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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 utilise 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.

1. 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) (reviewed in 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

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revealed a large array of extracellular pectinolytic enzymes in *A. niger* (Martens-Uzunova and Schaap, 2009; Coutinho et al., 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,b; Benen et al., 1996; Pařenicová et al., 1998, 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, 2006; Hilditch et al., 2007; Mojzita et al., 2010; Wiebe et al., 2010; Liepins et al., 2006; 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 characterised 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 (Maldonado and Strasser de Saad, 1998; Bussink et al., 1990, 1992a; 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 utilisation (Kato et al., 1997, 1998; van Heeswijck and Hynes, 1991; 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 (Thön 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 utilisation 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 (reviewed in 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.

2. Materials 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 Lasure, 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 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). 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 °C.

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).