The effects of extracellular pH and of the transcriptional regulator PACI on the transcriptome of *Trichoderma reesei*

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**Abstract**

**Background:** Extracellular pH is one of the several environmental factors affecting protein production by filamentous fungi. Regulatory mechanisms ensure that extracellular enzymes are produced under pH-conditions in which the enzymes are active. In filamentous fungi, the transcriptional regulation in different ambient pH has been studied especially in *Aspergillus*, whereas the effects of pH in the industrial producer of hydrolytic enzymes, *Trichoderma reesei*, have mainly been studied at the protein level. In this study, the pH-dependent expression of *T. reesei* genes was investigated by genome-wide transcriptional profiling and by analysing the effects of deletion of the gene encoding the transcriptional regulator *pac1*, the orthologue of *Aspergillus nidulans pacC* gene.

**Results:** Transcriptional analysis revealed the pH-responsive genes of *T. reesei*, and functional classification of the genes identified the activities most affected by changing pH. A large number of genes encoding especially transporters, signalling-related proteins, extracellular enzymes and proteins involved in different metabolism-related functions were found to be pH-responsive. Several cellulase- and hemicellulase-encoding genes were found among the pH-responsive genes. Especially, genes encoding hemicellulases with the similar type of activity were shown to include both genes up-regulated at low pH and genes up-regulated at high pH. However, relatively few of the cellulase- and hemicellulase-encoding genes showed direct PACI-mediated regulation, indicating the importance of other regulatory mechanisms affecting expression in different pH conditions. New information was gained on the effects of pH on the genes involved in ambient pH-sensing and on the known and candidate regulatory genes involved in regulation of cellulase and hemicellulase encoding genes. In addition, co-regulated genomic clusters responding to change of ambient pH were identified.

**Conclusions:** Ambient pH was shown to be an important determinant of *T. reesei* gene expression. The pH-responsive genes, including those affected by the regulator of ambient pH sensing, were identified, and novel information on the activity of genes encoding carbohydrate active enzymes at different pH was gained.

**Keywords:** *Trichoderma reesei*, Ambient pH, *pac1*, Gene regulation, Gene expression, Carbohydrate active enzymes, Cellulase, Hemicellulase, Transcriptional profiling

**Background**

Filamentous fungi can adjust to changing ambient pH of their habitat by an intracellular pH homeostatic system and by controlling the synthesis of gene products directly exposed to the surrounding environment (e.g. cell surface proteins, secreted proteins). Ambient pH regulation of filamentous fungi has been most extensively studied in *Aspergillus nidulans* and has been shown to be mediated by the wide-domain zinc finger transcription factor PacC together with a signal transduction cascade composed of the products of six pal genes (*palA, palB, palC, palF, palH* and *pall*) (reviewed by [1]).

Ambient pH is signalled through a plasma membrane complex [2-4]. The components of the pal pathway and of an ESCRT complex are subsequently recruited to the plasma membrane-associated structure. The proposed model for pH signalling can be found from [5]. There are three different forms of PacC in *A. nidulans*. In acidic
conditions the full-length form predominates. At alkaline pH, activation of the pal pathway leads to two subsequent cleavage steps resulting in two shorter forms of PacC [6]. The product of the PalB-mediated cleavage step, PacC53, is further cleaved by the proteasome to create the active form of PacC (PacC27) [7]. The active PacC binds to the core target sequence 5′-GCCARG-3′ in the promoters of its target genes [8].

PacC of A. nidulans acts as a repressor of acid-expressed genes and as an activator of alkaline-expressed genes [9]. In accordance, null mutations in pacC or in the pal genes [10,11] lead to a phenotype mimicking that displayed in acidic conditions, whereas a constitutively active PacC results in activation of alkaline-expressed genes and repression of acid-expressed genes regardless of pH [9,10]. PacC controls e.g. genes encoding acid and alkaline phosphatases [10], permeases [12] xylanases (xlnA and xlnB) [13] and an α-L-arabinofuranosidase (abfB) [14]. PacC has also been shown to regulate the expression of the pacC gene itself, resulting in an abundance of pacC mRNA at alkaline pH [9]. In a recent study, a negative feedback loop attenuating the first cleavage step of PacC during a longer exposure to alkaline conditions was proposed to exist [15].

Trichoderma reesei is an industrial producer of especially cellulases and hemicellulases for various applications and is also a widely used host for the production of heterologous proteins [16-18]. In order to create better production strains with enhanced enzyme production properties, the physiological and environmental factors and regulatory mechanisms affecting enzyme production by T. reesei have been widely studied. Several transcription factors regulating the expression of cellulase and hemicellulase genes have been identified and characterized in detail (for reviews see [18,19]). The availability of a complete genome sequence of T. reesei has made it possible to use genome-wide methods to study the various factors affecting protein production [20]. However, the effect of ambient pH on the production of cellulases and hemicellulases has received less attention, although previous studies indicate efficient production of xylenases at high pH and cellulases at low pH [21]. Recently, endoglucanase production by T. reesei was suggested to be highest at pH4.5, whereas exoglucanase and β-glucanase production reached their highest values at pH5 and 5.5, respectively [22]. However, another recent study suggests that there are differences in the optimal pH for cellulase and hemicellulase secretion between different T. reesei strains [23].

In this study, effects of the changing ambient pH on the transcriptome of Trichoderma reesei were studied by transcriptional profiling. The pH-responsive genes were screened from the genome using the transcriptome data and classified in order to identify the major gene groups affected by the extracellular pH. In addition, the effects of the regulator PACI were studied by constructing a deletion strain. The effects of the changing pH conditions on the members of specific gene groups were investigated in more detail. These genes included the ones encoding carbohydrate active enzymes, genes encoding the components of the pH signal transduction pathway and known and candidate regulatory genes of cellulase- and hemicellulase-encoding genes. The transcriptome data also enabled identification of co-regulated genomic clusters containing especially secondary metabolism genes.

Results

pH responsive genes of T. reesei according to microarray analysis

A recombinant strain from which the pacI open reading frame had been replaced by a hygromycin resistance cassette was constructed. This pacI deletion strain (designated as ΔpacI) was cultivated in a bioreactor on a medium containing Avicel cellulose at pH6 in parallel with the parental strain QM9414. In addition, the parental strain QM9414 was cultivated in a bioreactor on the same medium in different pH conditions (pH3, pH4.5 and pH6). Three biological replicates of the strain QM9414 and ΔpacI were cultivated in each case, and samples collected from two different time points of each culture were subjected to transcriptional analysis using the microarray method. The microarray platform used in this study includes probes for the transcripts of approximately 10 000 genes, including genes from the genome versions 2.0 and 1.2 [24,25], and for putative novel genes detected in a previous study [26]. Statistical analysis of the differentially expressed genes at different pH and in comparison of the cultures of the ΔpacI strain and its parental strain was carried out using Limma (R, bioconductor [27,28]) with a fold change log2 > 0.4 and a p-value < 0.01 as a threshold.

The statistical analysis revealed that in the strain QM9414 the expression of 940 genes (~9% of the transcripts in the array) responded significantly to the change of pH in pair-wise comparisons between pH6 and pH3 and/or pH6 and pH4.5 and/or pH4.5 and pH3 at both the time points analysed (for details, see Additional file 1). 346 genes were up-regulated at high pH (expression increased significantly in a comparison between a higher pH and a lower pH) and 584 were up-regulated at low pH (expression decreased significantly in a comparison between a higher pH and a lower pH) (Figure 1A). In addition, eight genes had a positive response to the change of pH from 3 to 4.5 but a negative response to the change of pH from 4.5 to 6, and two genes showed the opposite expression trend having the lowest signal at pH 4.5.

In total 189 genes (~2% of the transcripts in the array) were differentially expressed in the cultures of the ΔpacI strain as compared to the parallel cultures of the parental strain at pH6 (Additional file 2). Of these genes 77 were up-regulated and 112 down-regulated in the deletion
Figure 1 (See legend on next page.)
strain as compared to the parental strain. Distribution of genes showing differential expression either at different pH and/or in the Δpac1 strain as compared to its parental strain is shown in the Figure 1A. The gene set was explored for the ones most likely to be under direct PACI regulation using the Limma analysis of differential expression and based on the knowledge in PacC-mediated regulation in Aspergillus. The PacC transcription factor of A. nidulans is activated at alkaline pH. The active regulator represses the transcription of acid-expressed genes and activates the transcription of alkaline-expressed genes [9]. Deletion of the pacC gene leads to a phenotype mimicking acidic conditions. Therefore, the hypothesis was that the T. reesei genes directly repressed by PACI are only active at acidic pH and thus are more highly expressed in the parental strain at pH3 as compared to pH6. In the pac1 deletion strain, gene expression mimics that of the parental strain in acidic conditions and therefore the expression of the PACI repressed genes is higher as compared to the parental strain when grown at pH6. Similarly, the T. reesei genes induced by PACI are active at alkaline pH and thus are more highly expressed at pH6 as compared to pH3 in the parental strain. In the deletion strain, expression of the PACI-induced genes is lower as compared to the parental strain grown at pH6. According to these criteria, 30 and 38 genes were found to be repressed and induced by PACI, respectively (for details, see Additional file 2). When the expression data was clustered using the Mfuzz clustering method [29], the majority of the genes activated by PACI were assigned to a common Mfuzz-cluster whereas the PACI repressed genes were more evenly divided to several clusters.

The binding site of T. reesei PACI in the promoters of its target genes has not yet been characterised. However, the binding sites of the orthologues in A. nidulans and in the yeast Saccharomyces cerevisiae have been identified, and the core binding sites found to be very similar, GCCARG [8], suggesting conservation of the motif in evolution. The presence of the binding motif GCCARG in the promoters of T. reesei was tested. DNA sequences 1500 bp upstream from the gene start site (the start sites as in [24]) were analysed for the presence of the motif. The motif was significantly enriched (p < 0.01) in the set of genes that are likely to be under PACI regulation. 78% of the genes (53 out of 68 genes) harboured the motif. Especially, the motif was enriched in the set genes that are candidates for PACI-induced genes, 87% of the genes containing the motif (33 out of 38 genes, p < 0.0025). However, the motif is frequent in the T. reesei genome. 65% of the genes harbour the site in their promoters.

Classification of the pH-responsive genes

The pH-responsive genes were divided into different functional classes according to the Eukaryotic orthologous groups (KOG) classification (Figure 1B). The genes encoding glycoside hydrolases, carbohydrate esterases and polysaccharide lyases were classified according to the updated annotations [30]. A substantial proportion of the pH-responsive genes was classified as having a general function prediction only, an unknown function or did not have a KOG or CAZy (carbohydrate-active enzymes, http://www.cazy.org/) classification. A putative function could be assigned to a number of these genes according to the manual annotations in the Joint Genome Institute (JGI) T. reesei v2.0 database [24] or according to functional InterPro domains. Classification of the pH responsive genes is presented in Additional file 1. Analysis of the differentially expressed genes in the Δpac1 strain as compared to the parental strains revealed genes essentially from the same functional groups as the pH-responsive genes (Figure 2A). However, the proportion of candidate PACI-regulated genes varies within the functional classes. The functional groups “Inorganic ion transport and metabolism”, “Post-translational modification, protein turnover, chaperones” and “Amino acid transport and metabolism” contain the highest percentage of candidate PACI-regulated genes (28%, 18%, and 17% of the pH-responsive genes in the class, respectively), whereas the glycoside hydrolase class, which is the most abundant pH-responsive functional class, contains only 4% of candidate PACI-regulated genes. Classification of the genes affected by the pac1 deletion and the candidate target genes for PACI-mediated regulation is presented in Figure 2A and Figure 2B, respectively (for detailed information, see Additional file 2).

The effect of pH on the components of the pH-signalling pathway

The pH-signalling pathway leading to the activation of PacC in A. nidulans consists of the six pal genes (palA, palB, palC, palF, palH and palI) together with the components of the ESCRT-complex (for example Vps20, Vps23, Vps24 and Vps32). The T. reesei homologues of the A. nidulans pal-genes and vps-genes were searched from the
genome and the behaviour of these genes in the transcriptome data on different pH conditions was studied. Comparison of the three pH conditions showed a significant increase of \( \text{pac1} \) expression with increasing pH (using a fold change log2 > 0.4 and a p-value < 0.01 as a threshold) and a decrease in the expression of \( \text{palF} \) (gene ID 56605) with increasing pH (p-value < 0.05 as a threshold) (Figure 3A). The analysis did not detect significant changes in expression of the other \( \text{pal} \) or \( \text{vps} \) homologues, suggesting a rather steady expression of these genes regardless of pH in \( T. \) reesei.

In the \( \Delta \text{pac1} \) strain, the expression of the \( \text{palC} \) homologue (gene ID 80523) was significantly lower (p-value < 0.01) as compared to the expression of the gene in the parental strain QM9414 (Figure 3B). The result suggests that the \( \text{palC} \) homologue could be, at least in part, under positive PACI-mediated regulation. Furthermore, both \( \text{pac1} \) and \( \text{palC} \) have multiple consensus motifs (GCCARG) in their promoter for putative PACI binding. The expression of \( \text{palF} \) showed the opposite trend by being expressed at a slightly higher level in the \( \Delta \text{pac1} \) strain as compared to QM9414 (Figure 3B). However, the difference in the \( \text{palF} \) expression between the strains was not statistically significant.

The effect of pH on genes encoding carbohydrate active enzymes and on the regulators of cellulase- and hemicellulase-encoding genes

Due to the fact that \( T. \) reesei is an exceptionally efficient producer of cellulases and hemicellulases, the effect of pH on the expression of genes encoding carbohydrate active enzymes (CAZy, [31,32]) was studied in more detail (microarray data on the genes encoding carbohydrate active enzymes is presented in Additional file 3). The majority of the enzymes involved in the degradation of plant cell wall material belong to the classes of glycoside hydrolases (GH), carbohydrate esterases (CE) and polysaccharide lyases (PL). Of the pH-responsive genes, 50 are classified as genes encoding glycoside hydrolases, 10 encoding carbohydrate esterases and one encoding a polysaccharide lyase (Figure 4). Of these genes, 23 and 38 were determined to be up-regulated at high pH and at low pH, respectively.

Genes encoding members of the CAZy families GH16, GH18, GH27, and GH55 were especially abundant among the pH-responsive genes, but also a group of genes encoding CAZy enzymes with activities against cellulose or hemicellulose polymers were found among the pH-responsive genes. Especially, many of the genes for xylanolytic enzymes were differentially expressed at different pH. In addition, differential expression within a CAZy family or within a group of genes encoding
enzymes with a similar type of activity was detected. Especially, the multiple xylanases and acetylxylan esterases, as well as CAZy families GH3, GH16, GH18 and GH55, include both members that are up-regulated at low or high pH.

Expression of the genes encoding the major cellulases in *T. reesei* (*cbh1, cbh2, egl1* and *egl2*), was not significantly affected under the different pH conditions studied, but endoglucanases of families GH5 and GH12 were among the differentially expressed ones. For details of the CAZy family members encoded by the differentially expressed genes, see Table 1.

Expression of relatively few of the genes encoding CAZy enzymes was affected in the Δpac1 strain (Table 2). Seven glycoside hydrolase genes and one carbohydrate esterase gene were down-regulated in the Δpac1 strain, and one glycoside hydrolase gene was up-regulated. Only two of the glycoside hydrolase genes and one carbohydrate esterase gene had an expression pattern that suggested PACI-mediated regulation. These include genes encoding a candidate β-glucosidase (*bgli3i*), a candidate GH76 α-1,6-mannanase (gene ID 122495) and a candidate CE9 N-acetyl-glucosamine-6-phosphate deacetylase (gene ID 79671). No GH or CE genes under negative PACI regulation were detected with the statistical selection criteria used. However, clustering of the expression data shows an expression pattern expected for PACI-regulated gene for a set of CAZy genes. The vast majority of the PACI-repressed pH-responsive genes were assigned to three different Mfuzz clusters, and PACI-induced genes to one cluster (Additional file 2). In total 23 CAZy genes were found in these clusters (Table 3).

The expression patterns of the genes encoding for the candidate or characterized (*xyr1, ace1, ace2 and cre1*) regulators of cellulase and hemicellulase genes were also studied and compared to those of the cellulase- and hemicellulase-encoding genes. The characterized regulators were not pH-regulated according to the Limma test but some of the novel candidate regulatory genes identified during a previous study [33] (genes 111742 (pMH14), 120120 (pMH22) and 123019 (pMH30)) were up-regulated at low pH (Additional file 1). A heatmap representation from the fold changes of the expression levels of the genes encoding cellulolytic and hemicellulolytic enzymes and of the characterized or candidate regulatory genes for these enzyme genes is shown in Figure 5. Genes encoding the components of the pH-signalling pathway were also included. Genes forming the heat map are listed in Additional file 4. The candidate regulatory genes 111742 (pMH14), 120120 (pMH22), 123019 (pMH30) and 122523 (pMH29) [33] group together with the genes up-regulated at low pH. Interestingly, the *ace3* gene encoding a novel regulator of especially cellulase genes [33] cluster together with *cel3c*, a candidate β-xylosidase/α-L-arabinofuranosidase...
| CAZy family | Up-regulated genes at high pH | Up-regulated genes at low pH |
|-------------|-------------------------------|-----------------------------|
| AA9         | 22129 Polysaccharide monoxygenase, 31447 Polysaccharide monoxygenase | 107850 Candidate esterase, 107268 Candidate S-formylglutathione hydrolase |
| CE1         | 123940 cip2 Glucuronoyl esterase | 103825 Candidate acetyl esterase |
| CE15        | 44366 Candidate esterase, 70021 Candidate acetyl xylan esterase | 44214 axe2 Candidate acetyl xylan esterase, 60489 Candidate cutinase |
| CE9         | 79671 N-acetyl-glucosamine-6-phosphate deacetylase | |
| GH3         | 47268 bgλi3 Candidate beta-glucosidase | 104797 bgλj3 Candidate beta-glucosidase, 58450 xyλ6 Candidate beta-xylanase |
| GH5         | 71554 Beta-1,3-mannanase/endo-beta-1,4-mannosidase, 82616 celλ5b Candidate membrane bound endoglucanase | |
| GH10        | 120229 xyn3 Endo-beta-1,4-xylanase | |
| GH11        | 123818 xyn2 Endo-beta-1,4-xylanase | 74223 xyn1 Endo-beta-1,4-xylanase, 112392 xyn5 Candidate endo-beta-1,4-xylanase |
| GH30        | 69276 Candidate endo-beta-1,4-xylanase | 3094 Candidate glucan endo 1,6-beta-glucosidase |
| GH12        | 123232 egl3/cel12a Endo-beta-1,4-glucanase | 65333 Alpha-glucosidase (Glucoamylase and related) |
| GH15        | 65406 Candidate cell wall glucosyltransferase, 55886 Candidate glucan endo-1,3(4)-beta-D-glucosidase | |
| GH16        | 43873 chiλ8-12 Candidate chitinase, 59791 chiλ8-15 Candidate chitinase, 65162 endoT Endo-N-acetyl-beta-D-glucosaminidase | 39942 Candidate glucan endo-1,3-beta-glucosidase |
| GH17        | 102909 Candidate GH2 | 110317 chiλ8-17 Candidate chitinase |
| GH20        | 105931 Candidate N-acetyl-beta-hexosaminidase | |
| GH27        | 72632 agλ1 alpha-galactosidase, 72704 agλ3 alpha-galactosidase, 27219 Candidate alpha-galactosidase, 27259 Candidate alpha-galactosidase | |
| GH28        | 64827 Candidate raffinose synthase domain protein | 70186 Polygalacturonase/xylagalacturonan hydrolase |
| GH36        | 79960 Candidate alpha-1,2-mannosidase | |
| GH47        | 70845 Candidate beta-1,3-glucanase | 56418 Candidate beta-1 3-glucanase, 54242 Candidate beta-1,3-glucanase, 73248 Candidate exo-1,3-beta-glucanase |
| GH55        | 124175 Candidate endo-1,3-beta-glucanase | |
| GH64        | 123538 Candidate beta-1 3-glucanosyltransferase | 82633 Candidate beta-1 3-glucanosyltransferase |
gene (3739), egl2, cbi12, swo1, egl4 and two candidate regulatory genes (121121 and 56077), indicating similar expression of these genes under the prevailing conditions.

**Co-regulated genomic regions**

Mfuzz clustering of the expression profiles was performed in order to study the expression patterns of the genes in more detail and to identify co-regulated groups of genes. Co-regulated genes were also searched for in a genomic scale, identifying genes located close to each other in the scaffolds and being co-regulated. Co-expressed genomic regions were found by searching the genome for neighbouring genes that were assigned to the same Mfuzz cluster (at least 3 genes from 4 consecutive genes belonging to the same Mfuzz cluster) and of which at least one was pH-responsive according to the Limma analysis (in the strain QM9414 and/or in the deletion strain). In total 40 such clusters were found. The pH-responsive members of the clusters are marked in the Additional files 1 and 2.

Genomic clusters of co-regulated pH-responsive genes most probably involved in siderophore biosynthesis were identified. There is a tightly co-regulated genomic region in scaffold 46 containing six genes up-regulated at high pH. The genes are not under PACI regulation according to the criteria used. According to manually curated annotations, these genes putatively encode a non-ribosomal peptide synthase, a siderophore biosynthesis lipase/esterase, an ABC transporter, a siderophore biosynthesis acetylase, an enoyl-CoA hydratase/isomerase and a siderophore iron transporter. A very similar co-regulated cluster of six genes that are up-regulated at high pH and which is located in scaffold 20 includes genes annotated to encode an ABC transporter, an oxidoreductase, an MFS transporter, a siderophore biosynthesis acetylase, a long chain fatty acid acyl-CoA ligase and a non-ribosomal peptide synthase. Genes 67026 and 67189 belonging to this cluster are not pH-responsive according to the Limma analysis, although the *T. virens* homologues for these genes have been shown to be up-regulated at alkaline pH [34].

### Table 1 pH-responsive genes encoding CAZy family enzymes (glycoside hydrolases, carbohydrate esterases, polysaccharide lyases, and auxiliary activities) (Continued)

| Gene ID | Name | CAZy family | CAZy annotation | Expression at pH6 vs. pH3 | Expression in the strain Δpac1 vs. QM9414 at pH6 |
|---------|------|-------------|----------------|--------------------------|--------------------------------------------------|
| 47268   | bgl3i| GH3         | Candidate beta-glucosidase | pH6 > pH3               | QM9414 > ΔpacC                                    |
| 122495  | GH76 | Candidate alpha-1,6-mannanase | pH6 > pH3               | QM9414 > ΔpacC                                    |
| 79671   | CE9  | N-acetyl-glucosamine-6-phosphate deacetylase | pH6 > pH3               | QM9414 > ΔpacC                                    |
| 76227   | cel3e| GH3         | Candidate beta-glucosidase | QM9414 > ΔpacC                                    |
| 82235   | GH31 | Candidate alpha-glucosidase | QM9414 > ΔpacC                                    |
| 123226  | GH37 | Candidate alpha,alpha-trehalase | QM9414 > ΔpacC                                    |
| 71532   | GH71 | Candidate alpha-1,3-glucanase | QM9414 > ΔpacC                                    |
| 53542   | GH76 | Candidate alpha-1,6-mannanase | QM9414 > ΔpacC                                    |
| 69123   | GH76 | Candidate alpha-1,6-mannanase | ΔpacC > QM9414                                      |

Genes showing differential expression in comparison of different pH conditions (significant difference in expression, with p value < 0.01 and fold change log2 > 0.4, detected at both time points of the triplicate cultures). Gene ID number (JGI, http://genome.jgi-psf.org/Trire2/Trire2.home.html), common name of the gene and the CAZy family and activity of the encoded enzyme are shown.

### Table 2 Differential expression of genes encoding CAZy family enzymes (glycoside hydrolases, carbohydrate esterases, polysaccharide lyases, and auxiliary activities) in the Δpac1 strain

| Gene ID | Name | CAZy family | CAZy annotation | Expression at pH6 vs. pH3 | Expression in the strain Δpac1 vs. QM9414 at pH6 |
|---------|------|-------------|----------------|--------------------------|--------------------------------------------------|
| 47268   | bgl3i| GH3         | Candidate beta-glucosidase | pH6 > pH3               | QM9414 > ΔpacC                                    |
| 122495  | GH76 | Candidate alpha-1,6-mannanase | pH6 > pH3               | QM9414 > ΔpacC                                    |
| 79671   | CE9  | N-acetyl-glucosamine-6-phosphate deacetylase | pH6 > pH3               | QM9414 > ΔpacC                                    |
| 76227   | cel3e| GH3         | Candidate beta-glucosidase | QM9414 > ΔpacC                                    |
| 82235   | GH31 | Candidate alpha-glucosidase | QM9414 > ΔpacC                                    |
| 123226  | GH37 | Candidate alpha,alpha-trehalase | QM9414 > ΔpacC                                    |
| 71532   | GH71 | Candidate alpha-1,3-glucanase | QM9414 > ΔpacC                                    |
| 53542   | GH76 | Candidate alpha-1,6-mannanase | QM9414 > ΔpacC                                    |
| 69123   | GH76 | Candidate alpha-1,6-mannanase | ΔpacC > QM9414                                      |

Genes showing differential expression in comparison of the strain Δpac1 and QM9414 (significant difference in expression, with p value < 0.01 and fold change log2 > 0.4, detected at both time points of the triplicate cultures). For comparison the differential expression of the genes at different pH in the parental strain QM9414 is shown. Gene ID number (JGI, http://genome.jgi-psf.org/Trire2/Trire2.home.html), common name and the CAZy family and activity of enzyme encoded by the gene are included in the Table.
An additional gene cluster most probably involved in siderophore biosynthesis but not passing the criteria set for the co-regulated genomic regions is located in scaffold 31. The cluster includes a gene homologous to the \textit{T. virens} siderophore biosynthesis gene \textit{sidA} which is a gene expressed at high pH, as is the \textit{T. virens} counterpart. Similarly, the homologue of \textit{T. virens} non-ribosomal peptide synthase gene (\textit{tex10}) is clustered with the siderophore biosynthesis gene \textit{but is not co-regulated with it (and therefore is not assigned to the same Mfuzz-cluster).}

Three clusters possibly involved in cellulase signalling were found. In scaffold 17, a cluster of three genes that are up-regulated at high pH, included the \textit{oocl} gene encoding a secreted protein suggested to be involved in

### Table 3 Clustering of genes encoding CAZy family enzymes (glycoside hydrolases, carbohydrate esterases, polysaccharide lyases, and auxiliary activities) based on the expression array data

| Gene ID | Name  | CAZy family | CAZy annotation                                                                 | Co-clustering with candidate PACI induced genes | Co-clustering with cand. PACI repressed genes |
|---------|-------|-------------|--------------------------------------------------------------------------------|-----------------------------------------------|---------------------------------------------|
| 72072   | CE1   | Candidate esterase | Cluster 49                                                                 |                                               |                                             |
| 44366   | CE3   | Candidate esterase | Cluster 49                                                                 |                                               |                                             |
| 70671   | CE9   | N-acetyl-glucosamine-6-phosphate deacetylase | Cluster 49                                                                 |                                               |                                             |
| 108348  | GH    | Unknown     | Cluster 49                                                                 |                                               |                                             |
| 123818  | xyn2  | Endo-beta-1,4-xylanase | Cluster 49                                                                 |                                               |                                             |
| 65406   | GH16  | Candidate cell wall glucanoyltransferase | Cluster 49                                                                 |                                               |                                             |
| 109278  | GH24  | Candidate lysozyme | Cluster 49                                                                 |                                               |                                             |
| 47268   | bg13  | Candidate beta-glucosidase | Cluster 49                                                                 |                                               |                                             |
| 77942   | GH72  | Candidate beta-1 3-glucanoyltransferase | Cluster 49                                                                 |                                               |                                             |
| 122495  | GH76  | Candidate alpha-1,6-mannanase | Cluster 49                                                                 |                                               |                                             |
| 79921   | GH92  | Candidate alpha-1,2-mannosidase | Cluster 49                                                                 |                                               |                                             |
| 103825  | CE16  | Candidate acetyl esterase | Cluster 25                                                                 |                                               |                                             |
| 41248   | CE3   | Candidate acetyl xylan esterase | Cluster 25                                                                 |                                               |                                             |
| 65215   | CE4   | Candidate imidase | Cluster 25                                                                 |                                               |                                             |
| 4221    | GH105 | Candidate rhamnogalacturonyl hydrolase | Cluster 25                                                                 |                                               |                                             |
| 73101   | GH16  | Candidate glucan endo-1,3-1,4-beta-D-glucosidase | Cluster 25                                                                 |                                               |                                             |
| 70542   | GH16  | Candidate b-glycosidase (endo-beta-1,3(4)-beta-D-glucanase) | Cluster 20                                                                 |                                               |                                             |
| 110317  | ch18-17 | Candidate chitinase | Cluster 25                                                                 |                                               |                                             |
| 76852   | GH2   | Candidate beta-galactosidase/beta-glucuronidase | Cluster 20                                                                 |                                               |                                             |
| 72632   | agl1  | GH27 | alpha-galactosidase | Cluster 15                                                                 |                                               |                                             |
| 27219   | GH27  | Candidate alpha-galactosidase | Cluster 15                                                                 |                                               |                                             |
| 27259   | GH27  | Candidate alpha-galactosidase | Cluster 15                                                                 |                                               |                                             |
| 70186   | GH28  | Candidate polygalacturonase/xylagalacturonan hydrolase | Cluster 15                                                                 |                                               |                                             |
| 58450   | xyl3b | GH3 | Candidate beta-xilosidase | Cluster 15                                                                 |                                               |                                             |
| 69736   | GH30  | Candidate glucan endo 1,6-beta-glucanase | Cluster 25                                                                 |                                               |                                             |
| 80240   | bga1  | GH35 | Ceta-galactosidase | Cluster 20                                                                 |                                               |                                             |
| 73102   | GH39  | Candidate beta-xilosidase | Cluster 15                                                                 |                                               |                                             |
| 73248   | GH55  | Candidate exo-1,3-beta-glucanase | Cluster 15                                                                 |                                               |                                             |
| 69123   | GH76  | Candidate alpha-1,6-mannanase | Cluster 25                                                                 |                                               |                                             |
| 71394   | GH79  | Candidate beta-glucuronidase | Cluster 25                                                                 |                                               |                                             |
| 74198   | GH92  | Candidate alpha-1,2-mannosidase | Cluster 25                                                                 |                                               |                                             |
| 72488   | GH95  | Candidate alpha-L-fucosidase | Cluster 15                                                                 |                                               |                                             |
| 105288  | GHNA  | Unknown | Cluster 15                                                                 |                                               |                                             |
| 69189   | PL20  | Candidate endo-beta-1,4-glucuronan lyase | Cluster 25                                                                 |                                               |                                             |

Mfuzz was used for clustering of the microarray expression data, and genes that encode CAZy enzymes and co-cluster with the candidate PACI-regulated genes are listed.
cellulase signal transduction [35] together with a gene homologous to ooc1 and a gene of unknown function. ooc1 encodes a small acidic protein the expression of which is abundant on cellulose in strain QM9414 [35]. A cluster of three genes from scaffold 13 includes a major facilitator superfamily transporter gene 79202 possibly involved in cellulase signal transduction [36]. The two other genes belonging to the cluster encode proteins of unknown function. Only one of the unknown genes is pH-responsive (up-regulated at high pH), whereas expression of the other genes is not significantly affected by pH. These three genes belong to the same Mfuzz cluster as the majority of the genes induced by PAC1.

Another cluster of genes up-regulated at low pH in scaffold 29 includes a gene encoding GRDI that is a putative dehydrogenase associated with cellulase signal transduction [37]. The other members of the cluster include four genes with unknown functions and a fungal transcriptional regulatory protein (pMH14 [33]).

**Discussion**

The transcriptome of _T. reesei_ at different extracellular pH values was studied in order to elucidate the role of pH regulation in a saprotrophic cellulolytic fungus. Ambient pH was found to be an important determinant of _T. reesei_ gene expression. The gene groups abundant among the pH-responsive genes included e.g. genes encoding transporters, exported enzymes, signalling-related functions, regulatory proteins, and proteins involved in various metabolic reactions. Especially, the abundance of genes encoding the extracellular glycoside hydrolases among the pH-responsive genes indicates that adaptation to changing ambient pH conditions is an important determinant of the success of a saprotrophic fungus.
The regulatory mechanisms induced by ambient pH were also subjected to the study by deletion of the regulatory gene pac1, and analysis of the expression level of the genes encoding the components in the pH signalling pathway. In T. reesei, the expression patterns of the pac1 deletion strain and comparison of the different pH conditions suggested a possible role for PACI in their regulation pH-dependent regulation of pal genes have been studied in the fungus *Magnaporthe oryzae* [38]. The only pal gene influenced by pH and by the deletion of the pacC paralogue was MOPALF (a homologue of palF). The expression of the MOPALF gene decreased significantly with increasing pH from 5 to 8. Deletion of MOPACC (a homologue of pac1) indicated MOPACC-mediated repression of MOPALF. In previous studies, it was concluded that expression of the genes of the Pal-signalling pathway is not regulated by ambient pH in the fungi *A. nidulans* and *A. niger* [39,40]. These contradictory results suggest that either these fungi use partially different mechanisms for the regulation of pH signalling or is difficult to detect using traditional significance tests.

The expression patterns of the genes encoding enzymes involved in the degradation of cellulose and hemicellulose polymers were studied in more detail. Especially, the genes encoding hemicellulases appeared to be differentially regulated depending on the ambient pH. As expected for genes that encode for enzymes with related activities but functioning at different pH, the gene set contained both genes that were up-regulated at low pH and genes that were up-regulated at high pH. The xylanase genes of *T. reesei* are a good example of this. The genome of *T. reesei* encodes four characterized and two candidate xylanases involved in the degradation of the xylan chain of hemicellulose. The *xyn2*, *xyn3* and a candidate GH30 xylanase gene (69276) are abundantly expressed at high pH, whereas *xyn1* and *xyn5* are more highly expressed at a lower pH. In addition, PACI was suggested to have a slight inducing effect on *xyn2* according to the Mfuzz clustering, and a putative PACI binding site was found on the promoter of *xyn2*. The pH optima for the characterized *T. reesei* xylanases have been determined and the data support the results of the transcriptional analysis. XYNIII has a rather high pH optimum (6–6.5), whereas the pH optima of XYNI and XYNII are 4.0–4.5 and 4.0–6.0, respectively [41–44]. In lactose medium, XYNI and XYNII are preferentially produced at pH4 and pH6, respectively [45], and XYNII is produced under both pH conditions.

Although a variety of CAZy genes respond to change of pH, only a few are clearly under PACI regulation. It is also possible that other regulatory mechanisms have a stronger effect and mask the effect of PACI-regulation. For example, the promoter of the *Aspergillus tubingensis* xylanase gene *xlnA* contains overlapping binding sites for the activator XlnR and PacC, suggesting that PacC could be competing with the activator [46]. Several other activators and repressors besides XYRI [47] are involved in the regulation of cellulase and hemicellulase genes of *T. reesei*. These include for example ACEI, ACEII and CREI [48–51]. Of the novel candidate regulatory genes identified during a previous study [33], the genes 111742 (pMH14), 120120 (pMH22) and 123019 (pMH30) were up-regulated at low pH. Over-expression of these genes has been shown to result in slightly declined production of cellulase activity [33]. Interestingly, the novel regulator of especially cellulase genes, ace3 [33], was assigned to the same Mfuzz cluster and/or heat map branch as several genes that were already previously shown to cluster together with ace3 in a dataset from a transcriptional analysis with several different inducing carbon sources [33]. These genes included egl2, egl3, cbh2, swo1, cip2, xyn3, cell1b and cell3c. These results indicate that regardless of the environmental conditions (different inducing substrates or different ambient pH), the genes most probably under ace3 regulation are co-expressed with ace3.

The genomic cluster containing the *grd1* gene which is believed to be involved in cellulase signal transduction also included the gene 111742 encoding a transcriptional regulator. Both genes were more highly expressed at low pH as compared to high pH. The over-expression of gene 111742 (pMH14) had a negative effect on the production of cellulase activity and especially on endoglucanase activity [33]. GRDI, however, has been shown to have a positive effect on cellulase activity [37]. These results make this genomic cluster an interesting target for the study of cellulase signalling in *T. reesei*.

**Conclusions**

In this study, the pH-responsive genes from the genome of *T. reesei* were identified, including genes affected by the regulator of ambient pH sensing, PACI. The effect of ambient pH on the genes of the pH-signalling cascade leading to activation of PACI was investigated and novel information was gained from the pH-regulation of the carbohydrate active enzyme genes and especially genes encoding cellulose- and hemicellulose-degrading enzymes. In addition, several co-regulated genomic clusters responding to changes in ambient pH were identified.

**Materials and methods**

**Strains, media and culture conditions**

*Escherichia coli* DH5α (*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 relA1 endA1 thi-1 hsdR17*) was used for propagation of the plasmids and *Saccharomyces cerevisiae* FY834 (MATα his3ΔA200 ura3-52 leu2Δ202 lys2Δ202 trp1Δ63) for yeast recombinational cloning. *Trichoderma reesei* QM9414 (ATCC 26921, VTT-D-74075) was obtained from VTT Culture Collection and
maintained on potato-dextrose agar plates (Difco). Spore suspensions were prepared by cultivating the fungus on potato-dextrose plates for 5 days, after which the spores were dislodged, suspended in a buffer containing 0.8% NaCl, 0.025% Tween20 and 20% glycerol, filtered through cotton and stored at −80°C. In order to collect mycelia for DNA isolation, the fungus was grown in a medium containing 0.2% proteose peptone (BD Biosciences), 2% glucose, 7.6 g/l (NH₄)₂SO₄, 15.0 g/l KH₂PO₄, 2.4 mM MgSO₄, 7H₂O, 4.1 mM CaCl₂·2H₂O, 3.7 mg/l CoCl₂, 5 mg/l FeSO₄·7H₂O, 1.4 mg/l ZnSO₄·7H₂O, and 1.6 mg/l MnSO₄. 7H₂O, pH 4.8. Flask inoculum for the bioreactor cultivations was prepared by cultivating 8 × 10⁷ spores for 3 days in 400 ml of medium containing 25 g/l Avicel cellulose (Fluka BioChemika), 6 g/l proteose peptone (BD Biosciences), 7.6 g/l (NH₄)₂SO₄, 15.0 g/l KH₂PO₄, 2.4 mM MgSO₄, 7H₂O, 4.1 mM CaCl₂·2H₂O, 3.7 mg/l CoCl₂, 5 mg/l FeSO₄·7H₂O, 1.4 mg/l ZnSO₄·7H₂O, 1.6 mg/l MnSO₄. 7H₂O, pH adjusted to 5.2 with KOH. Bioreactor medium contained 25 g/l Avicel cellulose, 6 g/l proteose peptone, 13.6 g/l (NH₄)₂SO₄, 4 g/l KH₂PO₄, 0.2 ml/l Tween80, 0.6 g/l MgSO₄·7H₂O, 0.8 g/l CaCl₂·2H₂O, 3.7 mg/l CoCl₂, 5 mg/l FeSO₄·7H₂O and 1 ml/l of antifoam agent (Dow corning 1500). pH of the medium was adjusted before inoculation to pH3, 4.5 or 6. The bioreactor containing 0.9 l of medium was inoculated with 0.1 l of flask inoculum. Bioreactor cultivations were performed in Sartorius Qplus reactors at 28°C with a dissolved oxygen saturation level of >30%, agitation of 500-1200 rpm and a constant air flow of 0.6 l/min. pH was controlled with 15% KOH and 15% H₂PO₄. Samples from the cultivations of the QM9414 strain at different pH values were collected at time points of approximately 8 h, 18 h, 28 h, 44 h, 68 h, 93 h and 119 h. Samples from the cultivation of the QM9414 and Δpac1 strains at pH6 were collected at time points 17 h, 24 h, 40.5 h, 69 h, 95.3 h, 116.5 h and 139.3 h.

**Construction of the pac1 deletion strain**

Deletion cassette for the knock-out of the pac1 gene was constructed by yeast recombination cloning [52] in the pRS426 plasmid [53]. The construct contained a hygromycin resistance cassette flanked by 1890 bp fragments from 5′ and 3′ ends of the pac1 gene. Fragment 5′ from the pac1 open reading frame was PCR amplified with the oligos 5′- GTAAAGCCAGGTTTTCCCCAGTACAC GACGTTTTAACCACCTACACGACCTGTTT GCA-3′ and 5′- ACCGGGATCCATTAAGGTTACT GAAATCGGAGTCTGTTCGGGCTTGCATT-3′. Fragment 3′ from the pac1 open reading frame was PCR amplified with the oligos 5′- ATGGCCGAAAGAGTCCACCGGTCACTGTA CTAAGAATAC-3 and 5′- GCCGATACAAATTTC ACACAGGAAACAGCGTTTAACCCGACGACGCA GCATTGCTTGAGGCGG-3′. Hygromycin resistance cassette was PCR amplified with the oligos 5′- AGA CAATGCGGACGGCCAGTTCGATTTT GCACGTTAAGT-3 and 5′- TGGGTGTATTGT GGA AGAATGGTCTAATTACGTTGACGTACCT-3′. All the PCR products were purified and combined with the vector backbone. The resulting plasmid was digested with Pmel and SsPl enzymes (New England bio-labs) and the deletion cassette was transformed to T. reesei QM9414 by polyethylene glycol mediated protoplast transformation [54]. The hygromycin resistant transformants were initially selected from plates containing 150 µg ml⁻¹ of hygromycin B (Calbiochem). Stable transformants were obtained by streaking on plates containing 125 µg ml⁻¹ of hygromycin B for two successive rounds. Single colonies resulting from uninuclear transformants were obtained by plating dilutions of spore suspensions. Transformants with the expression cassette at the correct locus were identified by PCR and integration of only one copy of the cassette was further verified by Southern blot analysis. Genomic DNA for Southern blot was isolated using Easy-DNA Kit (Invitrogen) according to the manufacturer’s instructions. Southern blotting and hybridisation on nitrocellulose filters (Hybond N, GE Healthcare) were carried out according to standard procedures [55]. The signals were detected using a phosphorimager (Typhoon imager, GE Healthcare). The deletion of the pac1 gene was confirmed by Northern blot analysis. Total RNA was isolated from the mycelium samples using the Trizol® Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) essentially according to the manufacturer’s instructions. Northern blotting and hybridisation on nitrocellulose filters (Hybond N, GE Healthcare) were carried out according to standard procedures [55].

**Sample preparation and microarray analytics**

Mycelial samples collected at the time points of 28 and 44 hours from the cultures of QM9414 at pH3, 4.5 and 6, as well as samples collected at the time points of 17 and 24 hours from the parallel cultures of the strain Δpac1 and QM9414, were subjected to transcriptome analysis. Frozen mycelium was ground under liquid nitrogen, and total RNA was isolated with Trizol® reagent according to the manufacturer’s instructions. RNA was subsequently purified using RNeasy Mini Kit (Qiagen, Hilden, Germany) and RNA concentration was measured using NanoDrop ND-1000 (NanoDrop Technologies Inc. Wilmington, DE, USA). Integrity of the isolated RNA was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

Processing of the RNA samples for the microarray analysis was performed essentially according to the instructions from Roche Nimblegen. After synthesising
the double-stranded cDNA using Superscript Double-Stranded cDNA synthesis Kit (Invitrogen), the integrity of the double-stranded cDNA was analysed using an Agilent 2100 Bioanalyzer. The double-stranded cDNA was labelled with Cy3 fluorescent dye, after which it was hybridized to microarray slides (Roche-NimbleGen, Inc., Madison, WI, USA) and the slides were scanned using a Roche NimbleGen Microarray scanner according to the instructions of the manufacturer. The probe design and manufacturing of the microarray slides was carried out by RocheNimbleGen using an array design based on the *T. reesei* genome version 2.0 [24]. The design includes six 60mer probes for each of the genes.

The data was pre-processed using the R package Oligo and differentially expressed genes were identified with the package Limma [27, 28, 56]. Differentially expressed genes were identified by comparing the signals from the different pH-conditions and between the different strains at the corresponding time point. pH6 samples were compared to pH3 and pH4.5 samples and pH4.5 samples were compared to pH3 samples. Signals from the cultivation of the Δpac1 strain were compared to those of the QM9414 strain from the same cultivation conditions. Three biological replicates were analysed for each condition and each time point. The threshold used for statistical significance was p-value <0.01 and log2-scale fold change >0.4.

### Additional files

**Additional file 1: Transcriptional profiling data of the pH-responsive genes.** Gene identifiers are as in the *T. reesei* database version 2.0 and 1.2 (in the latter, v1_2 precedes the gene ID) or as in [26]. Signal intensities (log2 scale), fold changes (log2 scale) and significance test (R package limma, p < 0.01, log2 fold change > 0.4) are shown for the genes from two different time points of cultivations. Colour scales of yellow, red and green indicate different intensities of signals, red representing the strongest and green the weakest signals. The intensity of the red and blue colour indicates the strength of positive and negative fold change, respectively. In the significance test, 1 indicates induction and –1 repression. QM9414II indicates the control culture (QM9414) for Δpac1.Annotations of the genes are as in [30].

**Additional file 2: Transcriptional profiling data of the genes affected by the pac1 deletion.** Gene identifiers are as in the *T. reesei* database version 2.0 and 1.2 (in the latter, v1_2 precedes the gene ID) or as in [26]. Signal intensities (log2 scale), fold changes (log2 scale) and significance test (R package limma, p < 0.01, log2 fold change > 0.4) are shown for the genes from two different time points of cultivations. Colour scales of yellow, red and green indicate different intensities of signals, red representing the strongest and green the weakest signals. The intensity of the red and blue colour indicates the strength of positive and negative fold change, respectively. In the significance test, 1 indicates induction and –1 repression. QM9414II indicates the control culture (QM9414) for Δpac1. Annotations of the genes are as in [30].

**Additional file 3: Transcriptional profiling data of the CAZy genes.** Gene identifiers are as in the *T. reesei* database version 2.0 and 1.2 (in the latter, v1_2 precedes the gene ID) or as in [26]. Signal intensities (log2 scale), fold changes (log2 scale) and significance test (R package limma, p < 0.01, log2 fold change > 0.4) are shown for the genes from two different time points of cultivations. Colour scales of yellow, red and green indicate different intensities of signals, red representing the strongest and green the weakest signals. The intensity of the red and blue colour indicates the strength of positive and negative fold change, respectively. In the significance test, 1 indicates induction and –1 repression. QM9414II indicates the control culture (QM9414) for Δpac1. Annotations of the genes are as in [30].

**Additional file 4: Genes from the heat map visualisation.** Gene identifiers are as in the *T. reesei* database version 2.0. Annotations are shown according to the Eukaryotic orthologous group classification and as the manual annotations from the JGI *T. reesei* 2.0 database. Functional Interpro domain identifiers are as in the InterPro database. Annotations of glycoside hydrolase, carbohydrate esterase and polysaccharide lyase genes are as in [30].

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