Transcriptional control of pectin degrading enzymes in Aspergillus niger

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Chapter 4

The interaction of induction and repression mechanisms in the regulation of galacturonic acid-induced genes in *Aspergillus niger*

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Abstract

Aspergillus niger is an important industrial fungus expressing a broad spectrum of pectinolytic genes. The main constituent of pectin, polygalacturonic acid (PGA), is degraded into galacturonic acid (GA) by the combined activity of endo- and exo-polygalacturonases some of which are specifically induced by GA. The regulatory mechanisms that control the expression of genes encoding PGA-degrading enzymes are not well understood. Based on available genome-wide expression profiles from literature, we selected five genes that were specifically induced by GA. These genes include three exo-polygalacturonases (*pgaX, pgxB* and *pgxC*), a GA transporter (*gatA*), and an intracellular enzyme involved in GA metabolism (*gaaB*). These five genes contain a conserved motif (5'-TCCNCCAAT-3') in their promoter regions, which we named GARE (galacturonic acid-responsive element). Promoter deletion studies and site-directed mutagenesis of the conserved motif of the *pgaX* gene showed that the conserved element is required for GA-mediated induction. A set of promoter reporter strains was constructed by fusing the promoter region of the five above-mentioned genes to the *amdS* reporter gene. Expression of the *amdS* gene is quantitatively correlated with ability to utilize acetamide as an N-source, hence higher expression of *amdS* improves growth of the strain on acetamide and therefore can be used as an *in vivo* reporter for gene expression. Growth analysis of the reporter strains indicated that four genes (*pgaX, pgxB, pgxC*, and *gatA*) are specifically induced by GA. The *in vivo* promoter reporter strains were also used to monitor carbon catabolite repression control. Except for *gaaB,* all promoter-reporter genes analysed were repressed by glucose in a glucose concentration-dependent way. Interestingly, the strength of glucose repression was different for the tested promoters. CreA is important in mediating carbon catabolite repression as deletion of the *creA* gene in the reporter strains abolished carbon catabolite repression for most promoters. Interestingly, the *pgxC* promoter was still repressed by glucose even in the *creA* null background, suggesting a role for alternative repression mechanisms. Finally, we showed that low concentrations of GA are required to induce gene expression of *pgaX*, *pgxB*, and *pgxC* even under derepressing conditions. The results obtained are consistent with a model in which a GA-specific transcription factor is activated by GA or a GA-derivative, which binds to the conserved motif, possibly in combination with the HAP-complex, to drive GA-specific gene expression.

Keywords: pectin, pectinolytic genes, gene regulation, reporter genes, acetamidase, carbon catabolite repression, CreA

Introduction

Pectin represents a group of complex heterogeneous polysaccharides that are primarily present in the middle lamella of plant cell walls. The backbone consists mainly of α-1,4-linked D-galacturonic acid residues part of which are methyl-esterified. The polygalacturonic acid backbone can be further modified to form substructures with increasing complexity. Pectin is classified into four substructures: homogalacturonan (HGA), xylogalacturonan (XGA), rhamnogalacturonan-I (RG-I), and rhamnogalacturonan-II (RG-II) (Mohnen 2008). RG-II is the most complex structure and composed of up to twelve different types of sugars in >20 different linkages.

Aspergillus niger is an important industrial micro-organism and used as a cell factory for the production of enzymes and organic acids (Pel *et al.* 2007; Andersen *et al.* 2011). Genome mining has revealed a large array of extracellular pectinolytic enzymes in *A. niger* (Coutinho *et al.* 2009; Martens-Uzunova and Schaap 2009). The majority of the pectinolytic enzymes that act on the polygalacturonic acid backbone belong to the GH28 family of glycoside hydrolases, and include endo- and exo-polygalacturonases (Bussink *et al.* 1992a; Bussink *et al.* 1992b; Benen *et al.* 1996; Parenicova *et al.* 1998; Parenicova *et al.* 2000; Martens-Uzunova *et al.* 2006).

Metabolism of GA in fungi is well described and involves four enzymatic reactions to convert GA into glycerol and pyruvate. The genes encoding these enzymes (*gaaA*, *gaaB*, *gaaC*, and *gaaD* have been identified and the biochemical properties of the enzymes have been determined (Kuorelahti *et al.* 2005; Kuorelahti *et al.* 2006; Liepins *et al.* 2006; Hilditch *et al.* 2007; Mojzita *et al.* 2010; Wiebe *et al.* 2010; Zhang *et al.* 2011; Kuivanen *et al.* 2012). Specific sugar transporters that are able to transport GA over the plasma membrane have recently been identified and characterized in Neurospora crassa (Benz *et al.* 2014) as well as in *A. niger* (Sloothaak et al. 2014) and *Botrytis cinerea* (Zhang *et al.* 2014). The transporters identified in these studies are phylogenetically related and probably represent a subfamily of GA-specific transporters (Zhang *et al.* 2014).

Several studies have focused on understanding the transcriptional regulation of pectinolytic genes (Bussink *et al.* 1990; Bussink *et al.* 1992a; Maldonado and Strasser De Saad 1998; De Vries *et al.* 2002) and have shown that most pectinolytic genes are specifically induced by GA. In combination with a genome-wide expression study in *A. niger* (Martens-Uzunova and Schaap 2008) a conserved promoter element has been identified that is present in the promoter region of GA-induced genes. A promoter deletion study of the *pgaII* gene (encoding an endo-polygalacturonase) showed that this element is important for high level

expression of *pgaII* (Bussink *et al.* 1992b). The element (5'-TCCNCCAAT-3') is present in genes encoding extracellular enzymes that are specific for polygalacturonic acid hydrolysis (both exo- and endo-activities), in putative GA-transporter genes, and in genes encoding the enzymes for GA metabolism (*gaaA* to *gaaD*) (Martens-Uzunova and Schaap 2008). We propose to name this element GARE for galacturonic acid-responsive element. Benen and co-workers identified a second region (CCCTGA), which is present in promoters of many pectinolytic genes that might be important in activation of pectinolytic gene expression and has been named PecR (Benen *et al.* 1996; Coutinho *et al.* 2009). The GARE motif (5'-TCCNCCAAT-3') is similar to the binding site of the HAP2/3/4 complex. The HAP complex (as it is named in *Saccharomyces cerevisiae*) is a conserved multimeric transcription factor that regulates gene expression by binding to the consensus sequence CCAAT. In the filamentous fungi, the HAP complex consists of four subunits (HapB/C/E and HapX). The complex is required for the regulation of gene expression not only related to carbon or nitrogen source utilization (Van Heeswijck and Hynes 1991; Kato *et al.* 1997; Kato *et al.* 1998; Steidl *et al.* 1999) but also to other cellular processes like secondary metabolite production (Litzka *et al.* 1996), iron homeostasis (Hortschansky *et al.* 2015), and oxidative stress responses (Thon *et al.* 2010).

Several studies have shown that GA-induced genes are repressed by the presence of glucose (Bussink *et al.* 1991; De Vries *et al.* 2002) through carbon catabolite repression control (CCR). In filamentous fungi, the C2H2 type transcription factor CreA/CRE1, which is related to Mig1/Mig2/Mig3 proteins that mediate glucose repression in *S. cerevisiae* (Westholm *et al.* 2008), has been shown to act as a repressor mediating CCR (Dowzer and Kelly 1991; Ruijter and Visser 1997). CreA/CRE1 binds to the promoters of the respective target genes via the consensus motif 5'-SYGGRG-3' to repress expression. Disruption mutants in *creA* are viable in *A. nidulans* (Shroff *et al.* 1997) and *A. niger* (Yuan *et al.* 2006) and can be used to analyse transcriptional regulation under derepressed conditions.

For the efficient degradation and utilization of polymeric substrates, a synergistic and coordinated expression of the hydrolysing enzymes, sugar transporters, and enzymes involved in the intracellular metabolism is required. In filamentous fungi several substrate-specific transcription factors have been identified, which function as key regulators to control gene expression in response to the presence of a particular substrate (Kowalczyk *et al.* 2014). However, a specific GA-responsive transcription factor has not yet been identified. We suggest that an as yet unidentified transcription factor is responsible for GA-dependent

induction of the genes encoding the extracellular PGA-degrading enzymes as well as activation of genes that encode the intracellular enzymes involved in GA metabolism.

In this study, we show the importance of the GARE motif (5'-TCCNCCAAT-3') for GA-induced gene expression. We also constructed promoter-reporter constructs to analyse the regulation of these promoters *in vivo*. Using these reporter strains, we show that induction and repression of GA-induced genes is differentially fine-tuned in response to inducing and repressing conditions.

Material and methods

2.1 Strains and growth conditions

The *A. niger* strains used in this study are listed in Table 1. Strains were grown in liquid or on solidified (by addition of 2 % agar) minimal medium (MM), which contained 7 mM KCl, 8 mM KH_2PO_4, 70 mM $NaNO_3$, 2 mM $MgSO_4$ (pH adjusted to pH 5.5) as described by Bennett and L.L. 1991 (Bennett and L.L. 1991). MM was supplemented with a specific carbon source to a final concentration of 50 mM as indicated. Standard complete medium (CM) was also used and consisted of MM supplemented with 0.1% casamino acids and 0.5% $w.v^{-1}$ yeast extract and 50 mM glucose. MM agar plates containing 10 mM acetamide as sole nitrogen source were made as previously described (Arentshorst *et al.* 2012). Transformation of *A. niger* strains was also carried out as described in (Arentshorst *et al.* 2012). Targeted integration of reporter constructs to the *pyrG** locus was carried out as described previously by (Van Gorcom and Van Den Hondel 1988) or through via a recently developed *pyrG*-targeting vector pMA334 (named *pyrG**)* (Arentshorst *et al.* 2015). Fungal chromosomal DNA isolation was performed as described by Meyer *et al*. 2010 (Meyer *et al.* 2010). Two strains were used as a recipient for transformation and include AB4.1 and MA299.2 (Table 1). MA299.2 is derived from the *Δku70* mutant strain MA70.15 (*kusA::amdS*, *pyrG*$^-$) after curing the *amdS* marker by fluoro-acetamide selection (Arentshorst *et al.* 2012). The resulting strain MA299.2 (*kusA*$^-$, *pyrG*$^-$) was checked for proper removal of the *amdS* marker by diagnostic PCR. The growth of transformants was assayed by point-inoculating 4 µl of spore suspension (1×10^5 spores/µl) in the centre of the agar plate and incubating the plates for 7 days at 30°.

Table 1. Strains used in this study

Strain	Genotype	Reference
N402	*cspA*	Bos et al. 1988
AB4.1	*pyrG*$^{-}$ derivatie of N402	van Hartingsveldt et al. 1987
XY1.1	Δ*creA::pyrG* in AB4.1	Yuan et al. 2006
MA70.15	*ΔkusA::amdS, pyrG*$^{-}$ in AB4.1	Meyer et al. 2007
MA299.2	*ΔkusA*$^{-}$*, pyrG*$^{-}$ derivative of MA70.15	This study
MA211.15	*PpgaX(1203)-uidA-pyrG** in AB4.1[1,2]	This study
MA212.2	*PpgaX(1005)-uidA-pyrG** in AB4.1	This study
MA213.10	*PpgaX(688)-uidA-pyrG** in AB4.1	This study
MA214.14	*PpgaX(409)-uidA-pyrG** in AB4.1	This study
MA215.8	*PpgaX(380)-uidA-pyrG** in AB4.1	This study
CR01.6	*PpgaX (1210)* (TTTACCTTT)-*uidA-TtrpC-pyrG** in AB4.1[3]	This study
CR02.8; CR02.9	*PpgaX (688)* (TTTACCTTT)-*uidA-TtrpC-pyrG** in AB4.1	This study
CR03.1	*PpgaX (1210)* (TTTACCTTT)-*amdS-TtrpC-pyrG** in AB4.1	This study
CR04.2	*PpgaX (688)* (TTTACCTTT)-*amdS-TtrpC-pyrG** in AB4.1	This study
CR05.31	*PpgaX (1210)* (TTTACCAAT)-*uidA-TtrpC-pyrG** in AB4.1	This study
CR06.4; CR06.5	*PpgaX (688)* (TTTACCAAT)-*uidA-TtrpC-pyrG** in AB4.1	This study
CR09.1; CR09.20	*PpgaX (1210) (*TCCACCTTT)-*uidA-TtrpC-pyrG** in AB4.1	This study
CR10.1; CR10.6	*PpgaX (688)* (TCCACCTTT)-*uidA-TtrpC-pyrG** in AB4.1	This study
JC1.5; JC1.7; JC1.8	*PpgaX(1210)-amdS-pyrG***in MA299.2[4]	This study
JC3.6; JC3.7; JC3.9	*PpgxB(1250)-amdS-pyrG*** in MA299.2	This study
JC4.2; JC4.3; JC4.4	*PpgxC(1201)-amdS-pyrG*** in MA299.2	This study
JC5.1; JC5.2; JC5.3	*PgaaB(1194)-amdS-pyrG*** in MA299.2	This study
JC6.6; JC6.7; JC6.8	*PgatT(1208)-amdS-pyrG*** in MA299.2	This study
JN11.2	*PabfA(1266)-amdS--pyrG** in AB4.1	This study
JN29.2	*creA::hygB* in JC1.5	This study
JN31.3	*creA::hygB* in JC3.6	This study
JN32.1	*creA::hygB* in JC4.2	This study
JN33.1	*creA::hygB* in JC5.1	This study
JN34.3	*creA::hygB* in JC6.6	This study
JN16.1	*creA::hygB* in JN11.2	This study

[1] number in brackets indicates length of the promoter region.
[2] *pyrG** refers to targeting integration method of DNA constructs using the *pyrG* targeting method described by van Gorcom et al., 1988
[3] Underlined regions indicate mutations compared to the wild-type motif (TCCACCAAT).
[4] *pyrG*** refers to targeting integration method to the *pyrG* locus described by Arentshorst et al., 2015 (Arentshorst *et al.* 2015).

2.2 General DNA procedures

PCR amplifications were performed using phusion DNA polymerase (Finnzymes) and were carried out according to the manual provided by the manufacturer. *Escherichia coli* strain DH5α was used for all recombinant DNA experiments. *E. coli* was transformed using standard heat shock protocols as described by (Inoue *et al.* 1990). All endonuclease restriction enzymes were purchased from Fermentas or Sigma. DNA sequence analysis was carried out by Macrogen, Korea. Ligations were performed using the Rapid DNA ligation kit (Fermentas).

2.3 Construction of recombinant vectors

The plasmids used in this study are listed in Supplemental Table 1. *PgaX* promoter fragments with different lengths (1203, 1005, 688, 409 and 380 bp) were PCR amplified (see Supplemental Table 2 for primers) and cloned as *Not*I/*Eco*RI fragments in PagsA(30-bp)-*uidA-pyrG** (Damveld *et al.* 2005) to give pMA211, pMA212, pMA213, pMA214 and pMA215. Promoter fragments mutated in the CCNCCAAT box were constructed in either the large *pgaX* promoter fragment (1202 bp) or the short promoter (409 bp). Mutations were introduced by using oligonucleotides (Supplemental Table 2). PCR fragments were again cloned as *Not*I/*Eco*RI fragments in PagsA(30-bp)-*uidA-pyrG** to construct the pCRO plasmids (Supplemental Table 1).

Promoter regions of *pgxB*, *pgxC*, *gaaB*, *gatA* and *abfA* were amplified by PCR using the primers listed in Supplemental Table 2 and using *A. niger* N402 genomic DNA as template, and ligated into pJET1.2 (Fermentas). To construct the *pgaX-amdS* reporter constructs with the mutated CCNCCAAT box, primers Mut1-for and Mut1-rev were used (Supplemental Table 2). The promoter fragments containing the mutations were subsequently cloned in pJET1.2. From these intermediate vectors the promoter fragments were re-isolated as *Sal*I/*Eco*RI and subsequently ligated into the 3,662 bp *Sal*I/*Eco*RI-digested backbone of vector PagsA(2010)-AmdS (Damveld *et al.* 2008) to construct the *amdS* reporter constructs. For the *PabfA-amdS* reporter construct, the *pyrG** gene was cloned into this vector as a *Xba*I fragment to produce the final vector (Table 2). For the construction of promoter reporter plasmids for targeted integration through homologous integration via pMA334, a *Not*I site was introduced at the *Sal*I restriction site by inserting a *Not*I restriction site oligonucleotide (Supplemental Table 2) at the *Sal*I site. The promoter fragments were subsequently isolated as *Not*I fragments and cloned into pMA334 and transformed to MA299.2. Proper integration of the constructs was verified by Southern blot. For each promoter reporter construct three independent transformants were analysed by Southern blot and analysed phenotypically. For each construct a single strain was selected and the *creA* gene was deleted using the split marker method (Arentshorst *et al.* 2015) using primers listed in Supplemental Table 2 for amplification of *creA* 5' and 3' split marker fragments. For each reporter construct, three *creA* mutants were purified, analysed by diagnostic PCR and analysed phenotypically. All PCR amplified promoter regions and final constructs were verified by sequencing (Macrogen).

Table 2. Putative GA-responsive and putative CreA-binding site in GA induced genes.

Gene number	Gene name	Promoter length (bp)	Promoter element	
			GA-responsive element (CCNCCAAT)	CreA binding site (SYGGRG)
An12g07500	*pgaX*	1210	-389	3 (+) [*a] 5 (-)
An16g05390	*gaaB*	1194	-327	10 (+) 5 (-)
An03g06740	*pgxB*	1250	-299	7 (+) 3 (-)
An02g12450	*pgxC*	1201	-279	5 (+) 5 (+)
An14g04280	*gatA*	1208	-334[*b]	2 (+) 4 (-)
An01g00330	*abfA*	1266	Not present	8 (+) 6 (-)

*[a] + and - indicates a putative CreA in the forward (+) or reverse (-) orientation. The exact positions of the different putative *creA* binding sites are given in Supplemental Table 3.
*[b] GARE site in *gatA* does not perfectly matches consenses (TCCAGCAAT).

2.4 Analysis of transformants expressing GUS

The P*pgaX-uidA*-T*trpC-pyrG** vector variants (pMA211-pMA215 and pCRO plasmids) were transformed to *A. niger* AB4.1. Transformants were screened based on glucuronidase activity as described (Damveld *et al.* 2005). For each construct, two transformants expressing GUS from the *pyrG* locus were selected for further analysis. To perform expression analysis of *uidA* and to perform β-glucuronidase enzymatic assay (GUS assay). RNA and protein was isolated from mycelium that was grown for 18 hours at 30˚C in 100 ml CM supplemented with 50 mM GA as the sole carbon source. After growth, mycelium was harvested, frozen in liquid nitrogen, grinded and subsequently used for RNA isolation using TRIzol (Damveld *et al.* 2005) or for protein isolation. For protein isolation 200 mg of mycelium was resuspended in 1 ml Z-buffer (see above) to which a protease inhibitor cocktail (Sigma P8215) was freshly added. Fifty µl of the supernatant was mixed with 1 ml Z-buffer and 200 µl p-nitrophenyl α-D-glucuronic acid solution (PNP-Gluc, Sigma N-1377). Samples were incubated at 37˚C for 15 min or one hour after which the reaction was stopped by addition of two ml 100 mM Na_2CO_3. The ODs at 415 and 550 nm were determined using an Ultraspec 2100 pro (Amersham) spectrophotometer. The specific activity was determined in triplicate as described by (Roberts *et al.* 1989).

Results

3.1. A conserved motif in promoters of galacturonic acid-induced genes is required for *pgaX* induction

Genome-wide expression analysis of *A. niger* grown on various carbon sources has revealed specific induction of pectinolytic genes on galacturonic acid (GA) (Martens-Uzunova and Schaap 2008). These genes include among others exo-polygalacturonidases (*pgaX*, *pgxB*, *pgxC*), a putative galacturonic acid transporter (*gatA*) (Sloothaak *et al.* 2014), and genes involved in the intracellular metabolism of GA (*gaaA*, *gaaB* and *gaaC)*. Promoter analysis of these co-regulated genes identified a conserved motif comprising the consensus sequence 5'-YCCNCCAAT-3' (Martens-Uzunova and Schaap 2008) to which we will refer to as GARE (galacturonic acid responsive element) in this manuscript. To assess the importance of GARE, promoter deletion and site-directed mutagenesis studies were performed using the *pgaX* promoter as representative example. As schematically depicted in Figure 1, promoter deletions and promoter mutations were made in th*e pgaX* promoter and these promoter fragments were cloned in front of the β-glucuronidase (GUS) reporter. All promoter reporter constructs were targeted to the *pyrG* locus using the *pyrG** method (Van Gorcom and Van Den Hondel 1988) and Southern blots were performed to show single-copy, targeted integration of the reporter constructs (data not shown). For each reporter, two independently obtained single-copy transformants were analysed for *uidA* expression and β-glucuronidase activity when grown on GA. The promoter deletion studies showed that the conserved GARE motif is required for expression of the *pgaX* gene leading to GUS activity (Figure 1, and data not shown for the *uidA* expression analysis). As GARE (5'-TCCNCCAAT-3') contains a putative HAP-binding site (CCAAT), the role of the element was determined in more detail by introducing mutations in the specific motifs of the element. Mutations that abolish HAP complex binding (CCAAT to CCTTT mutations) eliminated the expression and induction of the *pgaX* gene. Importantly, also mutating the CC motif upstream of the CCAAT motif (CC to TT) abolished *pgaX* expression, indicating that it is not only the HAP binding site which is important for induction. Mutations in GARE were made either in the large *pgaX* promoter fragment (2012 bp) or the short *pgaX* promoter fragment (409), but in both cases no GUS activity could be detected. The results strongly suggest that both the CC motif and the CCAAT motif in the 5'-TCCACCAAT-3' box are necessary for *pgaX* expression. PecR (CCCTGA) is a second element which has been identified in promoter of GA-induced genes (Bussink *et al.* 1992b; Benen *et al.* 1996). The results presented in Fig.1 indicate that the pecR element is not necessary for *pgaX* induction by GA.

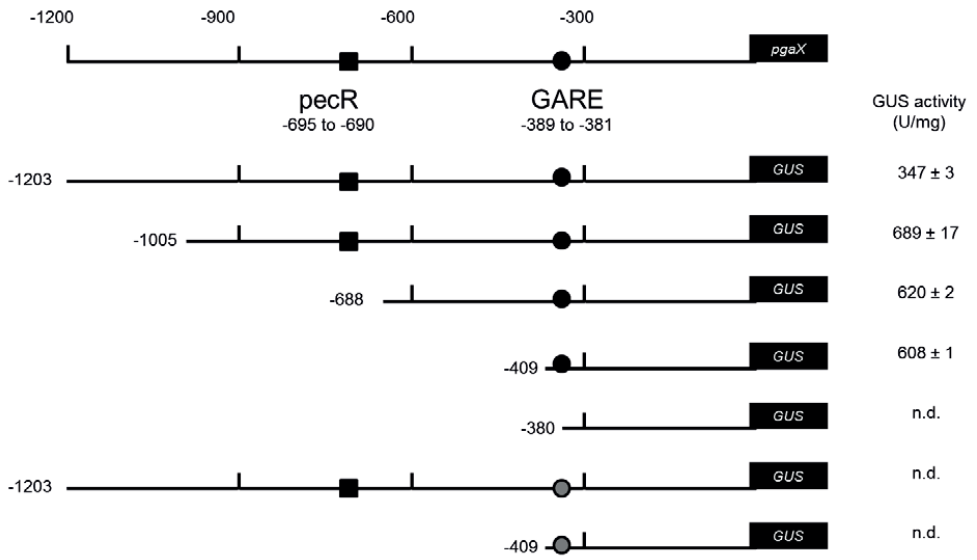

Figure 1. Schematic strategy to analyse the role of conserved promoter elements in the *pgaX* promoter. A) Promoter fragments with decreasing length were PCR-amplified and cloned in front of the β-glucuronidase (GUS) reporter. Mutations in the galacturonic acid-responsive element (GARE) in either the long promoter (1203 bp) or the shortened (409 bp) promoter version are indicated by the gray circle. For either the long or short promoter fragment three mutations were made (CCACCAAG to **TT**ACCAAG, CCACC**TT**G or **TT**ACC**TT**G) which all abolished expression if the *uidA* gene. On the right - GUS activity in mycelia of transformants grown on galacturonic acid as a sole carbon source.

3.2. Construction and analysis of *in vivo* promoter-reporter constructs to assess promoter activity

The acetamidase gene (*amdS*) of *A. nidulans* (Kelly and Hynes 1985) was used as a reporter for gene expression in *A. niger* (e.g. (Damveld *et al.* 2008; Punt *et al.* 2008) To analyse GA-specific expression *in vivo* on agar plates, we constructed several *amdS* reporter constructs, in which promoter sequences of GA-induced genes (*pgaX*, *pgxB*, *pgxC*, *gaaB* and *gatA*) were selected (Table 2). In addition, we included a promoter of *abfA* which is known to be an arabinose-induced gene (De Groot *et al.* 2003). The rationale of the assay is that expression of *amdS* from the various promoters can be assessed by the ability of the transformant to grow on medium containing acetamide as N-source. To allow comparison of promoter activity all promoter-reporter constructs were targeted to the *pyrG* locus either by the *pyrG** method (*abfA*) (Van Gorcom and Van Den Hondel 1988) or through a recently developed *pyrG*** targeting method ((*pgaX, pgxB, pgxC, gaaB* and *gatA*) (Arentshorst *et al.* 2015). Correct integration of the reporter constructs was verified by Southern blot analysis (data not shown). For each construct, three independent single-copy transformants with the expected integration

pattern were analysed for growth. Transformants were point-inoculated on minimal medium agar plates containing acetamide as a sole N-source and various carbon sources (Figure 2). Growth of the transformants containing the AmdS reporter fused to the promoter of *pgaX*, *pgxB*, and *pgxC*, was specific for GA, polygalacturonate (PGA) and pectin. On all other carbon sources these strains did not grow, indicating that *pgaX, pgxB, and pgxC* are specifically induced by GA. The growth of the transformant harbouring the *pgxC-amdS* reporter construct is reduced compared to the other strain, indicating that the expression from the *pgxC* promoter might be lower than expression from the other promoters. Growth of the *gaaB-amdS* reporter strain was less specific for GA, indicating that the *gaaB* gene is also expressed under non-inducing conditions. Growth analysis of *gatA-amdS* reporter suggests that the gene is not only expressed in the presence of GA, but also in the presence of rhamnose, arabinose, and fructose. As described in more detail in the next paragraph, the ability of the *gatA-amdS* reporter strain to grow on rhamnose, arabinose, and fructose is likely due to derepression. Growth of the *abfA-amdS* reporter strain nicely confirmed previous reports that *abfA* is specifically induced by arabinose (De Groot *et al.* 2003). The *abfA* promoter does not contain a GARE or pecR element. The *abfA-amdS* reporter strain also grows well on PGA and pectin indicating the *abfA* is also induced under these conditions. Whether the induction by pectin and PGA is AraR-dependent awaits further studies.

The *pgaX-amdS* reporter was also used to show the importance of the 5'-TCCACCAAT-3' motif. Mutating the GARE promoter in the wild type *pgaX* promoter (5'-TCCACCAAT-3' to 5'-TTTGTTGAT-3') and placing the mutated promoter in front of the *amdS* reporter abolished the ability of transformant containing the mutated reporter construct to grow on GA (data not shown). Collectively, by using both the *uidA* and *amdS* reporter constructs we show that *pgaX* induction is specific on GA and requires the GARE motif.

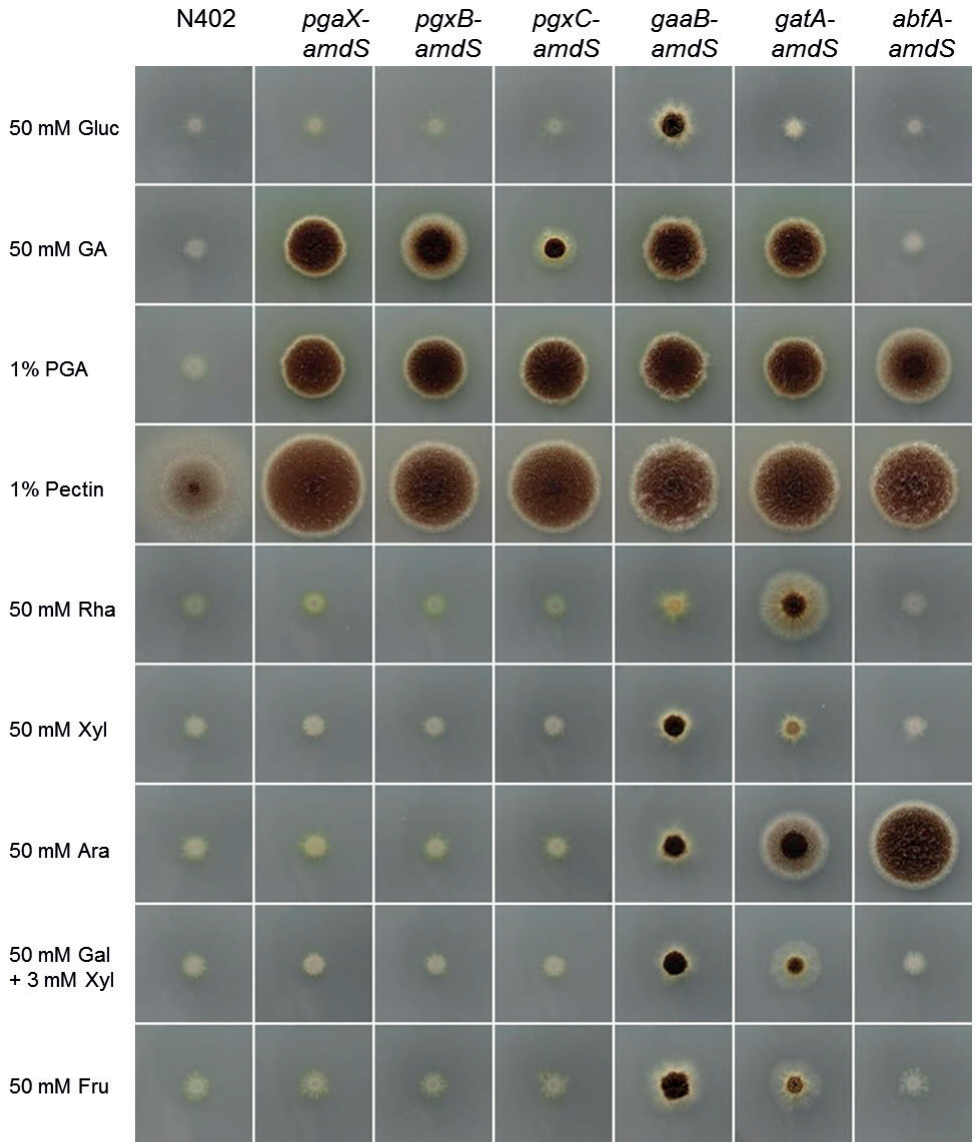

Figure 2. Growth analysis of promoter-reporter strains on various carbon sources. The wild-type (N402) and reporter strains were grown on MM-acetamide supplemented with 50 mM of a monomeric carbon source, or 1% w/v polymeric substrate.

3.3 Expression of galacturonic acid-induced genes is under carbon catabolite repression control and CreA-dependent

The reporter strains described above also offer the possibility to analyse *in vivo* the role of carbon catabolite repression on the GA-induced genes. First, *in silico* analysis of the promoter

sequences of all five reporter constructs for putative CreA binding sites (SYGGRG) showed that all promoters contain several putative CreA binding sites (Table 2 and Supplemental Table 3). To analyse the influence of glucose repression on the expression of the promoter-reporter genes, spores of the reporter strains were inoculated on MM-acetamide plates containing 50 mM GA and increasing concentrations of glucose (up to 50 mM) (Figure 3, columns A, C, E, G, I). Expression of *pgaX, pgaxB*, and *pgxC* was strongly repressed by the addition of glucose. Whereas expression of *pgxB* and *pgxC* was strongly repressed by the addition of 0.5 mM glucose, this concentration had less effect on *pgaX* expression. At glucose concentrations between 5 mM and 50 mM, however, *pgaX*, *pgxB*, and *pgxC* were all three fully repressed. The repression of *gatA* was only partial at the higher glucose concentrations (>5 mM) and the expression of *gaaB* was apparently not affected as growth of the reporter strains was not reduced on GA plates supplemented with glucose (Figure 4, column G).

To investigate whether CreA is responsible for carbon catabolite repression of GA-induced genes, the *creA* gene was deleted in the different AmdS-reporter strains. For efficient deletion of the *creA* gene in the reporter strains a *ku70* mutant (*kusA*) was used as a recipient strain (see material and methods). Deletion of *creA* in the reporters was very efficient (80-90%) and proper deletion of *creA* in all five reporter strains was verified through diagnostic PCR (data not shown). Growth and glucose repression of the strains were analysed as described above by inoculating spores on a GA/acetamide plate containing increasing concentrations of glucose. As shown in Figure 3 (columns B, D, F, H and J) deletion of *creA* abolished glucose repression in most of the reporter strains. Glucose repression of the *pgaX*, *pgxB and gatA* genes was completely lost (Figure 3; columns B, D, and J, respectively). Repression was still observed, although only at higher glucose concentrations (≥10 mM) in the *pgxC* reporter strain. The reduced growth of the *gaaB* reporter in the *creA* background at glucose concentration higher than 5 mM (compare in Figure 3, column G and H) is probably not directly related to *amdS* expression from the *gaaB* promoter but results from the general reduction in growth of the *creA* mutant compared to the wild-type strain as previously reported (Yuan *et al.* 2006). The analysis of the reporter strains indicates that for the *pgaX*, *pgxB* and *gatA* promoters CreA is required for glucose repression. The expression of *amdS* from the *pgxC* promoter is more complex as repression is still observed, only at high glucose concentration (Figure 3, column F). It will be of interest to discover the molecular basis for glucose repression in the *pgxC* gene at high concentrations as its repression seems to be independent of CreA.

3.4 GA-induced gene expression requires a specific activator

The promoter-*amdS* reporter strains in the *creA* mutant background also made it possible to assess the role of inducer molecules. In most cases, transcriptional regulation of networks involved in utilization of a specific carbon source is mediated via a network-specific transcription factor such as XlnR, AraR or AmyR (see introduction). These transcriptional activators are often activated via specific sugars derived from the substrate. For GA, it has been suggested that GA itself or a derivative of GA acts as an inducer (Mojzita *et al.* 2010). To establish if the presence of GA as an inducer was required to support growth, spores of the reporter strains in which the *creA* gene was deleted were point-inoculated on glucose medium (Figure 4, upper row). The reporter strains expressing *amdS* from the *pgaX*, *pgxB*, and *gaaB* promoters, still required the presence of GA as an inducer to activate gene expression. The *pgxC-amdS* reporter strain did not grow, indicating that the expression from the *pgxC* promoter is rather low, which was also observed in the wild-type background (Figure 2). The *gatA-amdS* reporter strain also grows on glucose/acetamide plates even without inducer indicating that derepression by deleting *creA* results in sufficient expression of *amdS* from the *gatA* promoter to support growth. As shown in Figure 4, an extracellular concentration of 100 µM GA was sufficient to induce expression of the GA promoter reporter constructs and to support growth.

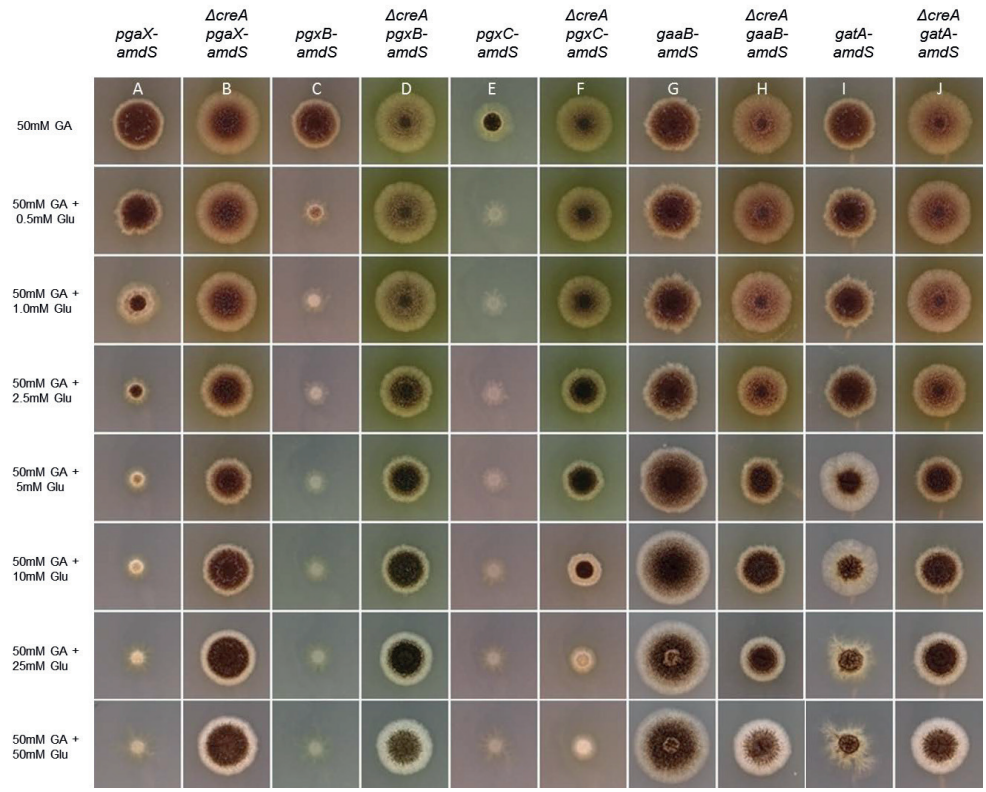

Figure 3. Galacturonic acid-induced gene expression is repressed by glucose in a *creA* dependent way. Growth of *A. niger* reporter strains was monitored on MM containing galacturonic acid/acetamide and increasing concentrations of glucose. Columns A, C, E, G, I represent promoter constructs in the wild-type background; columns B, D, F, H, J represent promoter constructs in the *ΔcreA* null background.

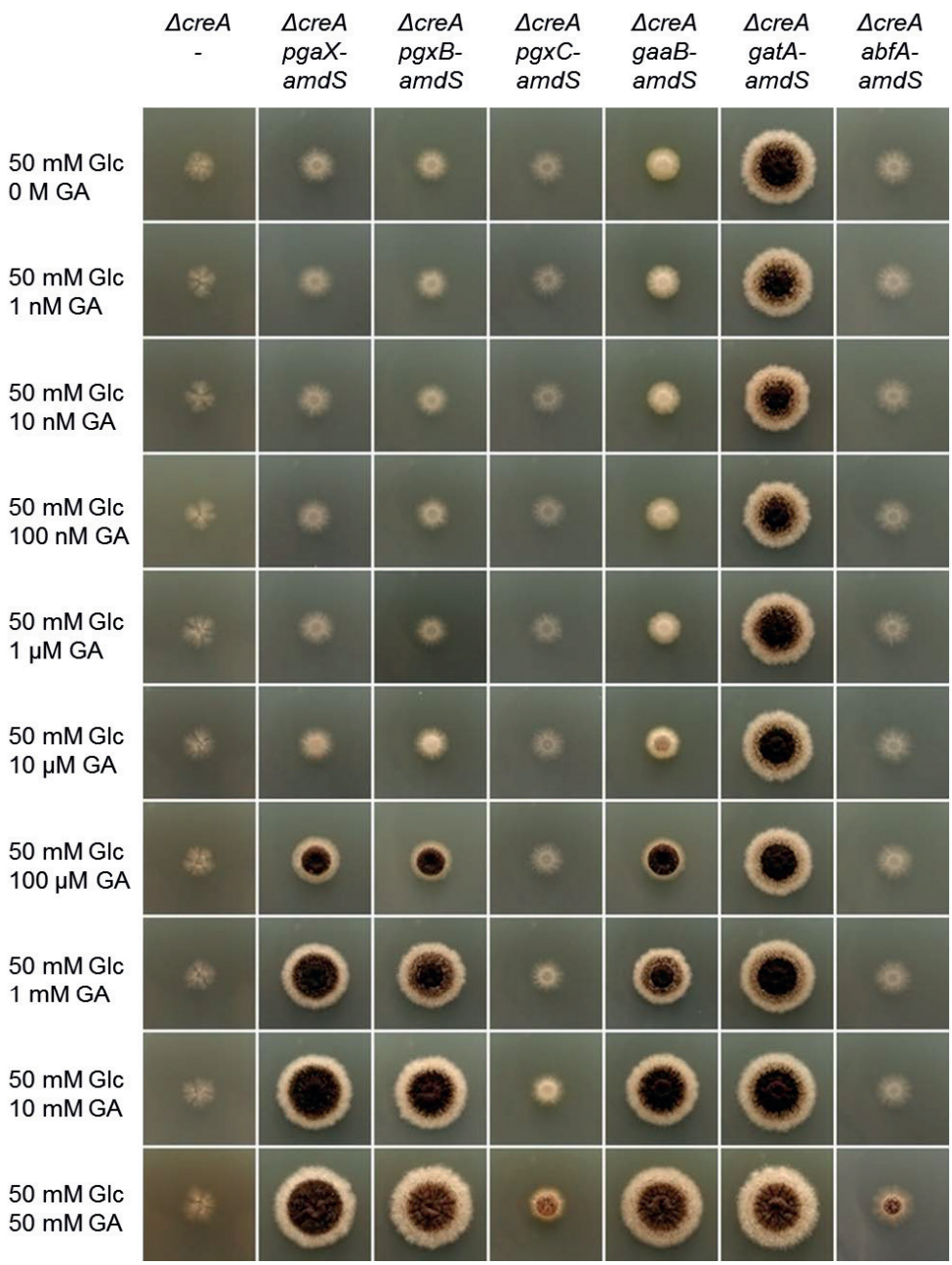

Figure 4. Galacturonic acid is required as an inducer. Growth analyses of the reporter strains on MM containing glucose/acetamide and increasing concentrations of galacturonic acid. No growth is observed for the reporter strains on MM containing glucose/acetamide without the addition of galacturonic acid, except for the *PgatA-amdS* reporter strain.

Discussion

The regulation of pectinolytic enzymes is likely to be complex and searches for transcription factor mutants that are unable to utilize galacturonic acid by targeted deletion of selected transcription factors have so far been unsuccessful (our unpublished data). The genome of *A. niger* harbours around 660 genes encoding transcription factors (Pel *et al.* 2007). We recently constructed a *A. niger* transcription factor knock-out mutants in which 240 transcription factor encoding gene were deleted. None of the transcription factor mutants in our collection showed a specific growth defect on GA, PGA or pectin (unpublished results). Besides the possibility that we missed so far a GA specific transcription factor, a possible explanation for why it is so difficult to obtain such mutants is genetic redundancy and/or overlapping GA degradation and utilization networks. In both cases, deletion of a single transcription factor does not result in strongly reduced growth on GA, PGA, or pectin. The co-regulation of a particular set of pectinolytic genes in *A. niger* supports however the idea that a specific GA-responsive transcriptional activator is involved (De Vries *et al.* 2002; Martens-Uzunova and Schaap 2008).

To study the regulation of pectinolytic genes, several promoters of GA-induced genes were selected based on their expression profile in microarray studies (Martens-Uzunova and Schaap 2008). By fusing these promoter regions to either the *uidA* or *amdS* marker gene, we could show that the induction of most of these genes was specific for GA and that the promoter was not induced on other monomeric carbon sources including those that are found in pectin, such as xylose, rhamnose and arabinose. Thus, it is likely that these promoters are not under control of e.g. the XlnR, RhaR and AraR transcription factors. The reduced growth of the *pgxC-amdS* reporter on GA/acetamide plates suggests a lower expression of *pgxC* compared to *pgaX* and *pgxB* (Figure 2). As the expression in liquid cultures of *pgaX*, *pgxB* and *pgxC* are comparable (Martens-Uzunova and Schaap 2008) it is well possible that the expression of *pgxC* is also dependant on the mode of cultivation (plate vs submerged) as has been shown for other genes encoding extracellular enzymes (Te Biesebeke *et al.* 2005) or sugar transporters (Fekete *et al.* 2012).

An important finding for future research is that the *pgaX*, *pgxB* and *pgxC* genes require GA as an inducer for expression (Figure 4). Even under derepressing conditions (in the *creA* mutant), the expression of *amdS* was not sufficient to allow growth. The *pgaX, pgxB* or *pgxC* reporter strains in the *creA* background can now be used in a genetic screen to isolate mutants that no longer require the presence of an inducer. Such mutants might contain a mutation that

causes constitutive activation of a GA-specific transcription factor. Identification of transcription factors involved in the activation of GA-responsive genes is important to optimize production of pectin-degrading enzymes in filamentous fungi.

Previous studies related to GA-induced gene expression identified two conserved promoter elements that could be involved in induction. The pecR site (CCCTGA) was identified by Benen and co-workers and it has been suggested that this element might bind a pectin-specific regulator (Benen *et al.* 1996; Coutinho *et al.* 2009). This element is present in the *pgaX* promoter at position -695 to -690. Our promoter deletion analysis showed, however, that this element is not required for the induction by GA. We also noticed the 50% reduction in GUS activity between transformants with the largest transcript (1203) and the transformants with the shorter transcripts (1005, 688 and 409 bp (Fig. 1). The reason for this difference and whether it has biological significance is currently not known.

Both the deletion study and the site-directed mutagenesis of the second conserved element (5'-TCCNCCAAT-3') indicate that this promoter element is important for induction and therefore we named this element GARE for galacturonic acid-responsive element. As previously noted (Bussink *et al.* 1992b; Martens-Uzunova and Schaap 2008), this motif includes a putative binding site (CCAAT) for the CCAAT-binding factor (CBF) complex, also known as AnCF or the HAP-complex(Kato *et al.* 1998; Steidl *et al.* 1999). The HAP complex is an important factor in activating gene expression as the complex is involved in recruiting specific transcription factors to their target sites. In *A. nidulans* the AmdR transcription factor is a $Zn(II)_2Cys_6$ binuclear cluster DNA- binding protein that is required for omega-amino acid induction of the *amdS* gene and the genes for omega-amino acid utilization (Andrianopoulos and Hynes 1988). Deletion of the *hapB* or *hapE* genes results in loss of omega-amino acid induction of *amdS* expression. The most likely explanation for the interaction between AmdR and the HAP-complex is that the HAP-complex facilitates AmdR binding to DNA *in vivo*. It has been suggested that HAP binding to its target sequence is a prerequisite for a change in chromatin structure necessary for AmdR binding (Steidl *et al.* 1999). A similar mode of action can be attributed to the regulation of *agdA* and *taaG2* in *A. nidulans* by AmyR. The gene *amyR* encodes a $Zn(II)_2Cys_6$ transcription factor specific for the induction of starch-degrading enzymes. Unlike AmdR, the AnCF-binding site and AmyR-binding site (starch-responsive element; SRE) are spaced by 100 nucleotides (Tani *et al.* 2001a; Tani *et al.* 2001b), suggesting that no direct interaction between AnCF and AmyR is necessary for activation. The binding of AmyR to the SRE is necessary for induction of gene

expression of both genes, whereas AnCF binding seems to be responsible for high affinity binding of AmyR. Our observation that a mutation in the CCAAT motif completely abolishes GA-induced gene expression, favours a mechanism similar to the AmdR example. In the case of GA-induced gene expression, the binding of a hypothetical GA-specific transcriptional activator (tentatively named GaaR) might require HAP for DNA binding. To examine the role of the HAP complex in *A. niger*, we disrupted the *hapB* gene. However, the deletion mutant has a very severe growth phenotype on a variety of different carbon and nitrogen sources tested (data not shown). Because of the very poor growth of the *hapB* mutant, no expression studies could be performed to analyse whether *pgaX* (or other pectinolytic genes) was induced in the *hapB* mutant. A additional example in which HAP binds to another transcription factor that binds in close proximity of the HAP complex, has recently been discovered for the HAP2/3/5-HapX complex. Both the HAP complex and the HapX protein interact with each other as well as with their DNA binding sites and both the protein-protein and DNA-protein interactions are required to regulate gene expression in order to maintain iron homeostasis (Hortschansky *et al.* 2015). HapX requires the minimal motif 5'-GAT-3', which is localized in a distance of 11 to 12 base pairs downstream of the respective CCAAT box (Hortschansky *et al.* 2015). To examine the possibility that additional conserved elements surrounding GARE were previously missed (Martens-Uzunova and Schaap 2008), we aligned promoter sequences of GA-induced genes and looked for conserved regions in regions including 45 bp up- and 45 bp downstream of the GAREs. The results are shown in Supplemental Figure 1A. As shown in this alignment, no additional motif was identified. We noted however, the preference of a TT motif 2 nucleotides upstream of the 5'-TCCnCCAAT-3 region. However, this motif was not entirely conserved, but could be relevant. To analyse the possible relevance of this TT motif, a cross *Aspergillus* analysis was performed. Since orthologous genes for *gaaA*, *gaaB* and *gaaD* are readily identified in *Aspergillus* species analysed (*A. nidulans*, *A. fumigatus* and *A. oryzae*), we performed our analyses on the promoter for these genes. Please note that *gaaA* and *gaaC* are expressed form the same promoter and therefore *gaaC* was not included separately. The analysis is shown in Supplemental Figure 1B and shows that the TT motif is conserved in the *gaaB* promoters among the four Aspergilli, but not conserved in the others genes (*gaaA* and *gaaD*). It should be noted that the GARE alignment as shown by Martens-Uzunova and Schaap 2008 (Martens-Uzunova and Schaap 2008), includes a putative GARE site in which the CCAAT motif in not completely conserved. For example, the GARE in the promoter of *gatA* 5'-TCCNGCAAT-3' or *pelA* 5'-TCCNCCTAT-3' do not match perfectly with the 5'-TCCNCCAAT-3' consensus. This suggests that small variants in CCAAT motif

are tolerable. Although the *abfA* promoter does not contain a perfect 5'-TCCNCCAAT-3' match, it does contain three variants (- 214 5'-TCCTCCACT, -470 5'-CCCTCCATT, -5'-899 TCCTCCGAT). Given the observation that the *amdS* gene us not expressed from the *abfA* promoter on GA (Fig. 2) we assume these variations are not allowed to mediate GA-induced gene expression. Further mutagenesis studies are required to clarify whether the TT motif, variations in the CCAAT motif, or other sequences surrounding the 5'-TCCNCCAAT-3' are required to function as GA-responsible elelements.

To address the question whether the 5'-TCCNCCAAT-3' motif was sufficient to induce GA-specific gene expression, the 5'-TCCACCAAT-3' motif, including four additional base pairs flanking the motif, was cloned upstream of the *A. nidulans gpdA* minipromoter. This system has been successfully used to identify a benzoate-responsive element from the *cprA* promoter (Van Den Brink *et al.* 2000). Two constructs were made consisting of either the empty mini-promoter (pMini-GUS) or the mini-promoter containing the motif 5'-TCCACCAAT-3' and transformed to the *pyrG* locus using the *pyrG** method. Transformants with targeted integration of the reporter constructs were verified by Southern blot analysis (data not shown). Expression analysis of the transformants revealed no specific induction of the GUS reporter in the presence of GA, indicating that the 5'-TCCACCAAT-3' domain is not sufficient to induce GA-specific gene expression.

Recently, the transcriptomes of A. niger grown on lignocellulose and during carbon starvation have been studied in detail (Delmas et al. 2012; Van Munster et al. 2014). This showed that a subset of genes involved in the degradation of lignocellulose is not only induced in the presence of an inducer (in this case xylose), but also by carbon starvation. The response to carbon starvation was shown to be dependent on CreA derepression, and independent of the xylanolytic regulator XlnR. The authors proposed a model in which carbon starvation induced the expression of scouting enzymes, independently of carbon source-specific inducers (Van Munster *et al.* 2014). These scouting enzymes are expressed with the purpose to sense the presence of plant cell walls by releasing small amounts of inducing sugars, to which the fungus can then respond by secreting additional hydrolases. Our *in vivo* reporter strains could well be used to verify the predictions based on the transcriptomic data. From our analysis using the promoter reporters, it is interesting to note that the GA-transporter gene is expressed under derepressing conditions even in the absence of GA as the inducer (Figure 4). The other promoters (*pgaX*, *pgxB*, *pgxC*) are not highly enough expressed

under derepressing conditions to sustain growth and this suggests that the corresponding enzymes will most likely not act as scouting enzymes.

As a working model (Figure 5), we postulate the existence of a GA-specific transcription factor (GaaR) that binds to the promoter of GA-induced genes. This transcription factor is most likely post-translationally activated by GA or a GA-derivative. Extensive co-expression network analysis based on a large collection of microarray data did not identify co-regulated transcription factors that upon disruption affected growth on GA or pectin (Homan, Alazi, de Vries and Ram, unpublished data), indicating that the activity of the proposed transcription factor is post-translationally regulated. The HAP complex is most likely involved DNA binding of the hypothetical GaaR transcription factor. Whether this directly involves the GARE box (5'-TCCNCCAAT-3') or whether GaaR requires HAP binding to bind to another motif(s) is currently unknown. Since the CC motif is essential for GA-induced gene expression, we considered it unlikely that induction by GA depends only on the HAP complex. We can not exclude the possibility that HAP interacts with some other non-DNA binding protein that gives specificity towards HAP to mediate GA-induced gene expression.

We also provided further evidence for the role of CreA to mediate glucose repression of pgaX, pgxB and to some extent of pgxC. As pgxC is still partially repressed by glucose even in de *creA* mutant, an alternative repression mechanism might play a role. Although we could not link the TupA general repressor protein to glucose repression in *A. niger* (Schachtschabel *et al.* 2013), we cannot exclude a possible involvement of TupA in *pgxC* repression and this possibility will be addressed in future experiments. Finally, the promoter reporter strains in the *ΔcreA* background that still require an inducer for growth (*pgaX*, *pgxB* and *pgxC)* allow forward genetic screens for inducer-independent mutants. One possible explanation for such a mutant is a mutant in which a galacturonic acid sensor protein or a galacturonic acid specific transcription factor is constitutively active, and experiments are ongoing to isolate and characterize such mutants.

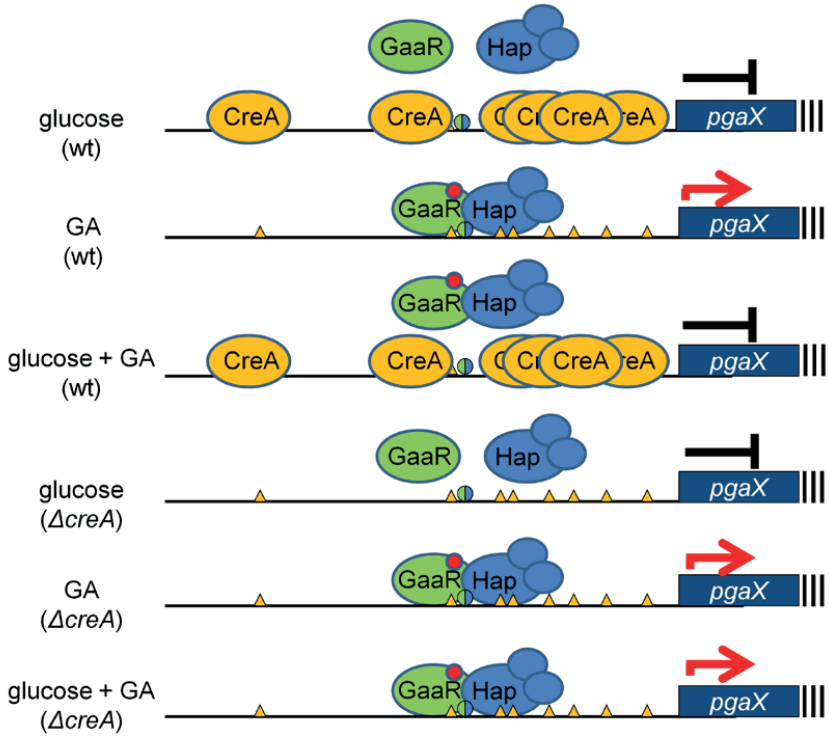

Figure 5. Speculative model for the regulation of galacturonic-acid induced gene expression in *A. niger*. GA-induced genes, poly-galacturonase X (*pgaX*) as an example, are regulated via competing inducing and repressing mechanisms. Induction of GA-responsive genes requires the presence of an inducer molecule (GA, or a metabolic derivative thereof, red circle) which is required for the activation of the putative GA-specific transcription factor (GaaR). The presence and importance of the HAP binding site as part of the GA responsive element (GARE, green/blue circle) suggests that HAP is also required for the expression, possibly by interacting with GaaR. Induction of *pgaX* is repressed by the presence of glucose and possibly other repressing sugars via CreA and putative CreA binding sites in the promoter region (brown triangles). Induction or repression of the *pgaX* gene under the conditions indicated in either the wild type (WT) or *ΔcreA* mutant are indicated by the red arrow or black symbol respectively.

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Supporting information

The supplementary material of this chapter are available via

http://www.sciencedirect.com/science/article/pii/S1087184515001516?via%3Dihub and comprises the following:

Supplemental Table 1. Plasmids used in this study

[1] *pyrG** refers to targeting integration method of DNA constructs using the pyrG targeting method described by van Gorcom et al., 1988, [2] *pyrG*** refers to targeting integration method to the pyrG locus described by Arentshorst et al., 2015.

Supplemental Table 2. Primers used in this study

*Restriction sites are underlined; **Letters in bold indicate mutations in the GARE element ((Mut1-3), ***bold letter indicate overlapping sequences for fusion PCR.

Supplemental Table 3. Positions of putative CreA bindingsites (SYGGRG) in the GA-induced promoters and *abfA*. Number of putative CreA binding sites in given in brackets. Distance from the ATG start codon in bp) is given.

Supplemental Figure 1. A) Promoter alignment of sequences surrounding the GARE motif of GA-induced genes in A. niger. GA-motifs were taken from Martens-Uzunova et al., 2008. B) Promoter alignments of gaaA, gaaB and gaaD orthologs in A. niger, A. nidulans, A. fumigatus and A. *oryzae*. Orthologs were taken from AspGD. (+) or (-) indicate whether the GARE motif was in forward or reverse orientation. The GARE motif, as well as the partially conserved TT-motif 5' upstream of GARE, are highlighted in yellow. * indicates that no CCNCCAAT motive was found in these promoter regions.