma strains. Biotechnology for Biofuels, 6, 1–11.

D’Icaro, J. E., Norville, J. E., Mali, P., Rios, X., Aach, J., & Church, G. M. (2013). Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems. Nucleic Acids Research, 41(7), 4336–4343. https://doi.org/10.1093/nar/gkt1135

Edwards, M. C., & Doran-Peterson, J. (2012). Pectin-rich biomass as feedstock for fuel ethanol production. Applied Microbiology and Biotechnology, 95, 565–575. https://doi.org/10.1007/s00253-012-4173-2

Gao, L., Li, Z., Xia, C., Qu, Y., Liu, M., Yang, P., ... Song, X. (2017). Combining manipulation of transcription factors and overexpression of the target genes to enhance lignocellulolytic enzyme production in Penicillium oxalici. Biotechnology for Biofuels, 10, 1–16.

Giles, N. H., Case, M. E., Baum, J., Geever, R., Huiet, L., Patel, V., & Tyler, B. (1985). Gene organization and regulation in the qa (quinic acid) gene cluster of Neurospora crassa. Microbiological Reviews, 49, 338–358.

Giles, N. H., Partridge, C. W., Ahmed, S. I., & Case, M. E. (1967). The occurrence of two dehydroquinases in Neurospora crassa, one constitutive and one inducible. Proceedings of the National Academy of Sciences of the United States of America, 58(5), 1930–1937. https://doi.org/10.1073/pnas.58.5.1930

Grant, S., Roberts, C. F., Lamb, H., Stout, M., & Hawkins, A. R. (1988). Genetic regulation of the quinic acid utilization (QUT) gene cluster in Aspergillus nidulans. Journal of General Microbiology, 134, 347–358.

Gruben, B. S., Zhou, M., Wiebenga, A., Ballering, J., Overkamp, K. M., Punt, P. J., & de Vries, R. P. (2014). Aspergillus niger RhaR, a regulator involved in L-rhamnose release and catabolism. Applied Microbiology and Biotechnology, 98, 5531–5540.

van Hartingsveldt, W., Mattern, I. E., van Zeijl, C. M., Pouwels, P. H., & van den Hondel, C. A. (1987). Development of a homologous transformation system for Aspergillus niger based on the pyrG gene. Molecular and General Genetics, 206(1), 71–75. https://doi.org/10.1007/BF00326538

Hesper, A. A. (2004). Function and mode of regulation of the transcriptional activator XlnR from Aspergillus. [dissertation]. Wageningen (The Netherlands): Wageningen University.

Hesper, A. A., Trindade, L. M., van der Veen, D., van Ooyen, A. J. J., & de Graaff, L. H. (2004). Functional analysis of the transcriptional activator XlnR from Aspergillus niger. Microbiology, 150, 1367–1375. https://doi.org/10.1099/mic.0.26557-0

Hawkins, A. R., Lamb, H. K., Moore, J. D., Charles, I. G., & Roberts, C. F. (1993). The pre-chorismate (shikimate) and quinate pathways in filamentous fungi: Theoretical and practical aspects. Journal of General Microbiology, 139, 2891–2899. https://doi.org/10.1099/00221287-139-12-2891

Heigwer, F., Kerr, G., & Boutros, M. (2014). E-CRISP: Fast CRISPR target site identification. Nature Methods, 11, 122–123. https://doi.org/10.1038/nmeth.2812

Huiet, L., & Giles, N. H. (1986). The qa repressor gene of Neurospora crassa: Wild-type and mutant nucleotide sequences. Proceedings
of the National Academy of Sciences of the United States of America, 83(10), 3381–3385. https://doi.org/10.1073/pnas.83.10.3381
Kashyap, D., Vohra, P., Chopra, S., & Tewari, R. (2001). Applications of pectinases in the commercial sector: A review. Bioresource Technology, 77, 215–227. https://doi.org/10.1016/S0960-8524(00)00118-8
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. https://doi.org/10.1146/annurev-food-030212-182525
Kowalczyk, J. E., Benoit, I., & de Vries, R. P. (2014). Regulation of plant biomass utilization in Aspergillus. Advances in Applied Microbiology, 88, 31–56. https://doi.org/10.1016/B978-0-12-800260-5.00002-4
Lamb, H. K., Newton, G. H., Levett, L. J., Cairns, E., Roberts, C. F., & Hawkins, A. R. (1996). The QUTA activator and QUTR repressor proteins of Aspergillus nidulans interact to regulate transcription of the quinate utilization pathway genes. Microbiology, 142, 1477–1490. https://doi.org/10.1099/13500872-142-6-1477
Marchler-Bauer, A., Derbyshire, M. K., Gonzales, N. R., Lu, S., Chitsaz, F., Geer, L. Y., ... Bryant, S. H. (2015). CDD: NCBI's conserved domain database. Nucleic Acids Research, 43, D222–D226. https://doi.org/10.1093/nar/gku1221
Martens-Uzunova, E. S., & Schaal, P. J. (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. https://doi.org/10.1016/j.fgb.2008.08.002
Niu, J., Alazi, E., Reid, I. D., Arentshorst, M., Punt, P. J., Visser, J., ... Ram, A. F. J. (2017). An evolutionarily conserved transcriptional activator-repressor module controls expression of genes for D-galacturonic acid utilization in Aspergillus niger. Genetics, 205, 169–183. https://doi.org/10.1534/genetics.116.194050
Niu, J., Homan, T. G., Arentshorst, M., de Vries, R. P., Visser, J., & Ram, A. F. (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. https://doi.org/10.1016/j.fgb.2015.06.006
Nedvig, C. S., Nielsen, J. B., Kogle, M. E., & Mortensen, U. H. (2015). A CRISPR-Cas9 system for genetic engineering of filamentous fungi. PLoS ONE, 10(7), e0133085. https://doi.org/10.1371/journal.pone.0133085.
de Ruiter-Jacobs, Y. M. J. T., Broekhuijsen, M., Unkles, S. E., Campbell, E. I., Kinghorn, J. R., Contreras, R., ... van den Hondel, C. A. M. J. (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. https://doi.org/10.1007/BF00391472
Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., ... Higgins, D. G. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Molecular Systems Biology, 7(539).
Song, L., Ouedraogo, J. P., Kolbusz, M., Nguyen, T. T. M., & Tsang, A. (2018). Efficient genome editing using tRNA promoter-driven CRISPR/Cas9 gRNA in Aspergillus niger. PLoS One, 13(8):e0202868. https://doi.org/10.1371/journal.pone.0202868
Thieme, N., Wu, V. W., Dietzschmann, A., Salamov, A. A., Wang, M., Johnson, J., & Benz, J. P. (2017). The transcription factor PDR-1 is a multi-functional regulator and key component of pectin deconstruction and catabolism in Neurospora crassa. Biotechnology for Biofuels, 10(1), 1–21.
Toushik, S. H., 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.
Zhang, L., Lubbers, R. J. M., Simon, A., Stassen, J. H. M., Ribera, P. R. V., Viaud, M., & van Kan, J. A. L. (2016). A novel Zn2Cys6 transcription factor BcGaaR regulates D-galacturonic acid utilization in Botrytis cinerea. Molecular Microbiology, 100, 247–262. https://doi.org/10.1111/mmi.13314
Zhang, L. & van Kan, J. A. L. (2013). Pectin as a barrier and nutrient source for fungal plant pathogens. In F. Kempken (Ed.), The mycota XI, Ed 2: Agricultural applications (pp. 361–375). Berlin, Germany: Springer-Verlag.
Znameroski, E. A., & Glass, N. L. (2013). Using a model filamentous fungus to unravel mechanisms of lignocellulose deconstruction. Biotechnology for Biofuels, 6(6).

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Alazi E, Niu J, Otto SB, et al. W361R mutation in GaaR, the regulator of D-galacturonic acid-responsive genes, leads to constitutive production of pectinases in Aspergillus niger. MicrobiologyOpen. 2018:e732. https://doi.org/10.1002/mbo3.732