Expression profiling of pectinolytic genes from *Aspergillus niger*

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Abstract **The expression of 26 pectinolytic genes from *Aspergillus niger* was studied in a wild type strain and a CreA derepressed strain, under 16 different growth conditions, to obtain an expression profile for each gene. These expression profiles were then submitted to cluster analysis to identify subsets of genes with similar expression profiles. With the exception of the feruloyl esterase encoding genes, all genes were expressed in the presence of D-galacturonic acid, polygalacturonate, and/or sugar beet pectin. Despite this general observation five distinct groups of genes were identified. The major group consisted of 12 genes of which the corresponding enzymes act on the pectin backbone and for which the expression, in general, is higher after 8 and 24 h of incubation, than after 2 or 4 h. Two other groups of genes encoding pectin main chain acting enzymes were detected. Two additional groups contained genes encoding L-arabinose and D-galactose releasing enzymes, and ferulic acid releasing enzymes, respectively. The genes encoding β-galactosidase and the L-arabinose releasing enzymes were not only expressed in the presence of D-galacturonic acid, but also in the presence of L-arabinose, suggesting that they are under the control of two regulatory systems. Similarly, the rhamnogalacturonan acetylesterase encoding gene was not only expressed in the presence of D-galacturonic acid, polygalacturonate and sugar beet pectin, but also in the presence of L-rhamnose. The data presented provides indications for a general pectinolytic regulatory system responding to D-galacturonic acid or a metabolite derived from it. In addition, subsets of pectinolytic genes are expressed in response to the presence of L-arabinose, L-rhamnose or ferulic acid.**

Key words: Pectin; Gene regulation; CreA; Expression analysis; *Aspergillus niger*

1. Introduction

Pectins are heteropolysaccharides with a complex and diverse structure [1,2]. Efficient biodegradation of pectin requires a wide range of enzymes, including enzymes that act on the main chain as well as enzymes acting on the side chains. *Aspergillus niger* is one of the microorganisms in which the pectinolytic system has been studied in most detail. A large number of *A. niger* enzymes that are involved in pectin degradation have been purified and the corresponding genes for most of these enzymes have been cloned [2].

Pectin, polygalacturonic acid, and D-galacturonic acid were found to induce expression of polygalacturonase (PG), pectin lyase, and pectin methylesterase (PME) encoding genes, while glucose repressed the expression of these genes [3–7]. The major carbon catabolite repressor protein (CreA) [8,9] was demonstrated to be involved in the repression of pectinolytic gene expression [10,11].

A promoter deletion study on one of the PG encoding genes (*pga*II) of *A. niger* resulted in the identification of a region (5′-TYATTGGTGGAA-3′), similar to the yeast HAP2/3/5 consensus binding site, which was important for high level expression of *pga*II [12]. A second region present in the promoters of many pectinolytic genes that might be involved in activation of pectinolytic gene expression has been reported (CCCTGA) [13]. However, the functionality of these regions has not been studied in detail. The expression of genes encoding α-L-arabinofuranosidase, endoarabinanase, and β-galactosidase was found to be induced by L-arabinose and L-arabitol [14,15]. This indicates that there may be two regulatory systems involved in pectin degradation. One system that responds to the presence of D-galacturonic acid and activates the expression of genes encoding enzymes active on the main chain of pectin, and another system that responds to the presence of L-arabinose and activates the expression of genes encoding α-L-arabinofuranosidase, endoarabinanase, and β-galactosidase.

The aim of this study was to identify subgroups of the 26 pectinolytic genes of *A. niger* based on a detailed analysis of their expression on pectin and pectin-derived carbon sources. We describe here a simple but effective approach using dot blots that is especially useful for the analysis of a select group of genes under a large number of different conditions. The conditions chosen in this study were based on previous expression studies of a smaller group of pectinolytic genes [7]. The analysis of the data was performed using principal com-

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ponent analysis (PCA), a well-established statistical method for the multivariate interpretation and visualisation of complex data [16–19]. PCA, based on the principle of linear combinations (using coefficients called the loadings) of the original measurements (e.g. genes), can summarise the data as a weighted sum of these loadings (the scores representing also the values of the linear combinations). The first principal component (PC; product of scores and loadings) is constructed in such a way, as to explain as much variance as possible from the data set (e.g. set of expression profiles). The subsequent PCs will analogously describe the still unexplained variance so that the explained variance per PC will decrease. When the original measurements show a correlation structure, only a few PCs will suffice to adequately describe the data. In the present case this means that only a few plots need to be inspected in order to find the subsets of genes that show correlated expression behaviour.

2. Materials and methods

2.1. Strains libraries and plasmids

Both strains used in this study were derived from *A. niger* wild type N400 (=CBS 120.49). N402 is a mutant with low conidiophores [20] and was used as a wild type. NW200 has a derepressed, but not a complete loss of function phenotype for its CreA function [21]. A low level of carbon catabolite repression is still present in this strain. *Escherichia coli* DH5αF′ was used for routine plasmid propagation.

2.2. Media and culture conditions

Minimal medium (MM) contained (per liter): 6.0 g of $NaNO_3$, 1.5 g of KH_2PO_4, 0.5 g of KCl, 0.5 g of $MgSO_4$, trace elements [22], and 1% (w/v) glucose as a carbon source unless otherwise indicated. For complete medium (CM), MM was supplemented with 0.2% (w/v) tryptone, 0.1% (w/v) yeast extract, 0.1% (w/v) casamino acids, and 0.05% (w/v) yeast RNAs. Liquid cultures were inoculated with 10^6 spores/ml and incubated at 30°C in an orbital shaker at 250 rpm. Agar was added at 1.5% (w/v) for solid medium. For the growth of strains with auxotrophic mutations, the necessary supplements were added to the medium.

In transfer experiments, strains were pre-grown in CM containing 2% (w/v) D-glucose as carbon source. After 16 h mycelium was harvested, washed with MM without carbon source and aliquots of 1.5 g (wet weight) were transferred to 50 ml MM containing carbon sources as indicated in Fig. 1. After the incubation time indicated in Fig. 1 mycelium was harvested, dried between tissue paper, frozen in liquid nitrogen and stored at −70°C.

2.3. Chemicals

D-Xylose, D-glucose, D-galactose, L-rhamnose and D-fructose were obtained from Merck (Darmstadt, Germany). D-Glucuronic and D-galacturonic acid were from Fluka (Buchs, Switzerland). L-Arabinose was obtained from Sigma (St. Louis, MO, USA). Polygalacturonate was from United States Biochemical (Cleveland, OH, USA). Sugar beet pectin was from Copenhagen Pectin A/S (Lille Skensved, Denmark). Ferulic acid was obtained from Acros (Oxon, UK).

2.4. Molecular biology methods

Standard methods were used for plasmid DNA isolations and DNA digestions [23]. RNA isolations were performed as described previously [21].

2.5. Dot blot Northern analysis

20 μl 20×SSC (3 M NaCl, 0.3 M trisodium citrate) and 17.5 μl formaldehyde was added to 1.5 μg total RNA (2.5 μl) after which the mixture was incubated for 10 min at 65°C. Samples were blotted on a Hybond N membrane, pre-wetted with 10×SSC, using a 96-well dot blot apparatus connected to a vacuum pump. Filters were hybridised at 42°C in a solution of 50% (v/v) formamide, 10% (w/v) dextran sulphate, 0.9 M NaCl, 90 mM Na_3-citrate, 0.2% (w/v) ficoll, 0.2% (w/v) polyvinylpyrrolidone, 0.2% (w/v) bovine serum albumin, 0.1% (w/v) SDS and 100 μg/ml single stranded herring sperm DNA. Washing was performed under strictly homologous conditions with 30 mM NaCl, 3 mM Na_3-citrate and 0.5% (w/v) SDS at 68°C, avoiding any cross hybridisation between the genes. Fragments used as probes for the pectinolytic genes are listed in Table 1. A 0.7 kb *Eco*RI fragment of the 18S rRNA subunit [24] was used as a probe for RNA loading control.

2.6. Quantification of the expression data

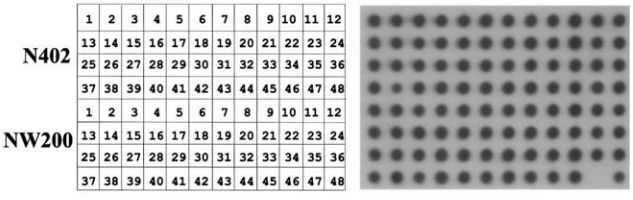
Scans of the autoradiograms were quantified using Imagene version 4.2.0 (Biodiscovery Inc., Los Angeles, CA, USA). The highest value on each autoradiogram was set as 100% and the other raw data was converted to relative values.

2.7. Expression data analysis

Analysis of the expression data was performed via a PCA (see Section 1) using MATLAB version 6.1 (Mathworks, Inc., Natick, MA, USA) and the PLS_Toolbox version 2.0.1b (Eigenvector Research, Manson, WA, USA) to visualise subsets of genes with similar expression profiles. Pre-processing of the data was performed by autoscaling in which each condition was centred on the average and divided by the standard deviation. This way the influence of each condition weighs equally in the PCA. This results in a PCA based on a correlation matrix, rather than a covariance matrix. For this study, only PC#1, PC#2 and PC#3 are analysed as they together explain 61.6% of the variance of the data.

3. Results and discussion

Expression profiles were obtained for the 26 genes encoding enzymes that act on either the main chain or side chains of pectin. Fig. 1 lists the transfers from which the RNA samples used for the expression analysis were obtained, the location of these samples on the dot blot and shows the RNA loading control of the samples. The complexity of these profiles varied from relatively simple, e.g. *faeA* (Fig. 2A), to very complex,



1-3: 25 mM D-glucose (glc); 2, 4 and 8 h
4-6: 25 mM D-xylose (xyl); 2, 4 and 8 h
7-9: 25 mM L-arabinose (ara); 2, 4 and 8 h
10-12: 25 mM D-galactose (gal); 2, 4 and 8 h
13-15: 25 mM L-rhamnose (rham); 2, 4 and 8 h
16-18: 25 mM D-galacturonic acid(gal.a.); 2, 4 and 8 h
19-21: 25 mM D-glucuronic acid (gluc.a.); 2, 4 and 8 h
22-24: 25 mM glc + 25 mM gal.a.; 2, 4 and 8 h
25-27: 25 mM xyl + 25 mM gal.a.; 2, 4, and 8 h
28-30: 25 mM ara + 25 mM gal.a.; 2, 4 and 8 h
31-33: 25 mM rham + 25 mM gal.a.; 2, 4 and 8 h
34-36: 1% polygalacturonate (PG); 2, 8 and 24 h
37-39: 1% PG + 25 mM glc; 2, 8 and 24 h
40-42: 1% sugar beet pectin (SBP); 2, 8 and 24 h
43-45: 1% SBP + 25 mM glc; 2, 8 and 24 h
46-48: 1% SBP + 0.03% ferulic acid; 2, 8 and 24 h

Fig. 1. Schematic drawing of the location of the RNA samples on the dot blot, and RNA loading control. The upper half of each dot blot (samples 1–48) contained samples from N402 and the lower half of the dot blot (samples 49–96) contained samples from the CreA derepressed mutant (NW200). Sample 95 of NW200 did not contain any RNA and thus served as a negative control. The times after transfer at which samples were taken are indicated for each carbon source. The result of probing with a fragment of the 18S rRNA gene [19] was used as an RNA loading control.

Table 1
Pectinolytic genes studied in this paper

Gene	Corresponding enzyme	Probe fragment	CCCTGA box	Reference
abfA	arabinofuranosidase A	2.0 kb *Pst*I	+	[35]
abfB	arabinofuranosidase B	2.8 kb *Pst*I	+	[36]
abnA	endoarabinanase	3.1 kb *Hin*dIII	+	[37]
faeA	feruloyl esterase A	0.5 kb *Eco*RV/*Xho*I	−	[38]
faeB	feruloyl esterase B	0.8 kb PCR fragment	−	[25]
galA	endogalactanase A	0.7 kb PCR fragment	ND	[50]
lacA	β-galactosidase	0.4 kb PCR fragment	+	[15], (R.P.d.V., unpublished data)
pelA	pectine lyase A	1.6 kb *Cla*I	+	[5]
pelB	pectine lyase B	1.1 kb *Sma*I	+	[6]
pelC	pectine lyase C	3 kb *Nsi*I	ND	[39]
pelD	pectine lyase D	0.7 kb *Xho*I	+	[40]
pelF	pectine lyase F	0.9 *Sst*I	ND	acc. nr. AJ489943
pgaA	polygalacturonase A	0.33 kb *Xho*I/*Xba*I	+	[41]
pgaB	polygalacturonase B	0.20 kb *Nsi*I/*Kpn*I	+	[41]
pgaC	polygalacturonase C	0.28 kb *Nsi*I/*Sma*I	+	[42]
pgaD	polygalacturonase D	0.13 kb *Cla*I/*Sst*I	ND	[43]
pgaE	polygalacturonase E	0.28 kb *Nsi*I/*Xho*I	+	[44]
*pga*I	polygalacturonase I	0.28 kb *Nsi*I/*Xba*I	+	[45]
*pga*II	polygalacturonase II	0.38 kb *Nsi*I/*Nco*I	+	[4]
pgaX	exopolygalacturonase	1.7 kb *Pst*I	ND	[46]
plyA	pectate lyase A	0.9 kb *Pst*I/*Xho*I	ND	[47]
pmeA	pectin methyl esterase	0.86 kb *Nsi*I/*Xho*I	ND	[48]
rgaeA	rhamnogalacturonan acetylesterase	0.7 kb *Nsi*I	ND	[34]
rglA	rhamnogalacturonan lyase	1.4 kb *Bam*HI	ND	acc. nr. AJ489944
rhgA	rhamnogalacturonan hydrolase A	1.2 kb *Eco*RV/*Hin*dIII	+	[49]
rhgB	rhamnogalacturonan hydrolase B	0.5 kb *Hin*dIII	ND	[49]

The presence of the CCCTGA sequence in the promoter of the genes was determined and is indicated. ND = not detected, as a result of insufficient promoter sequence available.

e.g. *rgaeA* (Fig. 2B). All the spots of the expression profiles were quantified and used in a cluster analysis to determine subsets of genes with a similar expression profile. A PCA was used for the clustering of the datapoints. The first two PCs (PC#1 and #2) explain 34% and 20% respectively of the variance observed in the expression profiles. By plotting the loading values of PC#1 against PC#2 (and therefore representing 54% of the variance) a clear correlation structure of a subset of genes was observed (Fig. 3A). Subset I consisted of *pga*I, *pga*II, *pgaB*, *pgaC*, *pgaD*, *pgaE*, *pelB*, *pelC*, *pelF*, *plyA* and *rhgA*, all encoding enzymes acting on the pectin main chain. All these genes are expressed in the presence of D-galacturonic acid, polygalacturonate and sugar beet pectin, and in general their expression levels did increase during the course of sampling. During growth, *A. niger* acidifies the media and therefore it can not be concluded at this point whether the increase in expression during the incubation is indeed time-related or related to the lower pH of the medium at a later stage of the incubation. The conditions that were most significant for the correlation between these genes were all obtained from the *creA* mutant and were: 1% polygalacturonate +25 mM glucose after 8 and 24 h (spots 86 and 87), 1% polygalacturonate after 24 h (spot 84), 1% sugar beet pectin +25 mM glucose after 24 h (spot 93), 25 mM xylose +25 mM galacturonic acid after 8 h (spot 75) and 25 mM galacturonic acid after 8 h (spot 66). This can be concluded from the position of these samples in the scores plot, which are positioned in the same direction as the earlier mentioned genes (Fig. 3B). It indicates that by eliminating the effects due to the different levels of CreA repression on the expression of the individual genes the similarity in induction of the gene expression becomes more obvious. Furthermore, expression after longer incubation times is significant for genes from subset I. The *rgaeA* gene is positioned slightly outside of this subset. The expression of this gene is similar to that of the genes of subset I in the presence of sugar beet pectin, polygalacturonate or D-galacturonic acid and *rgaeA* is therefore most likely co-ordinately regulated with these genes. In addition, *rgaeA* expression is also detected in the presence of L-rhamnose (Fig. 2), while the other genes of this subset are

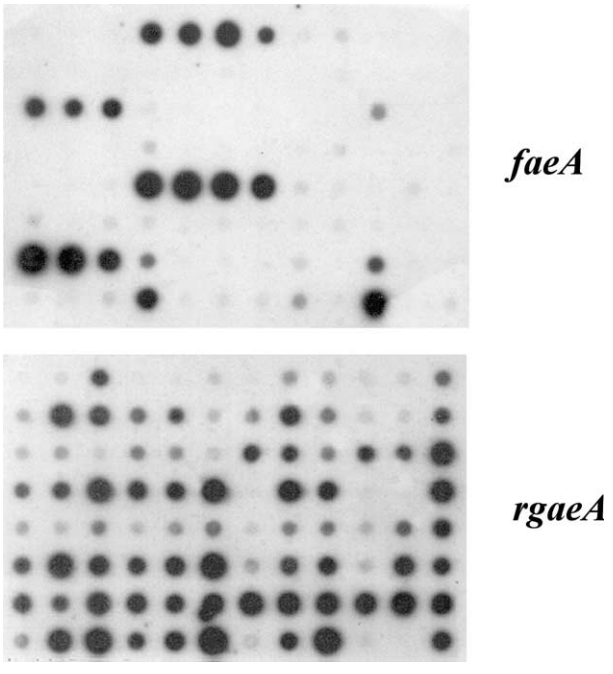


Fig. 2. Expression profiles of *A. niger faeA* and *rgaeA*. These are two representatives of the expression profiles obtained for the individual genes. Profiles varied from relatively simple (*faeA*) to very complex (*rgaeA*).

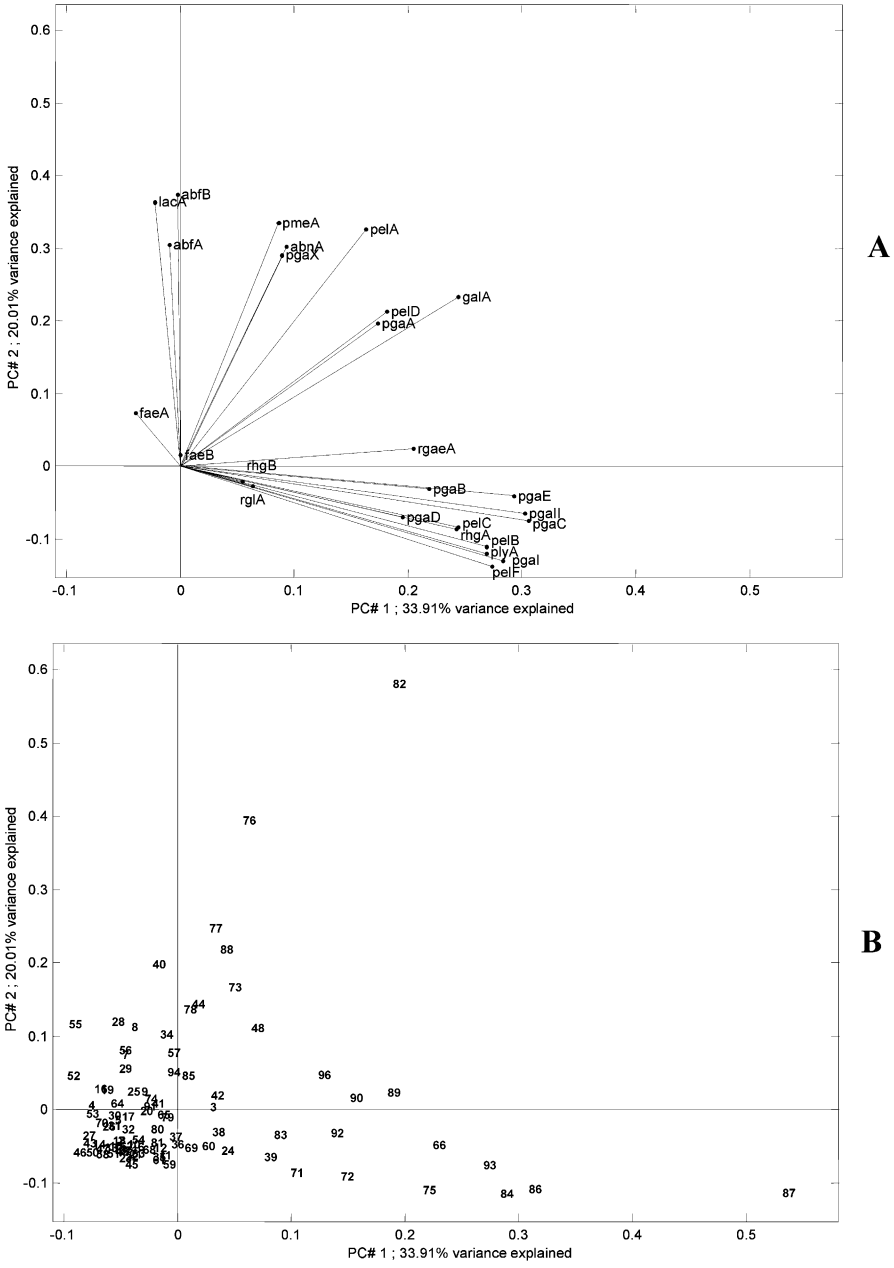


Fig. 3. Identification of a subset of genes involved in pectin degradation using PC values PC#1 and PC#2. This graph represents the first level of clustering of the expression data of the 26 genes tested in this study and is based on 53.92% of the variance. A: Loadings plot for PC#1 versus PC#2; B: scores plot for PC#1 versus PC#2. The numbers correspond to the conditions described in Fig. 1.

not. This suggests that a second positively acting system is involved in the regulation of this gene.

Further trends in gene expression involved in pectin degradation were analysed by plotting PC#2 to PC#3 (which explains an additional 7.7% of the variance). This resulted in four distinct subsets of genes (Fig. 4A), although the correlation between these subsets was weaker than observed for the major group described above. One subset, designated subset II, consists of *rglA* and *rhgB* (Fig. 4A) both encoding enzymes active on the pectin main chain. The conditions with the largest contribution to this group are: 1% sugar beet pectin +25 mM glucose after 8 h in the CreA mutant (spot 92) and 1% polygalacturonate +25 mM glucose after 8 h in the wild type (spot 38) (Fig. 4B).

Subset III contains *pgaA*, *pgaX*, *pelD*, *pelA*, and *pmeA* (Fig. 4A), again all encoding enzymes active on the pectin main chain. The conditions with the largest contribution to this group are: 1% polygalacturonate after 2 h in the CreA mutant (spot 82) and 25 mM arabinose +25 mM galacturonic acid after 2 h in the CreA mutant (spot 76) (Fig. 4B). This indicates that the expression of the genes in subset III is predominantly observed after a short incubation time, which is consistent with the raw data for the expression profiles (data not shown). This suggests that PelA, PelD, PgaA, PgaX and PmeA play a major role in the initial degradation of pectin. PmeA positively affects the activity of PGs in *Aspergillus aculeatus* [25], indicating that early expression of *pmeA* would increase the efficiency of pectin degradation. The early expres-

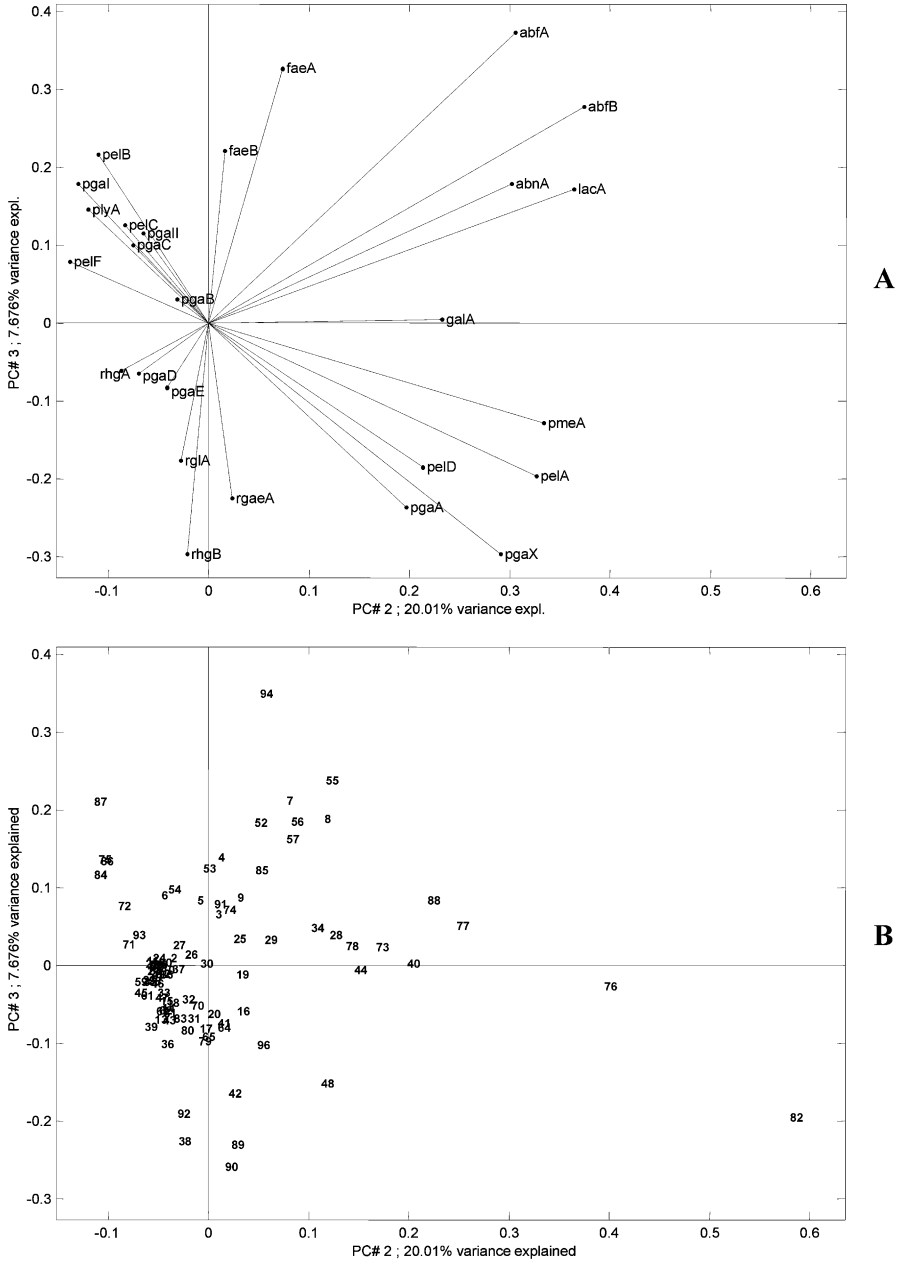


Fig. 4. Identification of additional subsets of genes involved in pectin degradation using PC values PC#2 and PC#3. This graph represents the second level of clustering of the expression data of 14 genes tested in this study and is based 27.7% of the variance. A: Loadings plot for PC#1 versus PC#2; B: scores plot for PC#1 versus PC#2. The numbers correspond to the conditions described in Fig. 1.

sion of *pgaX* is not surprising. The corresponding enzyme (PgxA) is an exopolygalacturonase that generates D-galacturonic acid monomers. Furthermore, the enzyme is able to hydrolyse digalacturonate to D-galacturonic acid. In view of the fact that *A. niger* is not able to import digalacturonate (L. Pařenicová, unpublished data), and the likeliness that D-galacturonic acid, or a metabolite derived from it, is the actual inducer of pectinolytic gene expression, PgxA activity would be essential to efficiently induce the pectinolytic system.

Subset IV consists of *lacA*, *abnA*, *abfA*, and *abfB* (Fig. 4A), all encoding enzymes active on the side chains of pectin. The conditions with the largest contribution to this group are: 25 mM L-arabinose after 2 and 4 h in both the wild type and the CreA mutant (spots 7, 8, 55 and 56), 25 mM L-arabinose +25 mM D-galacturonic acid after 4 h in the CreA mutant (spot 77) and 1% sugar beet pectin after 2 h in the CreA mutant (spot 88) (Fig. 4B). The expression of the arabinanolytic genes (*abfA*, *abfB*, *abnA*) on L-arabinose and L-arabitol was previously shown to be co-ordinately regulated [14], and studies in *Aspergillus nidulans* and *A. niger* have indicated that L-arabitol is the true inducer of this system as well as that of the L-arabinose catabolic pathway [26,27]. The data described in this paper suggest that *lacA* is also regulated by this system.

Subset V consists of *faeA* and *faeB* (Fig. 4A), both encoding enzymes active on the side chains of pectin. These genes respond to the presence of ferulic acid, especially in the CreA mutant (condition 94, Fig. 4B). For *faeA*, expression was also observed in the presence of D-xylose (Fig. 2). This is in accor-

dance with earlier studies. The expression of *faeA* in the presence of D-xylose has been attributed to the xylanolytic transcriptional activator XlnR [28]. The expression in the presence of L-arabinose was observed previously and it was suggested that this was caused by the small amount of D-xylose present in the L-arabinose preparation from Sigma [29]. The data in this paper strengthen this hypothesis as expression on L-arabinose was observed only after 2 h, while after longer transfers (when all D-xylose is consumed) no expression was detected (Fig. 2). The expression of *faeA* and *faeB* in the presence of ferulic acid has been reported before [29,30]. The absence of expression in the presence of ferulic acid after 8 and 24 h suggests that all ferulic acid is metabolised and the inducer is absent. Neither *faeA* nor *faeB* are expressed in response to the presence of D-galacturonic acid.

The only gene that did not fit in any of the above mentioned subsets is *galA*. Expression of this gene was detected in the presence of D-galacturonic acid, polygalacturonate and sugar beet pectin. Previously, expression of *galA* in a CreA derepressed strain was detected in the presence of L-arabinose (R.P.d.V., unpublished results). In this study, this was not confirmed. However, the growth conditions are different in that pre-growth was on D-glucose, rather than on D-fructose. Further study will be needed to determine whether *galA* is co-regulated with the arabinanolytic genes and *lacA* in the presence of L-arabinose.

The data in this paper provide strong indications for a general activating system for pectinolytic gene expression in *A. niger* responding to the presence of D-galacturonic acid or a metabolite derived from it. This system regulates both genes encoding enzymes acting on the main chain of pectin as well as genes encoding accessory enzymes. Regulation of the pectinolytic genes appears to be more complex than the regulatory system for the activation of xylanolytic gene expression in *A. niger* (XlnR) [28,31]. XlnR activates the expression of all xylanolytic genes simultaneously [28], whereas individual pectinolytic genes are expressed at different times and in response to different D-galacturonic acid containing carbon sources. Of the two promoter regions possibly involved in pectinolytic gene activation only the CCCTGA sequence was identified in the promoters of most of the pectinolytic genes that are induced in the presence of D-galacturonic acid (Table 1), whereas it is absent in the promoters of *faeA* and *faeB*. These two genes are not induced in the presence of D-galacturonic acid. For a number of the pectinolytic genes, insufficient promoter sequence is available to determine the presence of the CCCTGA sequence. Further studies are needed before conclusions about the functionality of this region can be made. Site-directed mutagenesis of the box will be the best approach to determine whether it is involved in D-galacturonic acid induction of pectinolytic genes.

Expression of all genes except *pgaX*, *pgaA*, *pgaB*, *pgaD*, and *rglA* were higher in the CreA derepressed strain (data not shown), indicating an important role for this repressor protein in the regulation of pectinolytic gene expression. The variation in the expression profiles of the pectinolytic genes suggests the involvement of factors other than induction in response to D-galacturonic acid and repression via CreA. pH regulation of pectinolytic gene expression has been reported in *Aspergillus kawachii* [32], indicating that pH may effect the expression of pectinolytic genes in *A. niger* as well. Potential pH regulatory sequences, PacC binding sites, have been detected in the promoters of several pectinolytic genes from *A. niger* [13], but their functionality has not been studied.

The regulatory systems responding to the presence of L-arabinose and ferulic acid appear to play a role in regulation of both xylan and pectin degradation. AbfA and AbnA are only active against pectin, whereas AbfB is also active against xylan [2]. The gene encoding arabinoxylan arabinofuranohydrolase (*axhA*), an enzyme only active against xylan, is also expressed in the presence of L-arabinose and L-arabitol [33] and is most likely co-ordinately regulated with *abfA*, *abfB*, and *abnA*. Both feruloyl esterase A and B were reported to be active against xylan and pectin [30,34].

The identification of subsets of pectinolytic genes with similar expression profiles in response to D-galacturonic acid, polygalacturonate and sugar beet pectin provides a good foundation for studying the pectinolytic regulatory system in more detail by comparing the promoters of these genes and isolating regulatory mutants with altered expression for co-ordinately expressed genes.

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