Assessment of the Pectinolytic Network of *Aspergillus niger* by Functional Genomics

Insights from the Transcriptome

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To Aernout, Christian and Maria ...

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Outline

More than a century ago, in 1889, A. Fernbach presented a detailed report about the invertase of *Aspergillus niger* in the third edition of “Annales De L'institut Pasteur”. Since then, many of the enzymes secreted by *A. niger* have found a broad range of applications, and today they are produced on an industrial scale. This filamentous fungus is also used as a primary source for the production of organic acids and other economically important metabolites. Although, many of these fermentation processes are well established, the underlying genetics are still not well understood. The recent determination of the genome sequence of *A. niger* illustrated the versatile metabolic capacities of this fungus and created the opportunity for challenging research aiming to unravel the fine tuned metabolic network and the full enzymatic potential of *A. niger*.

The work described in this thesis is focused on the genome mining and transcriptional profiling of the enzymatic network of *A. niger* involved in pectin degradation and utilization. Pectin was chosen because of the broad application of it and its derivates in food, feed, medicines, and cosmetics and the economical importance of this polymer in several technological processes. This study addresses several issues concerning pectin degradation by *A. niger*:

i) the complete inventory of the known and potential pectinolytic activities encoded in the genome of this fungus; **ii)** the understanding of dynamics of their gene-expression on (complex) carbon sources in order to unravel underling metabolic networks; **iii**) the evolutionary aspects of the pectinolytic system of *A. niger* and other filamentous fungi.

Chapter 1 of this thesis discusses the importance of fungi and *A. niger* for the biotech industry, and gives a brief introduction to the structural elements of pectin, the types of

enzymatic activities involved in its degradation, and the present knowledge of the metabolism of its major constituent – galacturonic acid. The current advances in fungal 'omics research are pointed out. **Chapter 2** presents the construction of pectin specific cDNA libraries from *A. niger*, and the annotation of more than 200 of the obtained expressed sequence tags. **Chapter 3** focuses on the roles of and the interactions between the 21 genes within glycoside hydrolase family 28 - the largest group of *A. niger* pectinases. A special emphasis is given to a new, previously unanticipated, group of exo-acting enzymes. **Chapter 4** describes the survey for all genes encoding pectinolytic activities in *A. niger* and their transcriptional profiles. In this chapter, a hypothetical model of the pectinolytic network of *A. niger* is proposed. **Chapter 5** focuses on the identification of the genes involved in galacturonic acid catabolism in *A. niger*, the verification of some of the enzymatic activities encoded by them, and elaborates on the presence of an evolutionary conserved galacturonic acid pathway operating only in filamentous fungi capable of plant cell wall degradation. In **Chapter 6,** the evolutionary conservation of transcriptional response of the pectinolytic system of *A. niger* is compared to that of the model organism *A. nidulans*. This chapter further describes the identification of the evolutionary conserved, regulatory core of functional orthologs involved in galacturonic acid utilization and metabolism. In **Chapter 7,** the results of the work described in this thesis are summarized and discussed. Supplementary data is provided at the end of the thesis.

Chapter 1

General introduction

Introduction

Fungi form a diverse lineage of eukaryotic organisms that spans over millions of years of evolution. Today they are employed in the manufacturing of medicines and a variety of agriculturally and industrially important products. Within the group of industrially important fungi, the black mold *Aspergillus niger* has a particularly pronounced economical value. For decades, its remarkable production capacities have been exploited in the manufacturing of useful metabolites and enzymes. *A. niger* is an excellent producer of vast variety of extracellular carbohydrate enzymes many of which have an important economic value. Among them, the commercial value of pectinases reaches approximately 20% of the annual sales of all industrial enzymes. Many of the pectinases produced by *A. niger* have been studied intensively and their structure, function and activities have been elucidated. However, only limited research has addressed the synergistic relationships between them and little is known about their regulation or about the fait of their metabolic products. The resent determination of the genomic sequence of *A. niger* and the availability of Affymetrix GeneChip microarrays provided the opportunity to study the efficient enzymatic network of this fungus in a comprehensive manner, by applying advanced biological techniques.

This introduction chapter emphasizes the particular importance of fungi and *A. niger* in biotechnology, provides a summary of the present knowledge of pectin structure and degradation, and gives a brief overview of the current advances in genomics and transcriptomics research addressing *A. niger*.

1.1 Fungi

Evolution and diversity

Fungi are eukaryotic, heterotrophic organisms that form a separate and diverse lineage of many different species, commonly described as mushrooms, molds, and yeasts. The fungal kingdom comprises more than 70, 000 described species distributed over 6 different clades - *Blastocladiomycota*, *Chytridiomycota*, *Dikarya*, *Glomeromycota*, *Microsporidia*, and *Neocallimastigomycota*. Modern Fungi have a monophyletic origin [1] and are probably a sister group of Animals. It is believed that the first fungi were monocellular, simple, aquatic forms with flagellated spores, similar to extant chytrids [2]. The oldest fungal fossils date back to the late Precambrian period - 650 to 543 million years ago. The first terrestrial fungi probably appeared around 460 million years ago as mutualistic symbionts with plants, similar to modern mycorrhizae, when eukaryotic organisms invaded prehistoric lands [3 97]. Devonian fossils from 412 million years ago reveal an already rich diversity of different free-living, symbiotic, and saprobic fungi including various species of chytrids and mycorrhizae, but also the earliest ascomycetes[*].

Nowadays, fungi are members of practically all ecosystems on Earth. They can grow under aerobic or anaerobic conditions, they are found from the desert to the sea [4]. Thermophilic fungi are the only eukaryotic organisms that can grow on temperatures above 45^{o}C [5]. Many fungi are free-living saprobic organisms, commonly found in the soil. Together with bacteria, they play a major role in decomposing organic material and therefore contributing to the biogeochemical cycles of the six “elements of life” – carbon, oxygen, hydrogen, nitrogen, phosphor, and sulfur. Fungi coexist with insects, plants, animals or other fungi. Although, their symbiotic relationships with other organisms are often recognized only in the “negative” form of parasitism, fungi do participate in a broad variety of mutualistic exchanges. Besides the well-known mycorrhizal fungi and the lichens, fungi live together with most plants as endophytes. There are several well-known mutualists of animals, such

[*] http://www.abdn.ac.uk/rhynie

as gilled mushrooms cultivated by ants or anaerobic fungal fermenters in the gut of many mammalian herbivores [4]. Less known are the vitamin-supplying fungal symbionts in the gut of aquatic insect larvae and marine crustaceans, or the yeasts that participate in sterol utilization and nitrogen recycling in land insects ([3], and references there in).

Importance of fungi in medicine and biotech industry

Fungi have been used for thousands of years for the traditional preparation of bread, and for the fermentation of beer, wine, tempeh and soy sauce. In medicine and in agriculture, fungi are often recognized as severe pathogens that cause serious diseases in humans, livestock and important agricultural crops. Nevertheless, fungi have their positive value, as they are good producers of many useful enzymes and different biologically active compounds. Today fungi are employed in the manufacturing of many medicines and a vast variety of agriculturally and industrially important products.

Starting with the discovery of beta-lactam antibiotics like penicillin and cephalosporin in the beginning of the 20^{th} century, modern medicine has been gaining increasing interest into the discovery and application of various fungal secondary metabolites with important therapeutic effects. A most successful example of a product that became the base for the developing of an entire range of hypolipidemic drugs is the polyketide lovastatin produced by *Aspergillus terreus*. Up to date fungal statins are the best cholesterol-lowering agents available. It is also thought that they have a positive effect in prevention of cardiovascular disease [6]. Another compound, produced by fungi, that is of established clinical relevance, is the non-ribosomal peptide cyclosporin, produced by *Tolypocladium inflatum*. Cyclosporin A is broadly used as an immunosuppressive drug after organ transplantation. Other fungal metabolites like bikaverin are being investigated as potential precursors in the developing of anticancer drugs [7]. In the last years, several reports have suggested that fungi can be used successfully to control malaria. [8]. The ability of fungi to produce powerful compounds with antibacterial and antiinsecticidal properties is explored in agriculture for the biological control of different pests. Today, besides for medicines, fungi

are used for the production of many other economically important compounds. These compounds include vitamins like riboflavin [9]; food grade additives like β-carotene and lycopene; and various organic acids like lactic acid, kojic acid, gluconic acid, fumaric acid or citric acid.

Fungi are excellent producers of extracellular enzymes and as such, they have been used for the production of practically all classes of industrially important enzymes. These enzymes are applied in detergents, textiles, and in the pulp and paper industry. They are also used for several applications in starch processing, for the production of bio-ethanol, in the synthesis of organic compounds, and in the food and feed industry [10]. The most important enzymes applied at an industrial scale include several different classes of hydrolases, oxidoreductases, transferases, lyases, and isomerases. Fungal xylanases, alpha-amylases, glucoamylases, and isomerases are used for the liquefaction, saccharification, and isomerization of starch to produce maltose, glucose and fructose corn syrups, for improving specific qualities of dough in bread baking, and for the production of ethanol. Proteases from fungal origin are used for the preparation of cheese, as an alternative for chymosin, and in the backing. Proteolytic enzymes from *Aspergillus oryzae* represent an alternative for acid hydrolysis of proteins to produce hydrolysates for soups, savory flavours and as ingredients in emulsified meat products [11]. Pectinases and other pectinolytic enzymes from *A. niger* and *Aspergillus aculeatus* are used in the food and feed production, for fruit juice liberation, clarification and stabilization, for fruit firming and maceration, for the extraction of ethereal oils and phenolic compounds; in the textile industries, for retting and de-gumming of fiber crops, and for quality paper production [12]. Several examples demonstrating the importance of fungal enzymes in industrial “non-food” processes include the application of fungal xylanases and lignin oxidizing enzymes in the processing and non-chlorine bleaching of pulp in the paper industry [13]. Cellulases and in particular endo-glucanases are now commercially used as “anti-pilling” enzymes in detergents and for the bio-polishing of cotton fabric in the textile industry. Yeast lipases are regular additive to detergent formulas. They are also used for the production of surfactants, emulsifiers and lipophilic antioxidants; for flavor improvement of several foods, and in the organic synthesis of several medicines [14]. Laccases produced by genetically modified *Aspergillus*

strains are used in the textile, dye, and printing industries for the decolorization of synthetic dyes, and in the pulp and paper industry for delignification [15].

Aspergillus niger

Within the group of industrially important fungi, the black mold *Aspergillus niger* has a particularly pronounced economical value. *A. niger* is a filamentous ascomycete fungus belonging to subphylum *Pezizomycotina*, class *Eurotiomycetes*, order *Eurotiales*, family *Trichocomaceae*. In older classification schemes *A. niger* is often described as a member of the form subdivision *Deuteromycotina* i.e. the Fungi imperfecti, because of the absence of a sexual reproduction cycle.

A. niger has been recognized for decades as an industrially important fungus since the U.S. Food and Drug Administration (FDA) has granted a GRAS (Generally Recognized As Safe) status to many substances produced by it. Its excellent production capacities have been exploited in the production of various useful metabolites and enzymes. This fungus is the main producer of citric acid for food, beverages and pharmaceuticals. World wide the amount of citric acid produced by *A. niger* reaches 1 billion pounds per year with a market value of US$ 1.4 billion. *A. niger* is also used as expression host of for the production of heterologous proteins [16]. Its ability to degrade a range of xenobiotics through various oxidative, hydroxylation and demethylation reactions provides potential for use in bioremediation [17]. Because of its saprobic nature, *A. niger* is an excellent producer of a wide spectrum of extracellular enzymes. This property has made this fungus a production organism for several commercial enzymes. Among the most important of them are acid-amylases, asparaginases, beta-galactosidases, glucoamylases, glucose oxidase, glycosidases, lipases, phospholipases, proteases, phytases and several hemicellulases including arabinases, arabinofuranosidases, pectin methylesterases, polygalacturonases, and xylanases. From these enzymes, glucoamylase is produced in higher tonnage than almost any other industrial enzyme, while the commercial value of pectinases is approximately 20% of the one billion US dollar annual sales of all industrial enzymes [12].

1.2 Pectin degradation as a model for the complex extracellular enzymatic network of *Aspergillus niger*

Pectin is the descriptive name for a diverse group of complex heteropolymers present in the middle lamella of the primary plant cell wall of dicotyledonous plants. Within the cell wall, pectin forms a negatively charged, hydrophilic network that together with other hemicelluloses and some proteins gives compressive strength to primary walls and plays an important function in cell-cell adhesion.

The structure, biosynthesis and biodegradation of this abundant plant polymer have been studied extensively both as a fundamental structural component of the plant cell wall and as an economically important component of food, feed and as a source of bioactive compounds. Because of its gelling properties, extracted pectin is widely used as a functional food ingredient, in the production of jams and jellies, fruit juice, confectionary products and bakery fillings [18, 19] and for the stabilization of acidified milk drinks and yogurts. Pectin has pronounced positive effects on health as a rich source of soluble fiber from fruit and vegetables. It has been demonstrated that pectin can lower both cholesterol and serum glucose levels, and that it may have anti-cancer activities [20-22]. Within all of these application areas, the fine structure of pectin profoundly affects its functionality, whereas commercial sources of pectin are limited to only few sources that have the required properties [23]. This leads to the need of enzymatic modification of pectin by the use of several different pectinolytic enzyme classes. The most commonly used industrial pectinases are polygalacturonases, pectin lyases, and pectin methylesterases from microbial origin. Many of them are produced by *A. niger* because of the proven safe use of the compounds produced by this fungus. Intensive studies have revealed their structure, function, and activities. In contrast, only limited research has addressed the synergistic relationships between enzymes from the pectinolytic system of *A. niger* [24] and little is known about their regulation or the fait of their metabolic products.

Pectin structure

Pectin is composed of a number of distinct polysaccharides, such as homogalacturonan (HG), xylogalacturonan (XGA), rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII) (Fig 3A). HG, also known as the smooth region of pectin, is a linear polymer composed of 1,4-linked α-D-galacturonic acid residues. Some of these residues can be, to different extent, methyl-esterified at the carboxylic acid group (*C*-6 position) and/or can carry acetyl groups on *C*-2 and *C*-3 [25, 26]. In XGA β-D-xylose residues are β-(1,3)-linked to the galacturonyl residues of the HG backbone. RGI is composed of a backbone of alternating α-(1,2)-linked rhamnosyl and α-(1,4)-linked galactosyluronic acid residues. The rhamnosyl residues are branched with *O*-4 attached neutral sugar side chains that can vary from a single galactose residue up to polymeric chains of glycosyl residues composed of

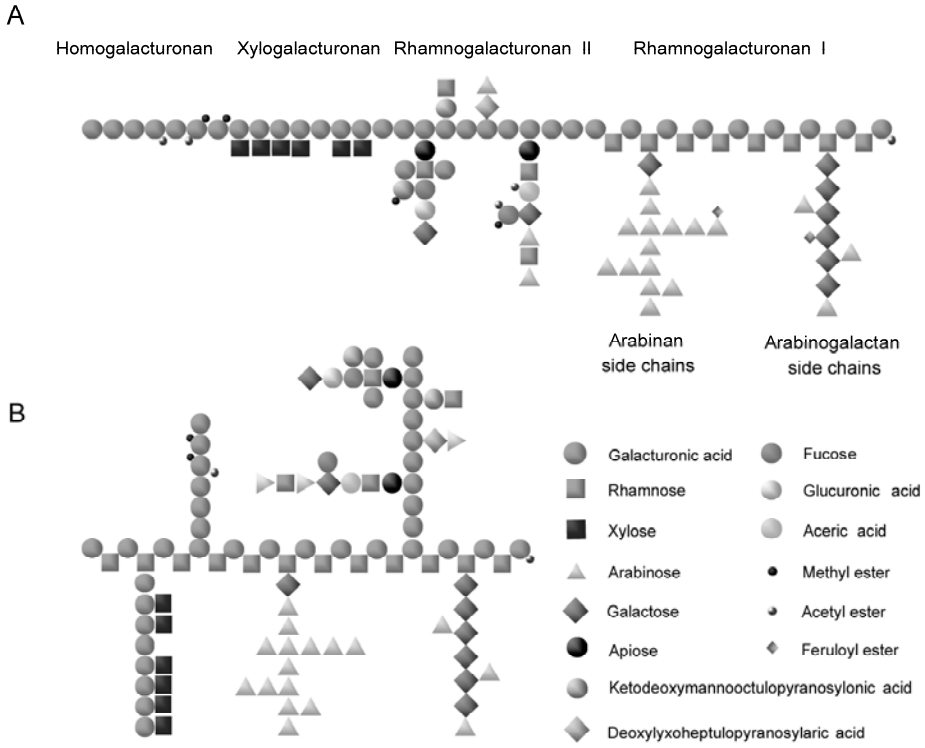


Figure 1. The schematic structure of pectin. **(A)** The Classical model**. (B)** A model recently proposed by Vincken et al. [26]. The illustrated polymers are only a cartoon representation of the major pectin components and do not depict definite molecular structures.

arabinose (arabinan side chains) and/or arabinose and galactose residues (arabinogalactan side chains).

The arabinan and arabinogalactan side chain residues can be substituted by feruloyl residues at *O*-6. Approximately 20-30% of the feruloyl residues in sugar beet pectin are attached to arabinan side chains, while the other feruloyl residues are attached to arabinogalactan side chains [27]. RGII is comprised of a backbone of approximately nine α-(1,4)-linked D-galactosyluronic acid residues that carry four side chains which consist of a number of rare sugars, such as apiose, aceric acid, 2-keto-3-deoxy-D-manno-octulosonic acid, and 2-keto-3-deoxy-D-lyxo-heptulosaric acid [26]. XGA, RGI and RGII are part of the branched or hairy regions of pectin [28]. Two pectin models exist, namely a pectin structure in which HG is an extension of RGI or vice versa and has neutral sugar side chains [29], and a pectin structure that is built from a backbone of RGI where HG and neutral sugars form the side chains [26] (Fig. 1A and 1B).

Pectin degradation

Pectin accounts for about one-third of the total cell wall material [30] and as such represents an important carbon source for bacteria and fungi that degrade decaying plant material. The complete biodegradation of the complex and heterogeneous structure of pectin by saprobic microorganisms requires the presence of many different enzymatic activities.

Pectinases of *A. niger*

With the classical model of pectin in mind, enzymes involved in the degradation of pectin can be formally grouped in two major classes – pectinases that attack the pectin backbone, and accessory enzymes that are involved in the degradation of the side chains of pectin. The group of pectinases comprises several different polygalacturonan-, rhamnogalacturonan- and xylogalacturonan hydrolases, pectin, pectate and rhamnogalacturonan lyases, pectin methylesterases and acetyl esterases (Fig. 2A).

Most *A. niger* pectic hydrolases that are active on the backbone of pectin belong to family

GH28 of the general classification system of glycoside hydrolases [31]. Lyases are grouped in families PL1 and PL4, and esterases in families CE1, CE8 and CA12 [17]. Exo- and endopolygalacturonan hydrolases, pectin lyases and pectate lyases degrade HG. XGA can be degraded by endoxylogalacturonan hydrolases and exopolygalacturonan hydrolases, whereas rhamnogalacturonan hydrolases and rhamnogalacturonan lyases degrade RGI. In addition, the complete enzymatic depolymerisation of pectin backbone requires the presence of different types of esterase activities.

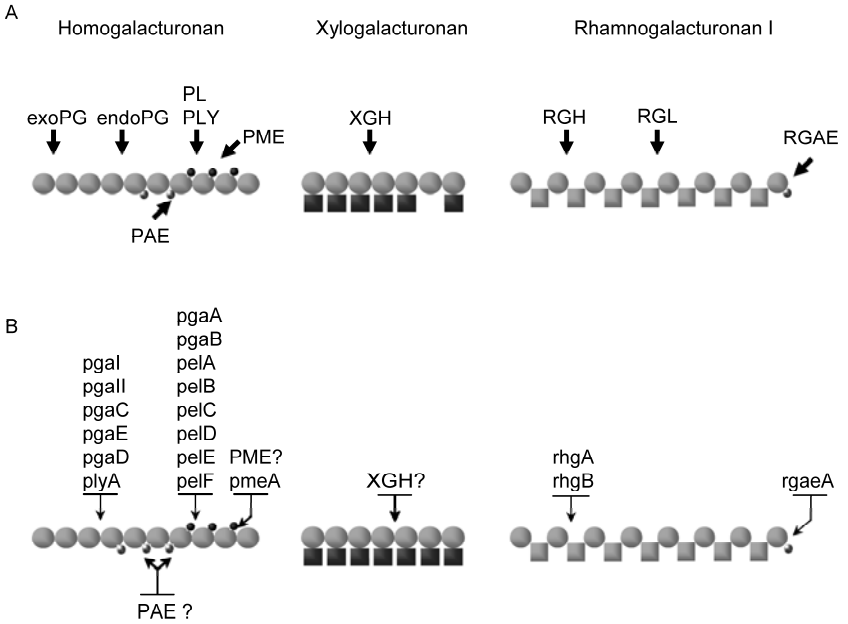


Figure 2. Overview of enzymatic activities involved in pectin degradation. (A) Points of attack in the backbone of pectin. A schematic drawing of pectin backbone according to the classical model is presented (For details see figure 3A). exoPG, exo-polygalacturonase; endoPG, endo-polygalacturonase; PL, pectin lyase; PLY, pectate lyase; PME, pectin methylesterase; RGH, rhamnogalacturonan hydrolase; RGL, rhamnogalacturonan lyase; RGAE, rhamnogalacturonan acetylesterase; HGAE, homogalacturonan acetylesterase; XGH, endoxylogalacturonan hydrolase. **(B) Known genes and enzymatic activities from *A. niger*.** Orphan enzyme activities for which the corresponding gene has not been cloned or characterized are given in capitals**.**

Polygalacturonases cleave α-1,4-D galacturonan linkages in homogalacturonan. Endopolygalacturonan hydrolase activities are assigned to EC 3.2.1.15, while exopolygalacturonan hydrolases depending on if the product produced is a mono- or digalacturonic acid to EC 3.2.1.67 and EC 3.2.1.82, respectively. Pectin lyases (EC

4.2.2.10) and pectate lyases (EC 4.2.2.2; EC 4.2.2.9) split α-1,4-D galacturonan linkages in HG by β-elimination and introduce a double bond between *C*-4 and *C*-5 of the newly formed non-reducing end. Xylogalacturonan hydrolases (EC 3.2.1.-) cleave α-1,4-D galacturonan linkages in XGA and require xylosyl side residues [32, 33].

Rhamnogalacturonan hydrolases (EC 3.2.1.-) cleave α-galacturonic acid-(1,2)-α-rhamnose linkages while rhamnogalacturonan lyases cleave α-rhamnose-(1,4)- α-galacturonic acid linkages by β-elimination. Pectin methylesterases (EC 3.1.1.11) cleave off methyl esters of the backbone of homogalacturonan. Pectin acetylesterases (EC 3.1.1.6) remove acetyl groups from *C*-2 or *C*-3 of the galacturonic acid residues in HG and RGI.

Several different genes encoding pectinolytic glycoside hydrolases produced by *A. niger* have been cloned and the corresponding enzymes have been characterized in detail (Fig. 2B). The list includes seven different endopolygalacturonan- and two endorhamnogalacturonan hydrolases [34, 35]. In addition six pectin lyases, a pectate lyase, two rhamnogalacturonan hydrolases, rhamnogalacturonan acetyl esterase, rhamnogalacturonan lyase, and a pectin methylesterase have been described, [24, 35-41]. Nevertheless, the genes encoding several known *A. niger* enzymatic activities towards specific linkages and/or saccharide motif's within the pectin molecule have not yet been identified [42-45].

The structures of *A. niger* endo-polygalacturonan hydrolases, PGI and PGII and pectin lyases PLA and PLB have been solved [46-49]. This, together with the biochemical data obtained from site directed mutagenesis of strictly conserved amino acids, allowed the identification of the residues involved in catalysis, substrate binding, substrate specificity and led to the elucidation of the mode of action of PGII and PLA [50-54].

Although differing in their substrate specificity (homogalacturonan, xylogalacturonan, rhamnogalacturonan) and mechanism of action (hydrolases, lyases, esterases), pectinases show surprising lack of structural variation. Independent of their bacterial, fungal or plant origin all these enzymes share the same single stranded right handed parallel β-helix topology characteristic for the pectin lyase-like superfamily. This type of protein structure binds the polymeric substrate to an exterior cleft that starts after five consecutive turns of the right-handed helix. As by other depolymerizing enzymes, e.g. proteases and nucleases,

the substrate-binding cleft of these proteins consists of multiple subsites, each of which makes contact with one monomeric unit of the polymeric substrate. The actual cleavage occurs between subsite -1 and +1 [55], while subsites distant from the bond that undergoes catalysis, modulate the activity, affinity and/or specificity of the enzyme. A correlated mutation analysis of a recently described structural alignment of proteins from the pectin lyase-like superfamily, containing over 1200 sequences, revealed surprising variation conservatism in several positions closely related to the catalytic residues in all examined enzymes [56]. In this alignment, alignment position (AP) 88 was detected as the one with

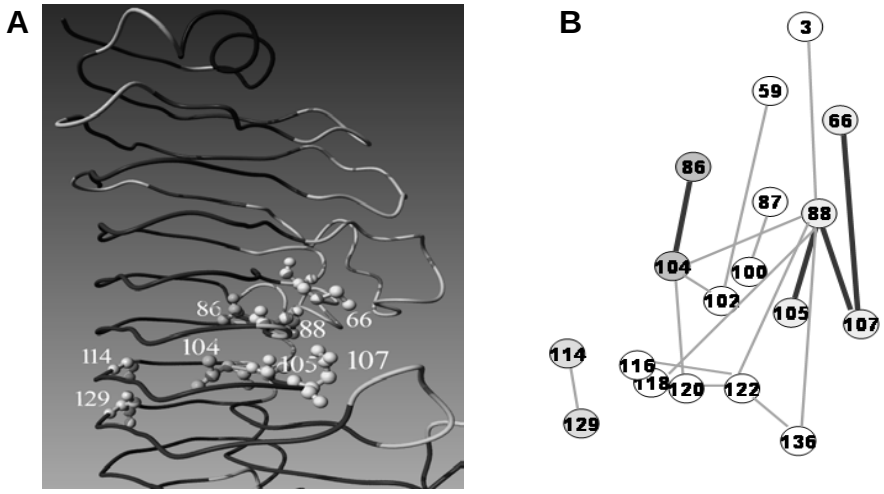


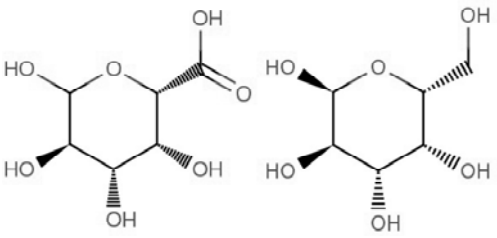
Figure 3. (A) Tube representation of endo-polygalacturonase II of *A. niger*. The β-sheets that form the right-handed β-helix fold are in dark gray. The positions with high CM scores are represented in ball and stick.
(B) Network of all alignment positions with correlations above 0.8. The nodes represent alignment positions. Note that their position is according to the 3D-configuration of the protein with the N-terminus on top. The coloring scheme of the nodes is according to CM score with the high correlating positions (>95) and the separate network formed by positions 114 and 129 in grey. The edges of the network are represented according to the height of the CM score (>95 is in bold). Covering three consecutive turns of the right-handed helix of the overall protein fold, the side chains of all amino acids in this network point to the exterior of the protein and are near each other surrounding position 88. Positions 114 and 129 form a small separate network (After Joosten, H.J. *et al*. [56]).

the highest correlated mutations (CM) score. In polygalacturonases, AP 88 is often an aspartate residue (Asp183 in *A. niger* PGII) that is involved in maintaining the proper ionization state of the catalytic aspartate residue at AP 85. Mutation of this residue strongly decreases both the specific activity and the affinity of the enzyme to its substrate [52]. In

pectin lyase A and B from *A. niger*, AP 88 is occupied by a tyrosine (Tyr215 PLA *A. niger* numbering) that together with several other Tyr and Trp residues is involved in increasing the basicity of the catalytic arginine at AP 102 (Arg236 PLA *A. niger* numbering) [54]. AP 88 is the center of a network of highly correlating positions (Fig. 3). The high CM scores, together with the structural positions of these residues strongly suggest a functional relation undetected in previous studies. Separately, a CM score above 0.95 was detected for AP 86 and AP 104. The side chains of the corresponding residues point to the interior of the parallel beta helix which suggests that amino acids at these positions serve a structural role, important for the proper orientation of the neighboring catalytic residues (at AP 85 and AP 102) in the substrate binding cleft. Interestingly, AP 104 in pectin lyases (Pro238 in PLB *A. niger*) and pectate lyases (Pro220 in PelC *Erwinia chrysanthemi*) corresponds to a proline with an unusual *cys* conformation [49]. Based on the fingerprints derived from these networks of correlated alignment positions, it is possible to classify pectinases in functional groups [56].

Utilization of galacturonic acid

D-galacturonic acid is an oxidation product of D-galactose, in which the 6-CH_2OH group has become a -COOH group (Fig. 4). D-galacturonic acid is the major constituent of pectin, where it exists in the form of a linear polymer (polygalacturonic acid) composed of 1,4-linked α-D-galacturonic acid residues. Galacturonic acid represents an important carbon source for bacteria and fungi living on decaying plant material. In bacteria, galacturonic acid is degraded in a five step pathway via D-tagaturonate, D-altronate, 2-keto-3-deoxy-gluconate and 2-keto-3-deoxy-6-phospho-gluconate resulting in the formation of pyruvate and glyceraldehyde-3-phosphate [57-60] (Fig. 5A).



D-galacturonic acid D-(+)-galactose

Figure 4. Structural formulas of D-galacturonic acid and D-(+)-galactose

In eukaryotes, the degradation of D-

galacturonic acid takes a different metabolic route. In the filamentous fungus *Aspergillus nidulans*, two mutants with strongly reduced growth on D-galacturonic acid were described [61] and an alternative non-phosphorolytic pathway leading to glyceraldehyde and pyruvate was proposed. It has been suggested that the so produced glyceraldehyde is further reduced to glycerol [62]. Enzymatic activities corresponding to D-galactonate dehydratase and 2-keto-3-deoxy-D-galactonate aldolase have been described in *Aspergillus terreus* [63] and *A. niger* [64, 65]. An NADPH dependent glycerol dehydrogenase that is specifically induced by galacturonic acid has been detected in *A. nidulans* [66]. In the fungus *Trichoderma reesei*, a pathway for the degradation of galacturonic acid was recently established. This pathway begins with the conversion of D-galacturonic acid to L-galactonate by a NADPH dependent D-galacturonic acid reductase [67]. An L-galactonate dehydratase further converts L-galactonate to 2-keto-3-deoxy-L-galactonate [68], demonstrating that indeed the degradation of galacturonic acid occurs via L-compounds. The 2-keto-3-deoxy-L-galactonate aldolase was also recently characterized [69]. An NADPH dependent glycerol dehydrogenase active towards L-glyceraldehyde and the corresponding gene have been proposed to be involved in the formation of glycerol in the last step of galacturonic acid degradation [70] (Fig. 5B).

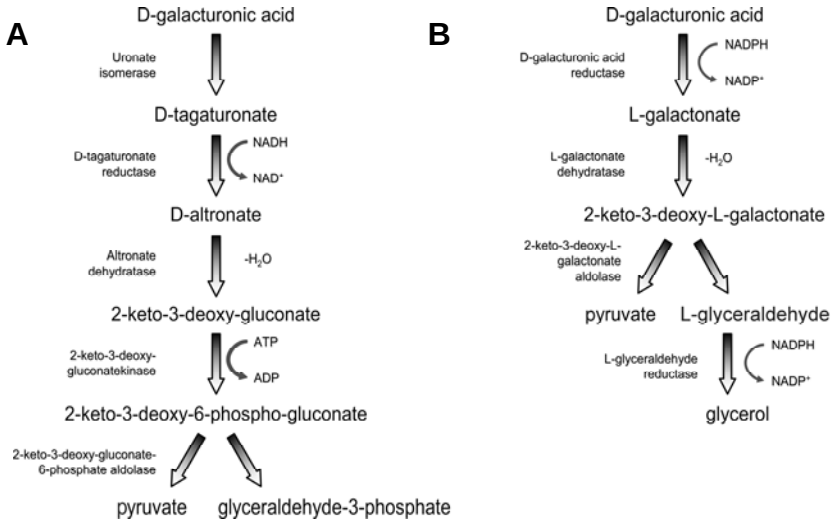


Figure 5. Metabolic conversion of D-galacturonic acid. (A) The Bacterial pathway for D-galacturonic acid catabolism**. (B)** The pathway for D-galacturonic acid catabolism in *Trichoderma. reesei*

Regulation op pectinases

The regulation of pectinase encoding genes has been well studied in prokaryotic species. In the plant pathogenic enterobacterium *E. chrysanthemi*, the differential expression of individual pectinase genes is under the regulation of several regulatory systems, including the KdgR repressor, which mediates the induction of all the pectinolytic genes in the presence of pectin catabolite. 2-keto-3-deoxy-gluconate, the second intermediate of the bacterial galacturonic acid catabolic pathway has been identified as an inducer of the pectinolytic system [71]. In this bacterium, in addition to KdgR, the expression of several genes is controlled in a catabolite repression way by cyclic AMP receptor protein (CRP) [72], and the PecS and PecT regulatory proteins that together modulate the pectinolytic system of *E. chrysanthemi*. Genome mining and expression profiling by targeted cDNA microarray analysis indicated that similar regulatory system operates in the hyperthermophilic bacterium *Thermotoga maritima* [73].

The pectinase regulation in fungi is still poorly understood. Evidence has been obtained that galacturonic acid can act as an inducer for some of the pectinolytic genes in *A. niger*. Three different expression profiles have been observed for genes encoding enzymes active on the homogalacturonan and rhamnogalacturonan parts of pectin. In the first group are genes encoding polygalacturonases with constitutive expression. The expression of another gene group is triggered by the presence of D-galacturonic acid or possibly an intermediate metabolite and it is typical for genes encoding enzymes active on homogalacturonan. The third type of pectinase induction is characterized by a high expression of genes on pectin later during growth, and it has been found for the rhamnogalacturonase and rhamnogalacturonan lyase genes [24]. Attempts to identify the transcriptional regulator(s) involved in pectinase regulation by genetic screening were not successful [74], however, the results of this study suggested that expression levels of pectinolytic genes may also be influenced by the presence of other sugars like L-rhamnose, D-xylose and D-glucose, although to a lower extent. Furthermore, evidence was found that pectin or its degradation products induce the D-galacturonic acid transport system [74].

In another study, promoter deletion analysis of the endopolygalacturonase gene *pga*II

demonstrated that the gene is subject to positive control by galacturonic acid and identified a 223 bp promoter fragment responsible for the high expression of PGII on pectin [75]. A comparison of this promoter DNA sequence with the yeast regulatory DNA sequences revealed a region of high identity with the upstream activation site UAS2 of the *Saccharomyces cerevisiae CYC1* gene, which is under control of the HAP2/3/4 system. A similar sequence was later identified in the promoters of two other polygalacturonase encoding genes (*pga*I and *pga*C) from which the consensus sequence, 5'-TYATTGGTGGA-3', was derived. The authors also reported the presence of an additional upstream *cis*-acting repressor region in the promoter of *pga*II [75] but no consensus sequence was reported for this repressor element.

1.3. Genomes and fungal genomes

Whole genome sequencing was initiated in 1977, when Frederick Sanger determined the complete sequence of all 5,375 nucleotides of the bacteriophage phi X174 by applying his revolutionary method of DNA sequencing based on controlled termination of enzymatic synthesis. This was the first complete genome sequence of any organism to be solved [76]. Five years later Sanger and co-workers published an adaptation of this method – "shotgun" sequencing, which they used to elucidate the 10 times bigger genome of bacteriophage lambda [77]. In the same year, GenBank was founded as a public repository database for DNA sequences. The first genome of a eukaryotic organism to be sequenced was the one of the yeast *S. cerevisiae* in 1996. Since then, the number of genome sequencing projects has been rapidly increasing (Fig. 6). So far 436 eukaryotic organisms are being sequenced and annotated and 23 of them are successfully completed (NCBI/genomes).

Up to date more than a quarter of the eukaryotic species with sequenced genomes are fungi (Fig. 6). In 2003, the first genomic sequence of a filamentous fungus was publicly released – that was the sequence of the red bread mold *Neurospora crassa* [78]. In June 2000, the Dutch Life Sciences company DSM announced the start of the sequencing project of *A. niger* CBS 513.88 the ancestor of range of improved enzyme production strains. During the

sequencing phase, a structured BAC approach was used and the genome sequence was completed in October 2001. The project resulted in a high quality DNA sequence of 8-x coverage that includes approximately 99 % of the 37 Mb genomic content of the fungus. The genome of *A. niger* was analyzed using a high quality process comprising an automatic gene annotation phase followed by a manual correction and verification. The completed sequence of *A. niger* CBS 513.88 was publicly released in 2006 [17]. Later that year a draft assembly of the genome of another strain of *A. niger*, the citric acid producer *A. niger* ATCC 1015 was released by the DOE Joint Genome Institute (JGI).

The benefit of a complete genome

The completion of a genome sequence from a particular organism followed by the location and the attachment of biological information, to all of the encoded genes is a scientific effort that provides the bases for a thorough analysis of all aspects of the metabolism of this organism. Fungal genomes are an ideal tool to study the complexity of the eukaryotic cell. They are small (up to 80 Mb), with relatively high coding density (37% to 61%) and a not to complex gene structure [79]. These features facilitate accurate *de novo* and comparative gene predictions and consequently allow a precise inventory of the encoded genetic content. Genome wide studies exploring the model system that *S. cerevisiae*, led to a much better understanding of fundamental biological processes like meiosis, unfolded protein response, RNA replication, cytoskeleton organization, cell-wall biosynthesis, chromosome segregation and DNA metabolism. In addition, genome wide studies of *S. cerevisiae* have been used to predict the possible cellular response to a variety of anticancer agents, antibiotics, antifungal agents and other target drugs [80].

The determination of the genomic sequence of *A. niger* and the comparison with other sequenced fungi revealed a remarkably large number of unique proteins involved in C-compound, carbohydrate, lipid, fatty acid, isoprenoid and secondary compounds metabolism, reflecting the versatility of *A. niger* as a cell factory [17]. A genome scale network of the central metabolism of *A. niger* CBS 513.88 was developed on the basis of its genome sequence and by combining it with data from the previously developed

stoichiometric model of the central carbon metabolism of *A. niger* [81]. This metabolic model consists of over 1000 reactions covering the central carbon metabolism processes. In addition, a vast number of transporter proteins from various classes were discovered, over 400 of which belong to the major facilitator superfamily. Their functional annotation

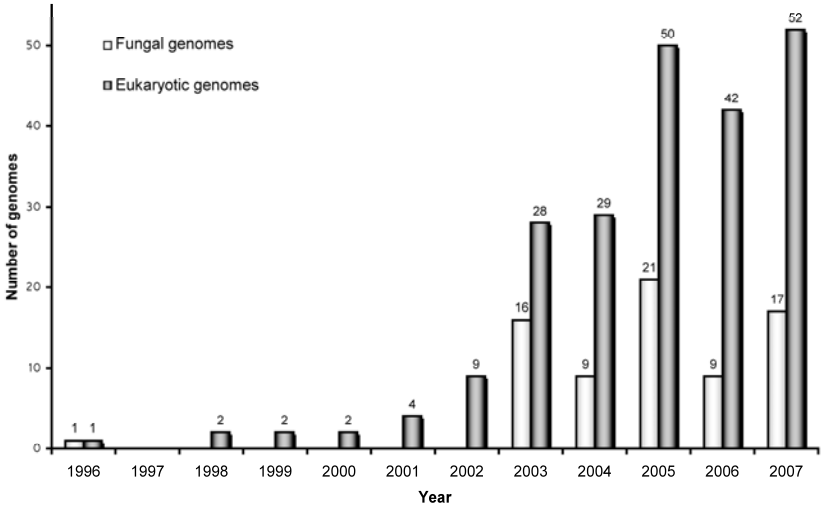


Figure 6. Increase of released assembled or finished eukaryotic genomes in the last decade. (Source: http://www.ncbi.nlm.nih.gov/genomes)

suggests that many of these proteins are involved in the transport of a broad range of substrates and that several of them may serve as nutrient sensors. The genome of *A. niger* CBS 513.88 is also enriched with GAL4 like zinc binuclear proteins which are predicted to regulate a variety of processes including primary and secondary metabolism and polysaccharide degradation. Compared to yeast, *A. niger* is a much better producer of extracellular proteins. This dissimilarity is reflected in the protein secretion machinery of *A. niger*, as the unfolded protein response signaling pathway, the ER associated protein degradation system and the glycosylation-dependant quality control system are clearly distinct from that of the well studied yeast *S. cerevisiae* [17]. The excellent secretory capacities of *A. niger* are reflected in the large number of extracellular biopolymer degrading enzymes. More the 130 different genes, putatively encoding secreted polysaccharide degrading enzymes from 41 different glycoside hydrolase families were

detected in its genome. Almost half of them are predicted to be involved in pectin degradation. Interestingly, the pectinolytic spectrum of *A. niger* is enriched with glycoside hydrolases from family 28, which reflects the acidifying properties of this fungus.

More genomes…

Currently, more than 70 fungal genomes have been sequenced, annotated, and deposited to NCBI. They include different human, plant and insect pathogen species, saprobes, as well as model organisms. Eleven of them are *Aspergillus* genomes (Table 1). More than other 40 fungal species are undergoing this process as well.

The rapidly increasing number of sequenced fungal genomes and the high conservation of their genomic content, despite the huge evolutionary distance of 900 million years between the most distant members, allows for the comparison of different fungi on a meta-species level, leading to better understanding of the evolutionary processes that these ancient eukaryotes have undergone. The possibility to perform comparative genomics between more than 40 species made it possible to reconstruct the exact fungal phylogeny using entire genomes instead of concatenated alignments of a limited number of genes [82-85].

The release of two *A. niger* genomes from different strains, the enzyme *producer A. niger* CBS 513.88 and the citric acid producer *A. niger* ATCC 1015, together with the sequencing of a third *A niger* strain, the wild type *A. niger* ATCC 9029 (N400; CBS 120.49) allowed a detailed analysis of the metabolic potential of this important organism. A second "high resolution" metabolic network of *A. niger* was reconstructed, based on the cross comparison of the genome sequences of the *A. niger* strains and other filamentous fungi, including *A. nidulans*, *A. oryzae*, *A. fumigatus*, *A. flavus*, *Fusarium graminearum* and *Neurospora crassa*, and to the yeast *Saccharomyces cerevisiae* [86]. The results of this study showed remarkable differences between *A. niger* and other *Aspergillus* species and demonstrated that unique genes account for around 4% of the total number of protein coding sequences in each one of the *A. niger* strains. However, we should bear in mind that their genome sequences are still near complete. The metabolic network reconstructed based on this analysis comprises twice as many (>2000) reactions, which demonstrates the power

of comparative genomics in the thorough elucidation of genome content. This network revealed that *A. niger* possesses a significant number of enzymatic activities involved in secondary metabolism or xenobiotics biodegradation. In addition, degradation pathways of different substrates like starch, cellulose, dextrin, glucan have redundant genes unique to *A. niger*. In total, more than 1000 unique *A. niger* genes were found, however, many of them have resulted from gene duplication events suggesting that gene duplication may be a key strategy for *A. niger* to adapt to different environmental conditions. Comparative genomics between different fungal species gave an answer to several important questions of fungal and eukaryotic biology. Juan E. Coronado and coworkers recently reported that cell wall proteins and proteins involved in the synthesis of the different components of the cell wall are lineage conserved and that the basic architecture of fungal walls precedes the divergence of ascomycete and basidiomycete species and shares similarities with cell wall synthesis in plants [91]. New insights have been gained about the regulation of pan-orthologous genes by analyzing their promoter regions for phylogenetically conserved regulatory elements [92-94]. Improved prediction of secretion signals and achieving from

Table 1. Sequenced *Aspergillus sp.*

Organism	Genome Size (Mb)	Coverage	Year	Status	Center/ Consortium	Reference
Aspergillus clavatus NRRL 1	35	11.4X	2005	Assembly	TIGR	
Aspergillus flavus NRRL3357	36	5X	2005	Assembly	TIGR	
Aspergillus fumigatus A1163	30	10X	2007	Assembly	TIGR	
Aspergillus fumigatus Af293	30	10.5X	2005	Assembly	TIGR	[87]
Aspergillus nidulans FGSC A4	31	13X	2003	Assembly	Broad Institute	[88]
Aspergillus niger ATCC 1015	37	8.9X	2008	In progress	DOE Joint Genome Institute	
Aspergillus niger CBS 513.88	37	8X	2006	Completed	DSM, The Netherlands	[17]
Aspergillus parasiticus			2008	In progress	University of Oklahoma	
Aspergillus terreus ATCC 20542	35		2003	Assembly	Microbia Broad Institute	[89]
Neosartorya fischeri NRRL 181	35		2005	Assembly	TIGR	
Aspergillus oryzae RIB40	37	9X	2005	Assembly	National Institute of Technology and Evaluation	[90]

this a better understanding of the fungal secretome has been addressed as well [95]. It became clear that modern fungi have complex gene structures that have undergone a great deal of intron rearrangements since they arose from their fungal-animal ancestor. In terms of eukaryotic evolution, these findings demonstrated the widespread importance of intron gain and loss, and made clear that new gene evolution models are needed. [96, 97].

From ORF to function

Genomics, and comparative genomics studies can predict the potential dynamics of the living cell, but they are restricted explicitly to the static content encoded in DNA.

Transcriptomics and proteomics, as means to study the entire populations of messenger RNA and proteins in a steady state manner can reveal the functional changes that the cell undergoes in response to the changes in environment, as they permit determination of which of the many genes or proteins present in the cell are functional under a certain condition.

The development of **transcriptomics** began in the 1980s, when Stephen P.A. Fodor and his team invented microarray technology [98]. The first microarray covering all of the encoded proteins of a eukaryotic genome was the one of *S. cerevisiae* in 1997 [99]. Since then, yeast DNA microarrays have been used to investigate differential gene expression during changes in various cellular processes and in response to environmental factors under more than 2400 different experimental conditions [100].

Up to date, microarrays have been produced for more than 20 different fungal species and the research questions addressed cover a broad range of areas including metabolism, development, pathogenesis, symbiosis and industrial applications [101]. Some examples of important findings that resulted from these studies are the identification of sugar and nitrogen responsive genes in *T. reesei* [102], *N. crassa* [103, 104], *Trametes gallica* [105], and *A. niger* [17]; the regulation of circadian clock in *N. crassa* [106-108]; the behavior of aging genes in *Metarhizium anisopliae* [109]; the identification of the gene clusters involved in the biosynthesis of aflatoxin in *A. parasiticus* [110-112] and fumonisin in *Fusarium verticillioides* [113]; the inventarisation and profiling of genes encoding

industrially important proteins in *T. reesei* [114], *A. oryzeae* [115] and *A. niger* [116], [117]; the identification of new transcriptional regulators in *A niger* [118], and the response of the fungal cell to environmental, antifungal or stress factors in *A. fumigatus* [87], *A. nidulans* [119], and *A. niger* [120-122].

Comparative transcriptome analysis between yeast, addressing the regulation of the cell cycle, has revealed a significant, but surprisingly small overlap between genes with conserved regulated expression [123]. This finding was later confirmed by comparative transcriptional analysis between these two yeast species and human [124]. Earlier this year, the first multiplex GeneChip microarray covering the genomes of three different *Aspergillus* species was made available. The simultaneous comparison of the transcriptomes of the model organism *A. nidulans* with the two industrial enzyme producers *A. niger* and *A. oryzae* in response to xylose and glucose was reported. As a result, 23 genes with a conserved response across all three *Aspergilli* were identified, including the xylose transcriptional activator XlnR together with a set of hemicellulose degrading enzymes and several transporters. This is the first comparative transcriptomics study of filamentous fungi [125]. The results of these studies indicate that, although biological systems appear to be evolutionary conserved on genome level in the form of orthologous structural proteins and transcription factors, the transcriptional regulation of the majority of them is not, and thus cannot be inferred by comparative genomics.

Starting with the sequencing of *S. cerevisiae* genome in 1996, fungal-biological studies rapidly entered the era of post genomics research. Within the last 5 years, the number of sequenced fungal genomes increased from 1 to 17, and more than 40 others are currently undergoing this process. The amount of data accumulated from whole genome, transcriptome, and proteome research in fungi, allows us now to answer several questions which were difficult (or impossible) to address by means of traditional methods. The presence of complete genome sequences for several fungal species makes it possible to unlock the complete potential of these fungi in terms of novel enzymatic activities and bioactive compounds. The combination of genomic and expression data permits the reconstruction of entire fungal metabolic and regulatory networks, the solving of concealed biochemical pathways and a better understanding of the dynamics of cellular response.

Disclosing the carbohydrate modifying network of *Aspergillus niger* by functional genomics

In 2002, as a part of the Dutch Innovation Oriented research Program (IOP) on Genomics, a research consortium called “Disclosing the carbohydrate modifying network of Aspergillus niger by functional genomics” (CarbNet) was set up between the universities of Wageningen, Leiden and Groningen and with the contribution of several Dutch biotech companies. The main goal of this project was to determine the complexity of the carbohydrate modifying enzyme network of *A. niger* involved in the degradation of the complex plant cell wall polysaccharide pectin and the plant storage polysaccharides starch and inulin in a comprehensive manner by means of a full-scale functional genomics approach (Fig. 7). Exclusive access to the genomic sequence of *A. niger* and the custom-made Affymetrix microarrays was provided by the Dutch life sciences company DSM, one of the industrial partners in the research consortium. The research described in this thesis was part of the CarbNet project and is focused on the genome mining and transcriptional profiling of the enzymatic network of *A. niger* involved in pectin degradation and utilization.

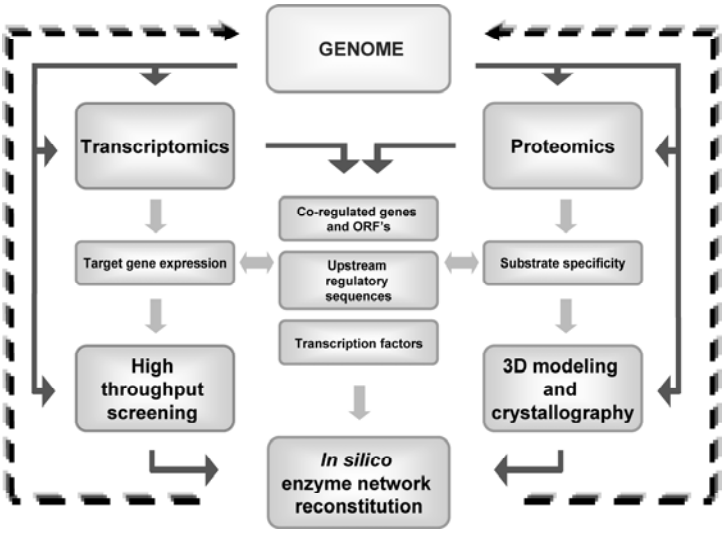


Figure 7. Schematic overview of the CarbNet project

Chapter 2

Construction and sequencing of cDNA libraries from *Aspergillus niger* grown on sugar beet pectin and apple pectin

Elena Martens-Uzunova and Peter Schaap

Three directional cDNA libraries were constructed from mRNA samples obtained from *Aspergillus niger* N400 cultures grown on apple and sugar beet pectin. At least 100, 000 clones per each single library were obtained. Restriction analysis, combined with 5' end sequencing of more than 200 randomly selected clones demonstrated that approximately 60% of them contain full-length inserts. Functional annotation of the sequenced clones using BLAST searches revealed a relative abundance of ESTs coding for proteins involved in central carbon metabolism. Among these, several were predicted to be involved in the catabolism of galacturonic acid.

Abbreviations

EST, expressed sequence tag; ORF, open reading frame; SBP, sugar beet pectin; AP, apple pectin; PGA, polygalacturonic acid.

Introduction

Pectin is a plant polycarbohydrate that can be used as a nutritional source by bacteria and fungi. The complete decomposition of pectin requires a vast set of different enzymatic activities. *Aspergillus niger* is a filamentous fungus commonly present on decaying plant material. Because of its saprobic nature, this fungus secretes a wide range of extracellular polycarbohydrate degrading enzymes, many of which can degrade pectin [126]. So far, more than twenty genes encoding pectinolytic enzymes from *A. niger* have been cloned and characterized [45]. However, the determination and annotation of the genomic sequence of *A. niger* CBS 513.88 [17], revealed a number of ORFs putatively coding for additional pectinase activities. It has been demonstrated that analysis of EST sequences can be an effective approach for gene discovery and annotation [127]. In an effort to isolate some of these putative genes, we constructed three pectin-specific cDNA snapshot libraries that were synchronized with high expression of known pectinases, and annotated over 200 clones.

Materials and Methods

Strains and Growth conditions

Wild-type *A. niger* N400 (CBS 120.49) was used in all experiments described in this study. For Northern blot analysis and for the construction of cDNA libraries, 300 ml minimal medium [128], pH 6.0, containing 0.1% (w/v) yeast extract and Vishniac trace elements [129] with 2% (w/v) D-glucose (Merck) as a sole carbon source was inoculated with 10^6 spores/ml and cultivated at 30°C at 250 rpm in an orbital shaker. After 18 h of incubation, mycelium was harvested on a Büchner funnel with nylon gauze, washed once with sterile 0.9% (w/v) NaCl, and aliquots of 1.5 g (wet weight) mycelium were transferred to 50 ml minimal medium, 0.1% (w/v) yeast extract, Vishniac trace element solution, pH6.0, and 1% (w/v) of the various sole carbon sources: D-glucose (Merck), polygalacturonic acid (United States, Biochemical Corp.), sugar beet pectin (GENU, Copenhagen pectin) and apple pectin

(GENU, Copenhagen pectin). The sugar and uronic acid content of the polymeric substrates *viz*. polygalacturonic acid, sugar beet pectin and apple pectin used in this study are given in Supplementary table 1. Mycelium was harvested at different time points after transfer on a Büchner funnel with nylon gauze and immediately stored at -70°C.

RNA isolation

Total RNA was isolated from frozen ground mycelia using TRIZOL® Reagent (Invitrogen™) according to the instructions of the supplier. Messenger RNA was isolated using FastTrack kit (Invitrogen, K1593). RNA concentrations were estimated using a NanoDrop® ND-1000 Spectrophotometer. Total RNA and messenger RNA quality and integrity were verified by analyzing aliquots on 1% TEA agarose gels and with the Agilent Bioanalyzer 'Lab-on-chip' system (Agilent Technologies).

Northern blot analysis

For Northern blot analysis 5 μg total RNA was denatured for 1 hour at 50 ^{o}C in a buffer containing 3.3 μl 6 M Glyoxal, 10 μl DMSO, 2.0 μl 0.1 M sodium phosphate buffer pH 7 and double distilled water to an end volume of 20 μl. RNA samples were separated on 1.2 % agarose gel in 10 mM sodium phosphate buffer pH 7.0 for 90 min at 400 mA, and transferred to Hybond-N filters (Amersham, UK) by capillary blotting using 10 * SSC as transfer buffer. Membranes were prehybridized at 68^{o}C in 0.9M sodium chloride, 90 mM sodium citrate, 1% (w/v) ficoll, 1% (w/v) polyvinylpyrolidone, 1% (w/v) bovine serum albumin, 10 mM EDTA, 0.5 % (w/v) SDS and 10 μg/μl single stranded herring sperm DNA. After 2 hours prehybridization, radioactively labeled and heat denatured probes were added to the prehybridization cocktail and incubation was continued for additional 16 hours. Blots were washed twice in high stringency washing buffer (30 mM sodium chloride, 3 M sodium citrate and 0.5 % (w/v) SDS) for 20 min at 68^{o}C, twice with low stringency washing buffer (3 mM sodium chloride, 0.3 M sodium citrate and 0.5 % (w/v) SDS) for 15 min at 68^{o}C and rinsed with 30 mM sodium chloride, 3 M sodium citrate to remove SDS. Probes for the detection of *A. niger* pectinolytic genes were generated by

PCR reactions of plasmids caring the different genes and subsequent gel extraction of the DNA fragments containing the sequence of interest (Table 1). Probes were labeled overnight at 37°C with α-^{32}P dATP using 5 units Klenow fragment of DNA polymerase I.

Construction of cDNA libraries, restriction analysis and sequencing of individual clones

Directional cDNA libraries on sugar beet pectin and apple pectin were constructed using the "Superscript plasmid system with Gateway technology for cDNA synthesis and plasmid cloning" (Invitrogen, 182480-013) following the instructions of the supplier. Shortly, mRNA was isolated directly from mycelium grown on sugar beet pectin for 4, 8, 24, 48, and 72 hours and on apple pectin for 24, 48 and 72 hours. In order to ensure highest abundance of low level mRNA of genes difficult to detect on sugar beet pectin an "early" library (from pooled 4, 8 and 24 hours derived samples) and a "late" library (48 and 72 hours derived samples) were constructed, whereas on apple pectin only a "late" library was prepared (24, 48 and 72 hours derived samples). First strand cDNA was synthesized with Superscript II RT polymerase from 3 μg messenger RNA primed with a NotI primer-adapter, containing 15 dT residues. After treatment of the resulting hybrid RNA/DNA products with RNaseH for the removal of the RNA strand, second strand cDNA was synthesized by DNA polymerase I. A SalI adaptor was added to the ends of the double stranded cDNA products, followed by digestion with NotI endonuclease. cDNAs carrying SalI-NotI termini were size fractionated to ensure the cloning of larger inserts and to remove residual adapters and unwanted digestion products. The so prepared inserts were subsequently ligated in pCMV-SPORT6 vector carrying ampicillin resistance gene for the selection of transformants. The obtained recombinant plasmids were used to transform *E. coli* ElectroMAX DH10B cells by electroporation. Primary transformants were frozen down in 40 % (v/v) glycerol and stored at -70°C. Aliquots from the transformation mixtures were plated out on LB medium with 100 μg/ml ampicillin for determination of transformation efficiency and for further screening.

Table 1. Plasmid templates and primers used for probe generation for the detection of *A. niger* N400 pectinolytic genes generated by PCR

Gene	Enzyme	Probe size	Template plasmid	Primer sequence
*pga*I	Polygalacturonase I	0.463 kb	pIM3700	FW 5'-CACTCTTCCACTCCTTGTTC-3' RV 5'-ACAGTCAGATCCTTACCACC-3'
*pga*II	Polygalacturonase II	0.308 kb	pIM3710	FW 5'-CTGCTCACACTGATGTCTAC-3' RV 5'-CACTCATGGAGATCAAGG-3'
*pga*A	Polygalacturonase A	0.381 kb	pIM3763	FW 5'-GACGCTGTCGAGATCTACAT-3' RV 5'-GGACTCAGTCTGCTTGGTTA-3'
*pga*B	Polygalacturonase B	0.335 kb	pIM3773	FW 5'-AGCTTCTCTCTAGTCCTTCC-3' RV 5'-GGAAGAGAGTTAGTTGGTCC-3'
*pga*C	Polygalacturonase C	0.440 kb	pIM3780	FW 5'-CGAGTCTTGTCCTCTCACTT-3' RV 5'-CCTAGTACATCTGTCAGCCA-3'
*pga*D	Polygalacturonase D	0.354 kb	pIM3793	FW 5'-GACTGAGTACGCTTCGATCT-3' RV 5'-TAGGCTCGTACTTGTCCTGT-3'
*pga*E	Polygalacturonase E	0.208 kb	pIM3803	FW 5'-AACCTTGATCTGCGAGTG-3' RV 5'-CACGAGGTCTTGGACTTG-3'
*pel*A	Pectin lyase A	1.507 kb	pIM3500	FW 5'-CTCTTACCTTGGAGACGATG-3 RV 5'-CTCGGCTATCCAGATTAGAC-3'
*pel*B	Pectin lyase B	1.252 kb	pIM3570	FW 5'-CTCTTACCTCGGAGATAACG-3' RV 5'-CAGCCTCCTTCACAGAATTG-3'
*pel*D	Pectin lyase D	0.702 kb	pIM3610	FW 5'-GTATCACCGTCAACTCCAAC-3' RV 5'-TGTCAGAGGAGAAGAGAGCA-3'
*ply*A	Pectate lyase A	0.954 kb	pIM3642	FW 5'-AGATTGGCAGCAACACCT-3' RV 5'-ATAGCTGTGCTGCAGATACC-3'
*rhg*A	Rhamnogalacturonan hydrolase A	0.759 kb	pIM836	FW 5'-AACTGGAGTGGAGGTTGTG-3' RV 5'-ACGAACCAGTCCAGTACCTA-3'
*rhg*B	Rhamnogalacturonan hydrolase B	0.500 kb	pIM850	FW 5'-TGACGAACAAGGACGAGT-3' RV 5'-GCGAAGTCCTCAATGGTA-3'

Quality assessment of cDNA libraries

The quality of each of the obtained cDNA libraries was assessed by restriction analysis of randomly selected clones and sequencing of their inserts. For that, individual colonies were cultured overnight at 37^{o}C in liquid LB medium containing 100 μg/ml ampicillin. Plasmid DNA isolated from these cultures was double digested with SalI and NotI endonucleases and the size of the obtained fragments was inspected using 1 % agarose gel electrophoresis in 1x TAE buffer.

Sequencing reactions were performed using a “Thermo Sequenase” fluorescent labeled primer cycle sequencing kit (Amersham Pharmacia Biotech, Little Chalfort, United

Kingdom), and an automated sequencer (A.L.F. Express; Amersham Pharmacia Biotech) according to the manufacturer's instructions. A Cy5 labeled SP6 promoter primer 5'-ATTTAGG TGACACTATA G-3' that binds 50 base pairs upstream from the SalI restriction site on the 5' end of each insert was used in all sequencing reactions. Obtained EST sequences were annotated by BLAST search against the *A. niger* genome and proteome, and in case of no match, against the NCBI non redundant database.

Results and discussion

Determination of optimal growth conditions for the generation of cDNA libraries

To examine the expression of the various genes in the wild-type strain *A. niger* N400 (CBS 120.49) that are involved in the degradation of pectin, different growth substrates were explored. For the investigation, the complex pectic substrates apple and sugar beet pectin, and the more defined substrate polygalacturonic acid were used. *A. niger* N400 wild-type strain was pregrown on 2 % glucose for 18 hours to gain sufficient mycelium mass; subsequently, aliquots were transferred to medium containing 1 % polygalacturonic acid, 1 % apple pectin, 1 % sugar beet pectin, or 1 % glucose, which served as a control substrate. The transcriptional profiles of 13 of the *A. niger* genes encoding known pectinases (Table 1), namely *pga*I [130], *pga*II [131] , *pga*A[132], *pga*B[132], *pga*C[75], *pga*D[34], *pga*E[133], *pel*A[37], *pel*B[38], *pel*D[36], *ply*A[39], *rhg*A[35] and *rhg*B[35] were assessed by examination of their mRNA levels at 2, 4, 8 and 24 h after transfer via Northern blot analysis. To prevent cross hybridisation, PCR fragments corresponding to the most variable regions of each examined gene were used as probes. From the tested 13 genes, only 6 had a signal detectable with Northern blot analysis (data not shown). Comparison of the expression patterns of these genes defined three distinct expression groups. The first group included the constitutively expressed gene *pga*B of which mRNA could be detected in the preculture sample and in mycelium cultivated on all tested carbon sources at all analyzed time points. Notably, at the latest time point (24 h), the hybridisation signal of mRNA

corresponding to the *pga*B gene on sugar beet and apple pectin and on polygalacturonic acid was profoundly lower than compared to earlier time points. The second group included the *pga*I, *pga*C and *pel*A genes. mRNAs of these genes were not detectable in mycelium cultivated on glucose as a carbon source but were present in mycelium cultivated for 4, 8 and 24 hours on sugar beet pectin, apple pectin, and polygalacturonic acid. The third group comprised the genes *pga*II and *rhg*A, which mRNA levels were detected only in samples obtained from mycelium grown for 24 h on 1% apple pectin, suggesting that these genes are induced in a relatively late stage of the cultivation. No signal corresponding to any of the other tested genes was detected, even after prolonged exposure of the blots, which is in agreement with previously reported data except for the *pga*A gene, previously found to be constitutively expressed [132]. The failure to detect *pga*A mRNA using Northern blot hybridization could be explained by the lower amounts of total RNA used for hybridization in our experiment in comparison to the cited study, since later assessments on expression of the *pga*A gene revealed that this gene is only weakly expressed [116].

Construction and analysis of sugar beet pectin and apple pectin cDNA libraries

Three separate *A. niger* N400 cDNA snapshot libraries were constructed. All three libraries were considered representative as at least hundred thousand *E. coli* clones were obtained for each library (Table 2). To evaluate their quality, at least 50 randomly selected colonies from each library were further analyzed for the size and completeness of their inserts using restriction analysis and sequencing of the 5' end of the target insert.

The combined results obtained from these analyses indicated an average insert size between1.6 kb and 2.1 kb varying between the three libraries. Approximately 60 % out of

Table 2. Evaluation of cDNA libraries. SBE, sugar beet pectin "early library" from pooled 4, 8 and 24 h samples; SBL, sugar beet pectin "late library" (48 and 72 h samples) ; APL, apple pectin "late" library (48 and 72 h samples).

Name	Transformation efficiency	Number clones	Average insert size	% clones		
				2.5 -3 kb	>3 kb	< 0.5kb
SBE	$0.40x10^7$	100 000	1.6 kb	5%	-	-
SBL	$1.76\ x10^7$	200 000	2.1 kb	10%	22%	12%
APL	$1.66x10^7$	110 000	1.8 kb	20%	4%	4%

the total of 227 sequenced clones demonstrated the presence of a putative ATG start codon in the same reading frame as the rest of the sequence, which together with the estimated insert size of the corresponding clone, indicated the presence of full length inserts. The inserts of 227 clones from the obtained cDNA libraries were annotated by BLAST search against the near-complete genome sequence of *A. niger* CBS 513.88 and the corresponding proteome. In case of no match search was performed against the NCBI non-redundant database. ESTs from 191 clones could be assigned to putative ORFs present in the genome of *A. niger* CBS 513.88 (Supplementary table 2). 126 clones were found to contain ESTs encoding proteins from 10 different functional categories. 65 ESTs were derived from genes coding for unclassified hypothetical proteins. From the remaining 36 clones, 5 contained inserts that were equivalent to ESTs from a cDNA library collection of *A. niger* N402, which was obtained from mycelium cultured on glucose, bran, maltose, xylan, xylose, sorbitol, and lactose [127]. Interestingly, from these 5, 3 clones, namely APL01, APL03 and SBE76C02, carried inserts which sequence matched genomic DNA form *A. niger* CBS513.88, but no ORF function was assigned to the corresponding region. In addition, another clone, APL04 had an insert, which sequence was found in the genome of *A. niger* CBS513.88, but was not assigned to an ORF.

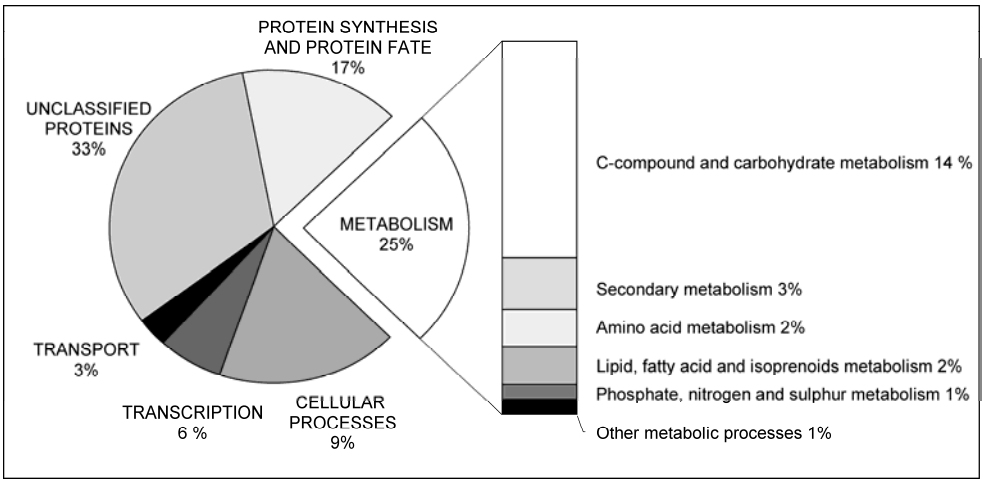


Figure 1. Functional category assignment of the 190 ESTs from analyzed cDNA library clones that were found to match corresponding *A. niger* CBS 513.88 annotated proteins. Genes that fall under the functional category "METABOLISM" are presented as a pulled out slice of the chart and are divided in subcategories.

Table 3. ESTs involved in C-compound and carbohydrate metabolism.

Library clone	Locus tag	Description
		Extracellular enzymes
SBE18	An08g01710	putative α-L-arabinofuranosidase
SBL26	An14g01800	putative α -galactosidase
SBL43	An01g10930	putative α -glucosidase with transferase activity
APL25	An01g10930	putative α -glucosidase with transferase activity
APL74	An18g03570	β-glucosidase *bgl*1
		Galacturonic acid metabolism and metabolism of C-compounds
SBE76B6	An16g05390	L-galacturonate dehydratase, *gaa*B
SBE76D4	An02g07720	2-keto-3-deoxy-L-galactonate aldolase, *gaa*C
APL65	An13g00920	putative rhamnonate dehydratase
SBE04	An10g00820	Oxaloacetate hydrolase, OAH
APL48	An04g05620	putative acetate-CoA ligase
APL40	An11g00510	putative ATP citrate lyase
SBL02	An16g01830	glyceraldehyde-3-phosphate dehydrogenase, *gpd*A
SBE76E07	An18g06760	mitochondrial NADP+-specific isocitrate dehydrogenase, *icd*A
SBE76H07	An07g02160	putative mitochondrial malate dehydrogenase
		Transporters and permeases
SBE05	An06g01370	putative Succinyl CoA: 3-oxoacid CoA transferase (mitochondrial)
SBE13	An07g02540	putative carboxylic acid transport protein , Major Facilitator Superfamily
SBE76B30	An18g01700	putative quinate transport protein
SBE76C06	An06g00560	putative hexose transporter
SBL40	An01g00850	putative xylose permease
APL21	An06g00260	putative hexose transporter
APL41	An08g01720	putative quinate transport protein
APL59	An02g00590	putative high-affinity glucose transporter
APL63	An15g04270	putative quinate transport protein
		Transcriptional regulators
SBE76G3	An17g01410	putative transcription regulator, similar to Snf2 from *S. cerevisiae*
APL14	An15g05810	xylanolytic transcriptional activator *xln*R
		Others
SBE76F07	An16g05970	putative UDP-glucuronosyltransferase
SBE76C05	An01g00620	putative p-cumic aldehyde dehydrogenase
SBE76A20	An08g06440	putative androgen-inducible aldehyde reductase
SBL9	An16g04220	putative 2-haloacid halidohydrolase
APL55	An08g05790	putative glycogen phosphorylase

From the remaining 30 clones, 10 contained an insert corresponding to the small subunit of mitochondrial ribosomal RNA, 12 clones carried no insert, five contained inserts matching regions from the genome of *Escherichia coli*, and eight had a sequence quality too poor to be used in BLAST search. (Supplementary table 2).

Within the 191 cDNA clones that returned significant BIT scores, particularly interesting was the group of genes encoding proteins belonging to the functional category "C-compound and carbon metabolism" comprising 14 % of the total (Fig. 1). This functional category included several carbohydrase activities such as β-glucosidase BGL1, a new α-galactosidase with strong similarity to guar plant α-galactosidases, a new arabinofuranosidase and a new α-glucosidase with strong transferase activity (Table 3). Furthermore, two ESTs encoded putative enzymes necessary for intracellular metabolism of galacturonic acid [134], and few enzymes involved in central carbon metabolism were found. Striking was the abundance of ESTs coding for several different putative sugar transporters as well as three transporters with high sequence similarity to the quinate transporter *qut*D from *A. nidulans* [135].

In addition, two genes encoding transcriptional regulators were identified, one of which was the well studied transcriptional regulator involved in the expression of xylanolytic genes, *xln*R [136]. The sequence of the other identified regulator displayed high similarity to the sequence of *snf*2 gene from *S. cerevisiae*, encoding a subunit of the SWI/SNF protein complex, required for many transcriptional activators to function in a chromatin context [137].

Conclusions

Sequencing of randomly selected clones from the tree pectin-specific cDNA libraries resulted in the identification of four previously unknown genes predicted to encode carbohydrate-modifying activities. In addition, a cDNA sequence encoding XlnR, the transcriptional factor regulating xylanolytic and hemicellulolytic enzymes attacking plant cell wall polymers [138] was identified. Although we ware not able to identify any

pectinases among the limited number of sequenced clones, several intracellular activities involved in the metabolism of galacturonic acid were detected.

Five of the identified clones contained inserts which sequence matched ESTs from a different cDNA library constructed from *A. niger* N402 [127]. Three of these 5 clones did not match predicted protein-coding regions in the near-complete genome of *A. niger* CBS 513.88, but we were able to identify their location in the genomic sequence of this strain (Supplementary Table 2). These results suggest that the corresponding genomic regions may actually encode active genes that have been missed during the annotation process of *A. niger* CBS 513.88.

Acknowledgments

We would like to thank Dr Joris Zandleven for the determination of sugar content of the polymeric pectic substrates used in this study.

Chapter 3

A New Group Of Exo-Acting Family 28 Glycoside Hydrolases Of *Aspergillus niger* That Are Involved In Pectin Degradation

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The fungus *Aspergillus niger* is an industrial producer of pectin-degrading enzymes. The recent solving of the genomic sequence of *A. niger* allowed an inventory of the entire genome of the fungus for potential carbohydrate-degrading enzymes. By applying bioinformatics tools, 12 new genes, putatively encoding family 28 glycoside hydrolases, were identified. Seven of the newly discovered genes form a new gene group, which we show to encode exoacting pectinolytic glycoside hydrolases. This group includes four exo-polygalacturonan hydrolases (PGAX, PGXA, PGXB and PGXC) and three putative exo-rhamnogalacturonan hydrolases (RGXA, RGXB and RGXC). Biochemical identification using polygalacturonic acid and xylogalacturonan as substrates demonstrated that indeed PGXB and PGXC act as exo-polygalacturonases, whereas PGXA acts as an exo-xylogalacturonan hydrolase. The expression levels of all 21 genes were assessed by microarray analysis. The results from the present study demonstrate that exo-acting glycoside hydrolases play a prominent role in pectin degradation.

[*] These authors contributed equally to this work.

Abbreviations

a-MHR-S, saponified apple modified ‘hairy’ regions; HG, homogalacturonan; HPAEC, high performance anion exchange chromatography; HPSEC, high performance size exclusion chromatography; MALDI-TOF MS, matrix-assisted laser-desorption ionization–time-of-flight MS; nano-RPLC/MS/MS, nano-reverse-phase liquid chromatography tandem MS; ORF, open reading frame; PG, polygalacturonan hydrolase; PGA, polygalacturonic acid; pnp-Rha, p-nitrophenyl-α-L-rhamnopyranoside; RG, rhamnogalacturonan; RGH, rhamnogalacturonan hydrolase; RT, reverse transcriptase; SBP, sugar beet pectin; XGA, xylogalacturonan; XGH, XGA hydrolase.

Introduction

Pectin is a complex heteropolymer present in the middle lamella of the primary cell wall of plants. This biopolymer accounts for about one-third of the total cell wall material [30] and as such represents an important carbon source for bacteria and fungi. Pectin is composed of a number of distinct polysaccharides [26], such as homogalacturonan (HG), xylogalacturonan (XGA), rhamnogalacturonan I (RG I) and rhamnogalacturonan II (RG II). Rhamnogalacturonan carries short and long, linear and branched side chains which are built up from neutral sugars (e.g. arabinose, galactose).

Two pectin models exist, namely a pectin structure in which HG is an extension of RGI or vice versa and has neutral sugar side chains [29], and a pectin structure that is built from a backbone of RGI where HG and neutral sugars form the side chains [26].

HG, also known as the smooth region of pectin, is an unbranched polymer composed of 1,4-linked α-D-galacturonic acid residues. XGA, RG I and RG II are part of the branched or hairy regions of pectin [28]. In XGA xylose is linked at *O*-3 of the galacturonyl residues of the HG backbone. Rhamnogalacturonan I (RGI) is composed of a backbone of alternating α-(1, 2)-linked rhamnosyl and α-(1, 4)-linked galactosyluronic acid residues [26]. The rhamnosyl residues are branched with *O*-4 attached neutral sugar side chains, which can vary, from a single galactose residue up to polymeric chains of glycosyl residues composed of arabinose and / or arabinose and galactose residues. Rhamnogalacturonan II (RGII) is comprised of a backbone of approximately nine α-(1, 4)-linked D-galactosyluronic acid residues that carry four side chains, containing rare sugars, such as apiose and aceric acid [26].

The biodegradation of this complex and heterogeneous structure of pectin requires many different enzymatic activities. Exo- and endo-polygalacturonan hydrolases (exo- and endoPG), pectin lyases (PL) and pectate lyases (PLY) degrade HG. XGA can be degraded by endo-xylogalacturonan hydrolases [32] and exoPG [139, 140], whereas rhamnogalacturonan hydrolases (RGH) [35] and rhamnogalacturonan lyases (RGL) [141, 142] degrade RG I. In addition, the complete enzymatic depolymerisation of pectin requires the presence of different types of esterase activities [126].

The industrially used saprobic fungus *Aspergillus niger* is an excellent producer of pectinolytic enzymes which, unlike those produced by many other fungi of the same genus, enjoy a GRAS status. So far 9 genes encoding pectinolytic glycoside hydrolases produced by *A. niger* have been cloned and the corresponding enzymes have been characterized in detail. The list includes seven different endopolygalacturonan hydrolases [34], and two rhamnogalacturonan hydrolases [35], all belonging to family 28 of the general classification system of glycoside hydrolases [31].

The structures of two *A. niger* endo-polygalacturonan hydrolases, PGI and PGII, have been solved [46, 47]. This, together with the biochemical data obtained from site directed mutagenesis of strictly conserved amino acids, allowed the identification of the residues involved in catalysis, substrate binding, substrate specificity and mode of action of PGII [50, 52, 53].

After the recent sequencing of the genome of *A. niger*, it became clear that only a part of the pectinase spectrum is currently explored. To obtain a complete inventory of pectinolytic glycoside hydrolases produced by *A. niger*, we applied bioinformatics and functional genomics tools. We were able to identify a total of 21 genes which belong to family 28 glycoside hydrolases. Their transcriptional levels were assessed using custom made Affymetrix gene chip DNA microarrays. Proteins from four newly discovered genes, encoding for novel exo-activities, have been overexpressed and their activities have been biochemically identified.

Materials and methods

Bioinformatics

Genome mining

The genome of *Aspergillus niger* strain CBS513.88, which is a natural derivative of strain NRRL3122, has been recently sequenced [143]. A list of accession numbers for currently available protein sequences belonging to family 28 glycoside was obtained from the CAZy web-server [144] at http://afmb.cnrs-mrs.fr/CAZY/. The corresponding 309 protein sequences were retrieved from the SWISS-PROT database [145] at

http://www.expasy.org/sprot/ and used to build a hidden Markov model profile using the HMMER package [146] from http://hmmer.wustl.edu/. The genome of *A. niger* was screened with the obtained profile using the WISE 2 package [147] from http://www.ebi.ac.uk/Wise2/.

Sequence analyses

2D alignments of identified protein sequences were performed using the T-coffee program [148] and manually curated. Dendrograms and distance measurements were performed using the Mega 3 software package [149]. Sequences were compared using the pairwise analysis algorithm with "Distances only computed" setting. Gaps/Missing Data were calculated by the pairwise deletion algorithm. The used substitution model was "Amino p-distance" with all substitutions included. Homogeneous pattern among lineages and uniform rates among sites were applied. Dendrograms were constructed by using the Neighbour-Joining Method with the same distance parameters as described above. The nucleotide sequences reported in this paper have been submitted to the DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession numbers DQ374422 to DQ374431, DQ417225 and DQ417226.

Verification of intron positions by RT-PCR

Total RNA was isolated from frozen ground mycelia using TRIZOL® Reagent (Invitrogen™) according to the instructions of the supplier. RNA concentrations were estimated using a NanoDrop® ND-1000 Spectrophotometer. cDNAs of *pgx*C and *rgx*A were amplified by two step RT-PCR. In the first step 1.5 µg total RNA from 2 h transfer cultures on D-galacturonic acid and L-rhamnose, respectively were used as a template in the reverse transcription reaction. Omniscript® reverse transcriptase (QIAGEN) was used as described by the supplier. Second step RT-PCR was performed with 1µl product from the reverse transcription reaction using 2.5 units SuperTaq Plus polymerase (SphaeroQ, Leiden, The Netherlands) under standard PCR conditions. Prior to sequencing, two overlapping fragments per messenger were amplified with the following primers: for the amplification

of pgxC 5'fragment: pgxCAFw 5'-CGTCATGTCTGTCTTCAAGG-3' and pgxCBRv 5'-TGATACTGTCGGTGATCTGG-3'; for the amplification of pgxC 3'fragment: pgxCDFw 5'-CACTGCGGTGCAGAATATAG-3' and pgxCCRv 5'-GCGTTCATGCAGATCAC-ACT-3'; for the amplification of rgxA 5'fragment: rgxAAFw 5'-GGTATC-GAGGTGAGCCAGGA-3' and rgxABRv 5'-TATGGTCGGATCGGTACGTG-3'; for the amplification of rgxA 3'fragment: rgxADFw 5'-GGCTGTTCTTGTCGATT-CCT-3' and rgxABRv 5'- CAGCGACAGCTCACGAATTG -3'; RT-PCR products were gel purified with QIAquick® gel extraction kit (QIAGEN) and outsourced to BaseClear Labservices (BaseClear B.V., Leiden, The Netherlands) for sequence determination.

Transcriptional profiling

Strains and Growth Conditions

Wild type strain *Aspergillus niger* N400 (CBS 120.49) was used in all transcriptional profiling experiments. 300 ml minimal medium [128], pH6.0, containing 0.1% (w/v) yeast extract and Vishniac trace elements [129] with 2% (w/v) D-fructose as a sole carbon source was inoculated with 10^6 spores/ml and cultivated at 30°C at 250 rpm in an orbital shaker. After 18 h of incubation mycelium was harvested on a Büchner funnel with nylon gauze, washed once with sterile 0.9% (w/v) NaCl, and aliquots of 1.5 g (wet weight) mycelium were transferred to 50 ml minimal medium, pH6.0, 0.1% (w/v) yeast extract, Vishniac trace element solution and 1% (w/v) of the various sole carbon sources: D-fructose (Merck), D-glucose (Merck), D-galacturonic acid (Fluka Chemica), L- rhamnose (ACROS organics), D-xylose (Merck), D-sorbitol (Merck), polygalacturonic acid (United States, Biochemical Corp.) and sugar beet pectin (GENU, Copenhagen pectin). At 2, 4, 8 and 24 h after transfer mycelium was harvested on a Büchner funnel with nylon gauze and immediately stored at -70°C. The amount of monomeric sugars remaining in the culture fluid was assessed by standard HPLC techniques [140].

RNA manipulations and microarray processing

Before and during microarray processing RNA quality was verified by analyzing aliquots with 1% TAE agarose gel electrophoresis and Agilent Bioanalyzer "Lab on chip" system

(Agilent Technologies, Palo Alto, CA). Messenger RNA levels were assessed using custom made "dsmM_ANIGERa_coll" Affymetrix GeneChip® Microarrays kindly provided by DSM Food Specialties (Delft, The Netherlands).

Probe labeling, hybridisation and scanning

Total RNA from mycelium samples was amplified, labeled and hybridized strictly following the Affymetrix protocols for "Eukaryotic Target Preparation" and "Eukaryotic Target Hybridization". For probe array wash and stain the "Antibody Amplification Washing and Staining Protocol" was used. Probe arrays were scanned with Agilent technologies G2500A Gene Array Scanner at pixel value 3 µm and wavelength 570 nm. Raw intensity measurements and present/absent calls were derived in Microarray Suite Software version MAS5 (Affymetrix, UK Ltd) after applying "Mask all outliers" algorithm. All chip data were scaled to an arbitrary target gene intensity of 500.

Cloning and overexpression of enzymes

PGXA, PGXB and RGXB were overexpressed in *A. niger* PlugBug® [150] and kindly supplied by DSM Food Specialties (Delft, The Netherlands).

Cloning and overexpression of PGXC

Molecular work was essentially carried out using standard techniques [151]. All enzymes were used according to the instructions provided by the supplier. *A. niger* genomic DNA was isolated as described [152] 1.5 kb PCR fragment containing the *rgx*C gene was amplified using primers PGXCFw 5'-TGG CAT GGC AAT TGG AGA CC-3' and PGXCRv 5'-GTG TGG CTT CCT GTG GAT GG-3'. The obtained product was used as a template in a subsequent nested PCR reaction using primers PGXC3NsiIFw 5'-GCA TCG TCA TGC ATG TCT TC-3'and PGXCNotIRv 5'-TGG CGG CGG CTG GAT GGC TTA-3' for the introduction of restriction sites NsiI and NotI at the ATG start codon and 73 bp downstream the stop codon of the pgxC gene respectively. Both PCR and nested PCR were performed using Pfu Turbo Polymerase (Stratagene). The nested PCR product was NsiI/NotI digested, gel-purified using QIAquick® gel extraction kit (QIAGEN) and ligated

in plasmid pIM3710 [153] resulting in pgxC overexpression vector pIM5150.
Transformation of *A. niger* NW188 (*csp*A1, *pyr*A6, *leu*A1, *prt*F28, *gox*C17) was performed as described [154] using 1 μg of pGW635 [155] and 20 μg of co-transforming pIM5150. Control transformation with 1 μg of pGW635 was performed in parallel. Positive transformants determination and enzyme production were performed as described [133] with the following modifications: STREAMLINE SP and Citrate buffer pH 3.8 were used instead of Sephadex DEAE and 10 mM piperazine/HCl. Following dialysis further purification steps were performed as described below.

Enzyme purification and gel electrophoresis

PGXA, PGXB, PGXC, and RGXB, were purified by dialysis overnight at 4°C against 50 mM NaAc (pH 5.7) and subsequent size-exclusion chromatography on a Hiload 16/60 Superdex 75 column (Amersham Pharmacia Biotech, Uppsalsa, Sweden) equilibrated with 50 mM NaAc (pH 5.7). The elution was carried out with the same buffer at a flow rate of 1 ml/min, and fractions of 2 ml were collected. Protein homogeneity was determined by SDS-PAGE on a 8-25% polyacrylamide gradient gel under reducing conditions, using the PhastSystem and the protocol of the supplier (Amersham Pharmacia Biotech, Uppsala, Sweden). The gels were stained by Coomassie Brilliant Blue. Low molecular mass standard proteins (14.4-94 kDa) were used to determine the molecular mass of the purified enzymes.

Substrates

PGA and XGA

Xylogalacturonan (XGA-25) was prepared from Gum Tragacanth by treatment with alkali and trifluoro acetic acid (TFA) and had a Xyl:GalA ratio of 1:4 [156]. Polygalacturonic acid (PGA) was from ICN Biomedials Inc., (Ohio, US). As substrates for rhamnosidase activity, p-nitrophenyl-α-L-rhamnopyranoside (pnp-Rha; Sigma Chemical Co, ST Louis, USA), naringin (Sigma Aldrich, Steinheim, Germany), and hesperidin (Fluka, Steinheim, Germany) were used.

Amidated and methylated pectin

Three commercial pectins, A, B and E extracted from lemon peels were obtained from Degussa Texturant Systems SAS (Baupte, France). These pectins are partially methyl-esterified with a degree of methyl-esterification (DM) of 74%, 72 % and 30% respectively, where the distribution of methyl-esters is blockwise for pectin A and random for pectin B [157].

Two other commercial pectins, O5 and O27, were obtained from DANISCO. These pectins have a degree of amidation (DA) of 5% and 27% respectively. Prior to use the methyl esters were removed from O5 and O27 by saponification [158]. Saponified amidated pectins are referred to as O5sap and O27sap.

Linear rhamnogalacturonan oligosaccharides

Saponified apple MHR (a-MHR-S) was prepared as described [159]. Enzymatic liquefaction of apples for the preparation of a-MHR-S was performed with a commercial enzyme preparation (Ultra-SP; Novozymes, Copenhagen, Denmark). This substrate was further treated with rhamnogalacturonan hydrolase (RGH) and fractionated by preparative size exclusion chromatography (HPSEC) as described [160]. Fractions 45-60, which contained oligomeric products, as determined by HPSEC analysis, were pooled and freeze-dried. As this material still contained some polymeric residue, further fractionation was performed by gel filtration on a Sephadex G50 column (GE Health Care, Uppsala, Sweden), using Millipore water as eluent at a flow rate of 1 ml/min. Fractions 89-100, which contained primarily oligosaccharides, as determined by HPSEC analysis, were pooled and subsequently freeze-dried. This pool is referred to as a-MHR-S-fr1. Since the activity of a RG-rhamnohydrolase and a RG-galacturonohydrolase towards rhamnogalacturonan oligosaccharides is hindered by Gal side chains [161], a-MHR-S-fr1 was treated with β-galactosidase [162] for 19 h at 40°C in 20 mM bis-Tris (pH 5). The final protein concentration was 2.2 μg/ml. The enzyme was inactivated by heating for 10 min at 100ºC. Subsequently, β-galactosidase treated a-MHR-S-fr1 (a-MHR-S-fr1-g) was desalted on a Biogel P2 column (BioRad, CA, U.S.A.) using Millipore water as eluens at a flow rate of 1 ml/min. After desalting, a-MHR-S-fr1-g was analyzed by MALDI-TOF MS [160], for the presence of rhamnogalacturonan oligosaccharides. Four linear rhamnogalacturonan

oligosaccharides were detected at m/z 539, m/z 685, m/z 861 and m/z 1007. Based on the mode action of RG hydrolase towards rhamnogalacturonan it is concluded that the produced rhamnogalacturonan oligosaccharides with a m/z at 685 and m/z at 1007 correspond to Rha-GalA-Rha-GalA and GalA-Rha-GalA-Rha-GalA respectively. The rhamnogalacturonan oligosaccharides at m/z 539 and at m/z 861 correspond to GalA-Rha-GalA and GalA-Rha-GalA-Rha-GalA. As these latter two oligosaccharides cannot be produced directly from RG hydrolase treated rhamnogalacturonan it is assumed that these oligosaccharides are formed due to side activities in the RG hydrolase preparation, in particular RG-rhamnosidase activity.

Enzyme activity measurements

PGXA, PGXB, PGXC and RGXB were analyzed for their activity towards 0.25% (w/v) PGA, 0.25% (w/v) XGA-25, and 0.25% (w/v) of the pectins, encoded as A, B, C, O5sap, and O27sap. ExoPG from *Aspergillus tubingensis* [163] was included as a reference. The action of RGXB was also studied towards 0.25% (w/v) narigin, 0.25% (w/v) hespiridin, 0.02% (w/v) α-L-pnp-Rha, and 0.5% (w/v) a-MHR-S-Fr1-g. For comparison, this latter substrate was also treated with rhamnogalacturonan galacturonohydrolase from *A. aculeatus* [164]. All digests were made in 50 mM NaAc (pH 4.0) for 16 h at 30°C. The final protein concentration was 1 μg/ml. Enzymes were inactivated by heating the reaction mixtures for 10 min at 100°C. All digests were analyzed by HPSEC and HPAEC, as described [33], except for RG-galacturonohydrolase or RGXB treated a-MHR-S-Fr1-g which were analyzed by MALDI-TOF MS as described [160]. Products released from XGA-25 and PGA after 1 hour of incubation with PGXA, PGXB, PGXC or Exo-PG were also analyzed by HPAEC. The specific activity of PGXA, PGXB, PGXC and ExoPG was determined towards 0.25% (w/v) PGA and 0.25% (w/v) XGA-25, as described [133]. Specific activity is in units per mg of enzyme (U/mg), where one unit is defined as one micromole of reducing sugar released per minute. The influence of pH on enzyme activity was determined as described [133].

Identification of RGXC protein sequence by MS analysis

Tryptic digest of RG-galacturonohydrolase from *A. aculeatus*

Protein concentration of RG-galacturonohydrolase [164] was determined by the Bradford assay (Bio-Rad, Hercules, CA) and digested with trypsin as described [165]. The tryptic digest was subsequently acidified to a pH 3.0 by the addition of 10% formic acid. The sample was then analyzed by nano-RPLC/MS/MS.

Nano-LC- nano-spray mass spectrometry

LC-MS/MS analysis of the tryptic peptides was performed on an integrated Famos/Switchos/Ultimate 1D/2D nano-flow HPLC system (LC Packings, a division of Dionex, San Francisco, CA, USA), as described [166] with the following adaptations: The peptides were first trapped on a LC Packings C18 precolumn (300 μm i.d. x 5 mm, 100 Å) and eluted after desalting onto a Pep Map C18 resolving column (3 μm, 100 Å, 75 μm I.D. x 15 cm, LC Packings, Dionex) using the following elution profile: a linear gradient of increasing acetonitrile concentration in water (5-30%) over 40 min, isocratic at 30% for 5 min, a linear gradient (30-95%) over 30 min, isocratic at 95% for 5 min. All eluents contained 0.05% formic acid as the ion-pairing agent.

The electrospray needle was operated with a voltage differential of 3.0 kV, and the heated desolvation capillary was held at 180^{o}C. The specific mass-to-charge value of each peptide sequenced by tandem mass spectrometry was excluded dynamically from reanalysis for 2 min.

Analysis of MS/MS and MS3 data

Data analysis of each raw tandem spectrum was performed as described [167], with adaptations. Processed tandem mass spectra were correlated with the various public protein databases and the *A. niger* protein database (DSM, Delft, The Netherlands) using the program Bio Works 3.1 (Thermo Finnigan). The allowed mass tolerance range between expected and observed masses for search was +/- 1.0 Da for MS peaks, and +/- 0.1 Da for MS/MS fragment ions. The correlation results were then filtered using the value of the cross-correlation score and the matched sequence for each spectrum. For singly charged peptides, spectra with a cross-correlation score to a tryptic peptide ≥ 1.5 were retained. For

multiply charged peptides, spectra with a cross-correlation to a tryptic peptide ≥ 2 were retained. All spectra with cross-correlation scores not meeting these criteria were eliminated from further consideration.

Results and discussion

Genome mining

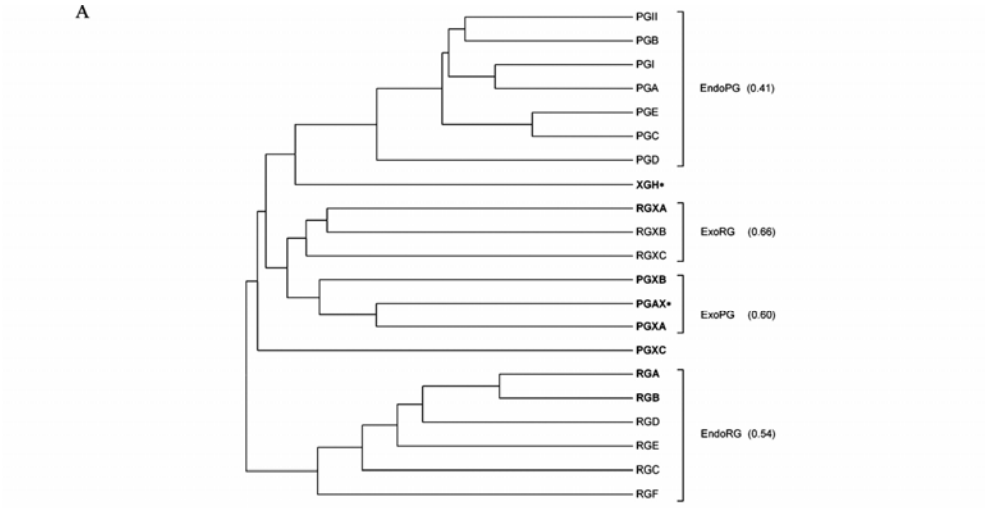
Screening the genome sequence of *A. niger* with a hidden Markov model profile for glycoside hydrolase family 28 returned 21 significant alignments from which only 9 correspond to already known *A. niger* proteins. The start and the end of each alignment were extended to the nearest in frame start-codon and nearest stop-codon respectively. Further analysis of the Gene Wise output indicated possible frame shifts in the sequences of *pgx*C and *rgx*A therefore the corresponding cDNAs were sequenced full length. Comparison of the obtained cDNA sequences with the predicted gene models demonstrated the presence of one frame shift in the genomic sequence of *rgx*A and 3 frame shifts in the genomic sequence of the *pgx*C. These corrected sequences were used for further analysis.

Comparison of *A. niger* glycoside hydrolases

The protein sequence alignment of the complete *A. niger* glycoside hydrolase family 28 and the corresponding sequence distance dendrogram are presented in Figure 1. Based on sequence similarity three major groups are observed (Fig. 1 A). The first group is composed of all previously known endopolygalacturonases (endoPG) of *A. niger*. No newly identified sequences are added to this group. The second group contains six newly discovered ORFs (open reading frames) of *A. niger* of which one designated PGAX is a very close homolog of *A. tubingensis* exo-polygalacturonase ExoPG [163] with 99% amino acid identity. Based

on sequence similarity, the six ORFs can be divided into two sub-groups (Fig. 1A) each comprising three proteins. PGAX forms a subgroup with PGXA and PGXB, therefore it is very likely that PGXA and PGXB are also exopolygalacturonan hydrolases. RGXA, RGXB and RGXC form the second distinct subgroup and evidence is presented that they are similar to rhamnogalacturonan galacturonohydrolase [164] (see below). The conservation of intron positions within ORFs of both sub-groups further underpins this subdivision. The most deviating sequence from all 21 identified proteins is PGXC. Also, there are no introns in the encoding gene *pgx*C to support its classification. However, exo-polygalacturonan hydrolase activity was determined for the encoded enzyme (see below). The last group includes two previously characterized endo-acting *A. niger* proteins – rhamnogalacturonan hydrolase A (RGHA) and rhamnogalacturonan hydrolase B (RGHB) as well as 4 putative enzymes which we propose to be endo-rhamnogalacturonan hydrolases. Finally XGH, the strongly conserved homolog (97% amino acid identity) of xylogalacturonan hydrolase of *A. tubingensis* [32] did not group together with any of the other sequences.

Detailed inspection of the amino acid conservation between all 21 sequences revealed that only one of the 4 disulfide bridges between columns 544 and 550 of the multiple alignment (Fig. 1 B) demonstrated by the protein structures of PGII of *A. niger* [46] and RGA of *A. aculeatus* [168] is well conserved within family 28 glycoside hydrolases of *A. niger*. The other three cysteine bridges are well conserved in the groups of endoPGs and endoRGs but not in the exo-hydrolase group. Inspection of the catalytic residues revealed that all previously identified catalytic Asp residues are conserved within the *A. niger* family except for Asp362 (Fig. 1B). In the group of rhamnogalacturonan hydrolases Asp362 is replaced by Glu which is characteristic for this type of enzymatic activity [168]. The substrate binding Lys at alignment position 428 is 100% conserved while the second substrate-binding residue Arg426 is present in all endoPG sequences and in only four of the seven proteins from the exo-group, *viz.* PGAX, PGXA, PGXB and PGXC. Armand and co-workers [50] have demonstrated by site directed mutagenesis that the corresponding Arg of *A. niger* PGII (Arg256) is essential for the proper orientation of the substrate in the catalytic cleft and mutations of this residue dramatically affect the substrate affinity of the enzyme. Interestingly, other amino acids shown to be involved in substrate binding at subsite +1,



B

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                340                 360                 380                 400                 420                 440
                *. *                .**                 .    +1 +1          .                   .   +1-1           .
PGII   ---GGHNT-AFDVGNSVGVNIIK-PWVHNQ-DCLAV--NSGENIWFTGGTCI-GGHGLSIGSVGDRSN--NVVKNVTIEHSTVSNSEN-----AVRI-TIS--------- :234
PGI    ---GGHNT-GFDISESTGVYISG-ATVKNQ-DCIAI--NSGESISFTGGTCS-GGHGLSIGSVGGRDD--NTVKNVTISDSTVSNSAN-----GVRI-TIY--------- :236
PGA    ---GGHNT-GFDISESTYITITG-ATVKNQ-DCVAI--NSGENIYFSGGTCS-GGHGLSIGSVGGRDD--NTVKNVTFIDSTVSDSEN-----GVRI-TVY--------- :235
PGB    ----GHNT-AFDIGQSTYITIDG-ATVYNQ-DCLAI--NSGEHITFTNGYCD-GGHGLSIGSIGGRSD--NTVNDVTISNSKVLNSQN-----GVRI-TIY--------- :233
PGE    --EAA-NT-GFDIGDSTYITITG-ANVYNQ-DCVAV--NSGENIYFSGGVCS-GGHGLSIGSVGGRSD--NTVKNVTFYDSEIKSSQN-----GVRI-TIY--------- :240
PGC    -DLAA-NT-GFDIGESTYITITG-AEIYNQ-DCVAI--NSGENIYFSASVCS-GGHGLSIGSVGGRDD--NTVKNVTFYDVNVLKSQQ-----AIRI-TIY--------- :241
PGD    GDPAAHNT-GFDIKQSDFLTLSN-SWVHNQ-DCVAV--TSGSSIVVDNLYCY-GGHGLSIGSIGGKSN--NTVDGVTFSNSQVINSEN-----GCRI-SNA--------- :381
XGH    --NPPKNT-GFDIGESTYVTITE-VTVVND-DCVALKPSS-NYVTVDTISCT-GSHGISVGSLGKSSD--DSVKNIYVTGATMINSTK-----AAGI-TYPSGG------ :274
PGXC   -GSPPFNT-GFD-VEGTNIQITD-SIMYNG-DAIAVGADSHDTLFTRNTIGY-QTHGMSIGSLGKDPTDFANISNIRFDDVTVVDGLY-----AAFF-SWSG-------- :275
PGXA   -DAPAKNT-GWDIYRSSNIVIQD-SRIVNT-DCVSFKPNSTQIVI-QNLDCT-GSHGISVGSLGQYQGETDIVEDLYIYNISMTDASD-----VARI-VWPGVPA----- :294
PGAX   -DNEAKNT-GWDTYRSNNIVIQN-SVINNG-DCVSFKPNSTNILV-QNLHCN-GSHGISVGSLGQYKDEVDIVENVYVYNISMFNASD-----MARI-VWPGTPS----- :289
PGXB   -SSDPANT-GMDSLDVDGVSFTN-MRIDVG-DCFSPKPNTTNIFV-QNMWCN-NTHGVSMGSIGQYAGEMDIIENVYIENVTLLNGQN-----GARL-AWAGQDV----- :306
RGXC   GDNWVQNT-GFDTMDATNIQLTN-FVYQGG-DCIAIKPRSYNIDI-QNVTCR-GGNGIAIGSLGQYL-EDSSVANIRVDKVNIIRYNED-MHNSAYL-TWVGALVPQSSY :284
RGXB   DGGSAQNT-GANTIYSKNITLTN-WEVVNG-DSISTKANSTDITI-ANCTFT-SGLGIAIGSIGQYNGAFETVERLKISNITYEKTTH-----AVYF-TWTGDQV----- :306
RGXA   EGEWVQNT-GIDTYRSDHITVTN-WVYQGG-DAVAFKGNSTNIHV-ENVTVY-GGPGIAFGSLGQYPDRTDIVENVTVRNVRVQPSFQRAMNSGVYF-SWIGVNY----- :288
RGA    ------GL-GIDVW-GSNIWVHD-VEVTNK-ECVTVKSPA-NNILVESIYCN-WSGGCAMGSLGAD----TDITDILYRNVYTWSSNQ-----MYMI-SN---------- :256
RGB    ------GL-GIDVW-SNNIWVHD-VEVTNK-ECVTVKSPS-KNILIESIYCN-WSGGCGMGSFGSD----TNVSDITYRNIYTWSSNN-----MMLI-SN---------- :256
RGD    ------GL-GIDIS-GANYWIHD-VEVTNG-ECVTVKSPS-ANVRVENVFCN-HSGGCAMGSLGTD----TNISNIEFENIYTYNSTQ-----MYMI-SN---------- :257
RGC    ------AT-AIDVW-GENMWPHD-IEVSNG-ECVTVKSPA-HNYLIENIYCN-LSGGTAIGSLGTG----TNISDIHYRNLYM-NQAD-----ACFL-SN---------- :255
RGE    ------GL-GIDLY-GGNIWIHL-VMVTNK-ECVTSKTNS-HNFLIENIYCN-PSGGCAIGSLGSS----VNVTNILYRNVYTWDSNQ-----MMMI-TN---------- :258
RGF    ------SY-GIDAI-GTNYYIHD-NEVTNR-ECVSVKSPS-HHALVENLVCNQAGSGVSIGSLNVS----AEISNIEARNISIIQGNN-----IAFI-TYP--------- :255

                460                 480                 500                 520                 540
                .                   +1.                 .                   .                   .   #   #
PGII   ---------GATGSVSEITYSNIVMSGISDYGVVIQQDYEDGKPT----GKPTNGVTIQDVKLESVTGSVDSGA----------------------TEIYLLCGS-GSC :307
PGI    ---------KETGDVSEITYSNIQLSGITDYGIVIEQDYENGSPT----GTPSTGIPITDVTVDGVTGTLEDDA----------------------TQVYILCGD-GSC :309
PGA    ---------DATGTVEDITYSNIQLSGISDYGIVIEQDYENGDPT----GTPSNGVTISDVTLEDITGSVDSDA----------------------VEIYILCGD-GSC :308
PGB    ---------GKTGTVENVKFEDITLSDISKYGIVVEQDYENGSPT----GTPTNGVKVEDITFKKVTGSVKSSG----------------------TDIYILCGS-GSC :306
PGE    ---------GDTGSVSEVTYKEITLSDITDYGIVVEQNYDDTS------KSPTDGITIEDFVLDNVQGSVESSG----------------------TNIYIVCGS-DSC :311
PGC    ---------GDTGSVSEVTYHEIAFSDATDYGIVIEQNYDDTS------KTPTTGVPITDFVLENIVGTCEDDD----------------------CTEVYIACGD-GSC :313
PGD    ---------DTTGEVYNVKYENITLSGISDYGIDIQQDYENGGAT----GDPTNGVKIENISFVNVKGTMSDGK----------------------DYIYLCGD-GSC :453
XGH    --------DHGTSTVSNVTFTDFTVDN-SDYAFQIQSCYGE-DDDYCE-ENPGNA-KLTDIVVSSFSGTTSDKY----------------------DPVVANIDCGSDGTC :351
PGXC   ----------GTGLVKNVTWNNIRVFNVT-FPIFVTQSYSDQGASRSGTVNASSAVMMEDFTWSDFAGSINTYQPGDGSCVSDPCWYNVGLPNLKHTEALIIECHTAQSC :374
PGXA   DTSGS-TSGGGLGRVRNVTYEHMQSENND-HIISVSQCYESKNQTMCDSY-P-SKLVIEDVLFKDFKGTTSK----------------------KYDPEIGELTCSSPDVC :379
PGAX   ALSADLQGGGGSGSVKNITYDTALIDNVD-WAIEITQCYGQKNTTLCNEY-P-SSLTISDVHIKNFRGTTSG----------------------SEDPYVGTIVCSSPDTC :375
PGXB   ----------GYGRINNVTYKNIQIQNTD-APIVLDQCYFDINATECAKY-P-SAVNITNILFENIWGSSSG----------------------KDGKIVADLVCSPDAVC :382
RGXC   E-SAGVPRGDGWGSIRNVLFSNFNVQGAS-AGPSISQDSGDN-----GSYAGTSKMSISNVAFVNFTGWVD----------------------TEKSVVSTVSCSEVHPC :365
RGXB   GYPPN-GGGGGLGYASDIVATNLKTNNLKGAPFTISQCTTFSGAS---GNCTNSKFQIRDLVFTDISGTTDS----------------------SDVASFQCSAVAPC :388
RGXA   GVPPN-GGGGGHGYVRNVSVENLRLKDVQ-LPVYIDTCLSYLFSENITQYCDTSTYEFEDLHFRNISGNGLA----------TVTDYPGKNISFAVALLCSEKAPC :382
RGA    ---------GGSGTVNNTVLENFIGHGNA-YSLDVDSYWSSMTA------VDGDGVQLSNITFKNWKGTEADGA----------------------ERGPIKVVCSDTAPC :329
RGB    ---------GGSGFVENVLLENFIGHGNA-YSLDIDSYWASMSA------VDGDGVQLSNITVKNWKGTEAYGA----------------------ERGPVKVVCADGAPC :329
RGD    ---------GGNGTVTNCSFKNFIGYSNA-YMLDLDTYWGDES--------DGDGIKYENIGFENWKGTSSNGI----------------------QRSPIRILCPDANPC :328
RGC    ---------NGDGIVKNIIWENVIVHGGP-YPLAIDEAWGDDRG--------SVGVQVSNLTFRNWHGESVSA----------------------SRPVIRLQCDSDVPC :325
RGE    ---------GGLGNVSNIVFENFIGHGNV-NSLDLDSYWSSMNA------IDGVGIYYHNITIYNWTGTAIDGE----------------------TRPPIRVICPEDMPC :331
RGF    ---------GGSGYVKDVTFENFRSLNSL-YGLDINQYWQNTWE------PDTGSVTLSNLVFKNFSGSVADGA----------------------LRPPLYLFASDLTFA :328
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His386 and Ser389 are conserved within the same subset of sequences. The same is true for the Tyr479, which, in PGII, is involved in the stabilization of the substrate at subsite +1 [50, 169]. The partial conservation of amino acids responsible for substrate specificity and binding within the exo-hydrolase group further supports the suggestion that only PGXA, PGXB, PGXC and PGAX have exo-polygalacturonan hydrolase activity.

Identification of RGXC as a close homolog of *A. aculeatus* RG galacturonohydrolase

A rhamnogalacturonan galacturonohydrolase activity, specifically removing terminal galacturonosyl residues from rhamnogalacturonan I, has been characterized in the commercial mixture Pectinex Ultra SP produced by *A. aculeatus* [164]. In order to identify a possible *A. niger* homolog of this enzyme a tryptic digest of the *A. aculeatus* protein was analyzed by MS. The obtained peptide masses were used to search the inferred *A. niger* proteome. At least 6 peptides perfectly matched the sequence of RGXC resulting in a total of 29% overall coverage of the protein (data not shown). Based on sequence similarity, RGXC is grouped together with RGXA and RGXB indicating that there are three candidate genes encoding proteins with exo-rhamnogalacturonan hydrolase activity.

Transcriptional profiling

The complex substrate sugar beet pectin (SBP) and polygalacturonic acid (PGA) were chosen as primary carbon sources to investigate expression of selected pectinolytic genes. Galacturonic acid, rhamnose and xylose represent the most abundant sugar residues present effects on gene expression caused by simple well defined carbon sources. Fructose as astrong repressor of the expression of genes that are under carbon catabolite regulation in homogalacturonan, rhamnogalacturonan and xylogalacturonan and were used to asses the

Figure 1. Comparative sequence analysis of *A. niger* family 28 glycoside hydrolases. (A) Dendrogram of *A. niger* family 28 glycoside hydrolases. EndoPG: endo-polygalacturonan hydrolases; XGH: xylogalacturonan hydrolase; ExoPG: exopolygalacturonan hydrolases; ExoRG: putative exo-rhamnogalacturonan hydrolases; EndoRG: endo- rhamnogalacturonan hydrolases. The average pair wise distance within the different functional groups of *A. niger* hydrolases is indicated in brackets. Biochemically identified enzymes are in bold. * - *A niger* enzyme amino acid sequence that are greater than or equal to 97% identical to that of a previously characterized *A. tubingensis* enzyme. (B) Excerpt of the multiple alignment of *A. niger* family 28 glycoside hydrolases**.** * - columns containing catalytic residues; # - Conserved cysteine bridge; +1 - columns containing residues involved in substrate binding at subsite +1; -1 - columns containing residues involved in substrate binding at subsite -1.

(CCR) and sorbitol as a non-inducing sugar like alcohol, which does not affect the CCR mechanisms were chosen as control substrates. After transfer from 18 h pre-culture on 2 % fructose, mycelium was sampled at four time points during 24 h growth on the different substrates. The corresponding total RNA samples were used in microarray experiments to monitor the mRNA levels of family 28 glycoside hydrolase encoding genes (Fig 2).

During growth on non-pectic carbon sources, *viz.* fructose and sorbitol, none of the genes encoding for enzymes from the exo-hydrolase group was transcribed. In contrast, transfer from fructose to galacturonic acid and PGA caused a strong induction peak in the first 2 h after transfer for *pga*X, *pgx*B and *pgx*C followed by a decrease to relatively moderate expression levels during the next 8 h and further reduction of the signal to background levels 24 h after transfer, coinciding with the depletion of carbon source. Although less strongly induced, *rgx*C displayed a similar transcriptional profile on galacturonic acid and PGA. This gene was also moderately expressed together with *rgx*A and *rgx*B after transfer to rhamnose (Fig. 2B). Interestingly *pgx*A showed only low expression levels on these carbon sources.

Transfer to SBP caused a more complex transcriptional pattern. *Pga*X was quickly induced and remained highly expressed reaching a maximum 8 h after transfer. *Pgx*C messenger levels reached a maximum 4 h after transfer. *Pgx*B and *rgx*C levels gradually increased and reached a maximum 8 h after transfer. The signals obtained for *rgx*C were much higher on sugar beet pectin compared to rhamnose or galacturonic acid as sole carbon source. SBP caused a similar effect on the induction of the *rgx*B gene. *Rgx*A messenger was hardly detected 8 h after transfer to SBP. In contrast to the strong induction observed for genes from the exo-group, the transfer to galacturonic acid and PGA did not cause a strong effect on the expression of endopolygalacturonases. SBP moderately induced *pga*I, *pga*B and *pga*C 4 h after transfer, coinciding with the slight accumulation of extra-cellular free galacturonic acid (data not shown). *Pga*A and *pga*B have been reported to be constitutively expressed [132] which is in agreement with the data obtained in this study (Fig. 2A). Yet a slight increase of *pga*B messenger was observed coinciding with the depletion of fructose during the time course of incubation suggesting that, although weak, *pga*B is under CCR control.

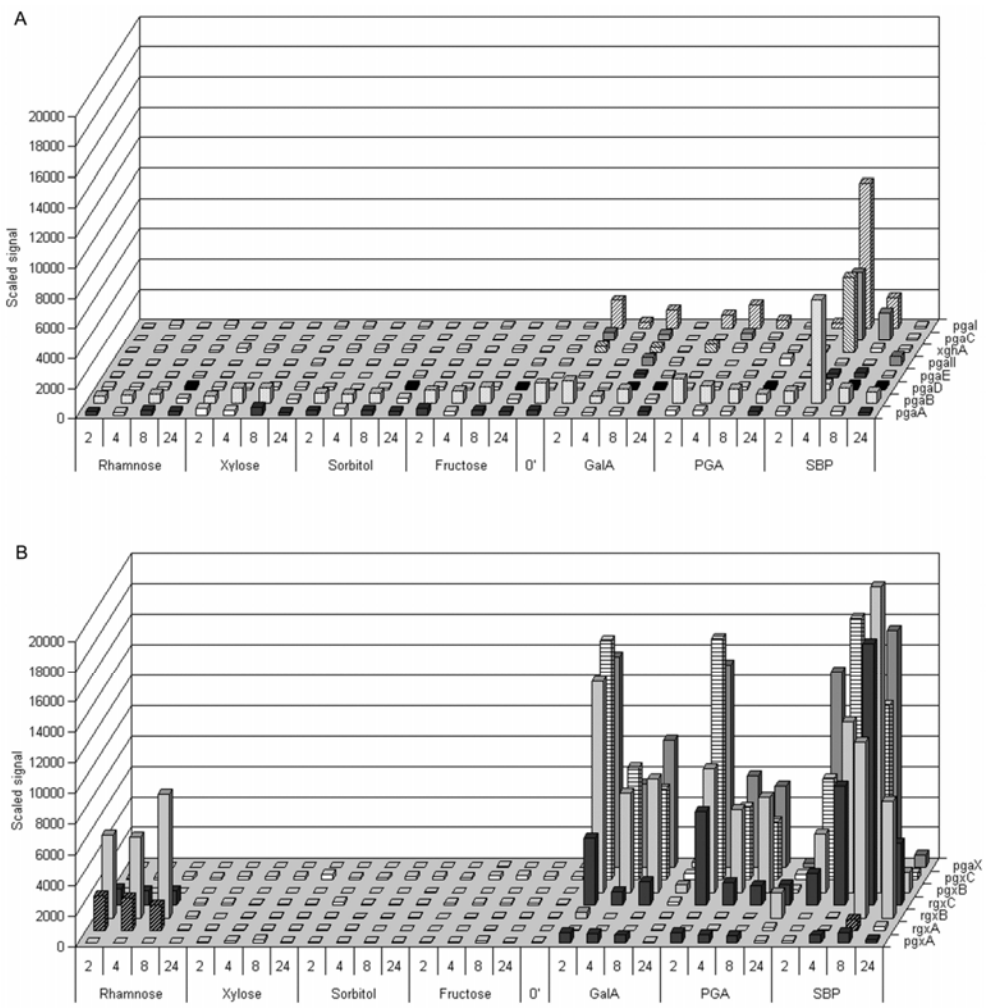

Figure 2. Transcriptional levels of *A. niger* glycoside hydrolases – 24 hours time course on different carbon sources. (A) endo-polygalacturonan hydrolases and endo-xylogalacturonan hydrolase. (B) – exo-acting hydrolases. Minor axis intervals - time points of harvesting after transfer from fructose (in hours);0' – 18 hours preculture on 2% (w/v) fructose; GalA – galacturonic acid; PGA – polygalacturonic acid; SBP – sugar beet pectin. White colour indicates absent call.

In contrast, when pectic substrates were used, the signal of *pga*B decreased in time to reach a background level at 24 h (Fig. 2A). Instead, low levels of *pga*D messenger were detected. PGD, the protein encoded by *pga*D gene, has been proposed to be a cell wall attached

oligogalacturonase with high tolerance towards methyl-esterified pectins [34]. HPLC measurements (data not shown) demonstrated that at the time points of expression of *pga*D free sugars are being depleted from the culture fluid, which together with its functional properties, suggests that the expression of *pga*D may in part be due to carbon starvation. Still, during incubation on galacturonic acid and sugar beet pectin *pga*D messengers could be detected slightly earlier, 8 h after incubation.

Despite many efforts including several genetic screens [74], no specific transcriptional factors regulating the expression of pectinolytic glycoside hydrolases have been identified so far. The strong discrepancy between the general expression profiles observed for the exo- and the endo-hydrolase encoding genes suggests separate or loosely linked regulation mechanisms for both groups. Exopolygalacturonases are well induced by galacturonic acid and the observed expression levels on pectin, although slightly more elevated, do not differ much from the levels observed on its monomeric substitute. Unexpectedly, galacturonic acid and PGA as a sole carbon source appeared to be only weak inducers for endopolygalacturonases, while SBP caused profoundly higher expression of endo-PG. In addition, the expression pattern of all studied genes on SBP has a much more complex character compared to monomeric sugars, which suggests that a multi-factorial regulation system and not just galacturonic acid, is responsible for the induction of pectinolytic genes.

Biochemical identification of PGXA, PGXB, PGXC and RGXB

Purified PGXA, PGXB, PGXC and RGXB appeared as a single band upon SDS-PAGE (results not shown) and had an apparent molecular mass of 78 kDa, 67 kDa, 79 kDa, and 82 kDa respectively (Table 1). The difference between the measured and calculated molecular masses of these enzymes is most probably caused by a high degree of glycosylation, which has also been demonstrated previously for the exo-polygalacturonase (Exo-PG) from *A. tubingensis* [163].

The action of PGXA, PGXB, PGXC and RGXB was studied towards PGA, XGA-25, and pectins with various degrees of methylation or amidation. As determined by HPSEC, only PGA and XGA-25 were degraded by PGXA, PGXB and PGXC. Similar results were

observed for Exo-PG, which indicates that PGXA, PGXB and PGXC have an exo-polygalacturonase activity. RGXB was not active towards these GalA-containing substrates, indicating that this enzyme is not a polygalacturonase. To investigate whether RGXB exhibits exo-rhamnogalacturonan hydrolase activity, a mixture of rhamnogalacturonan oligosaccharides was treated with RGXB and analyzed by MALDI-TOF MS. These oligosaccharides were also treated with exo-acting rhamnogalacturonan galacturonohydrolase (RG-galacturonohydrolase) from *A. aculeatus* [164] for comparison. As determined by MALDI-TOF MS (results not shown), rhamnogalacturonan oligosaccharides GalA-Rha-GalA-Rha-GalA and GalA-Rha-GalA, at m/z 861 and m/z 539 respectively, disappeared upon treatment with RG-galacturonohydrolase, which implies the expected removal of the non-reducing GalA residue from these oligosaccharides [164]. The enzyme RGXB did not degrade these rhamnogalacturonan oligosaccharides. Also the rhamnogalacturonan oligosaccharides Rha-GalA-Rha-GalA-Rha-GalA at m/z 1007 and Rha-GalA-Rha-GalA at m/z 685 respectively were not attacked (results not shown). From these results it is concluded that RGXB does not act towards these rhamnogalacturonan oligosaccharides from the non-reducing end nor from the reducing end. However, this is still unclear for rhamnogalacturonan oligosaccharides with a rhamnose residue at the reducing end.As RGXB was able to degrade pnp-Rha (results not shown), for now this enzyme will be named a pnp-rhamnohydrolase. In contrast to the pnp-rhamnohydrolase from *A. aculeatus* [161], RGXB did not hydrolyse hespiridin or naringin.

The enzymes PGXA, PGXB and PGXC were optimally active towards PGA at pH 3.5-4.0, pH 4.0-4.5 and pH 3.5-4.0, respectively (Table 1). pH optima for exo-polygalacturonases close to this value were previously reported by several investigators [163, 170, 171]. The specific activities of PGXA, PGXB, PGXC towards PGA and XGA-25 are presented in Table 1. Also the specific activity of *A. tubingensis* ExoPG towards these polymers is included in this table for comparison. PGXB, PGXC and Exo-PG have a similar activity towards PGA. The specific activity of PGXB and ExoPG towards XGA were less than towards PGA, which shows that these enzymes have a higher preference for PGA. However PGXC has no preference as it was equally active on both substrates. Compared to the other enzymes, PGXA has a significant lower specific activity towards PGA and it is the only

Table 1. Properties of PGXA, PGXB, PGXC, and Exo-PG. Specific activity of the enzymes were determined towards 0.25% (w/v) PGA and 0.25 % (w/v) XGA in 50 mM NaOAc (pH 4.0). The optimum pH for each enzyme was determined towards 0.25% (w/v) PGA in McIlvaine buffers (over a pH range of 2.5-7.5). n.d.: not determined. [1]As determined by Kester et al. (1996).

Enzyme	Molecular mass (kDa)	Calculated molecular mass(kDa)	Specific activity towards PGA(U/mg)	Specific activity towards XGA (U/mg)	pH-optimum towards PGA
PGXA	78	47	7	25	3.5-4.0
PGXB	67	48	242	33	4.0-4.5
PGXC	79	45	223	202	3.5-4.0
ExoPG	78[1]	47	230	17	4.2[1]
RGXB	82	50	n.d.	n.d.	n.d.

enzyme that has a higher specific activity towards XGA-25 than towards PGA. This was also demonstrated by HPAEC analysis (see further).As determined by HPSEC analysis (results not shown), PGA and XGA-25 were degraded by PGXA, PGXB and PGXC, respectively, without a dramatic decrease in the molecular weight of these polymers. These results were comparable to those obtained for Exo-PG and imply that PGXA, PGXB and PGXC degrade both polymers in an exo-fashion. This is further substantiated by analysis of the products by HPAEC. PGXA, PGXB and PGXC predominantly produce GalA from PGA, and a mixture of GalA and the disaccharide GalA-Xyl from XGA-25 at the early stage of the reaction (i.e. after 1 hour of incubation, see Fig. 3) as well as after prolonged incubation (results not shown). These results were similar to those observed for Exo-PG, thus, again confirming the exolytic mode of action of these enzymes as predicted from the sequence alignment.

A detailed inspection of the HPAEC results demonstrates that PGXA acts differently towards PGA and XGA-25 than PGXB, PGXC and Exo-PG. As shown in Figure 3A, the production of GalA from PGA (after 1 hour of incubation) was relatively low for PGXA, when compared to PGXB, PGXC and Exo-PG. These results are also in accordance with the specific activities of these enzymes towards PGA (Table 1). As illustrated in Figure 3B, compared to the other enzymes, PGXA also produced significantly more GalA-Xyl in ratio to GalA from XGA-25. This shows that PGXA prefers to act towards xylosylated GalA residues of the substrate XGA-25. It can be concluded that PGXA is primarily behaving as an exo-xylogalacturonan hydrolase (and not an exo-polygalacturonase), but is also able to degrade polygalacturonic acid.

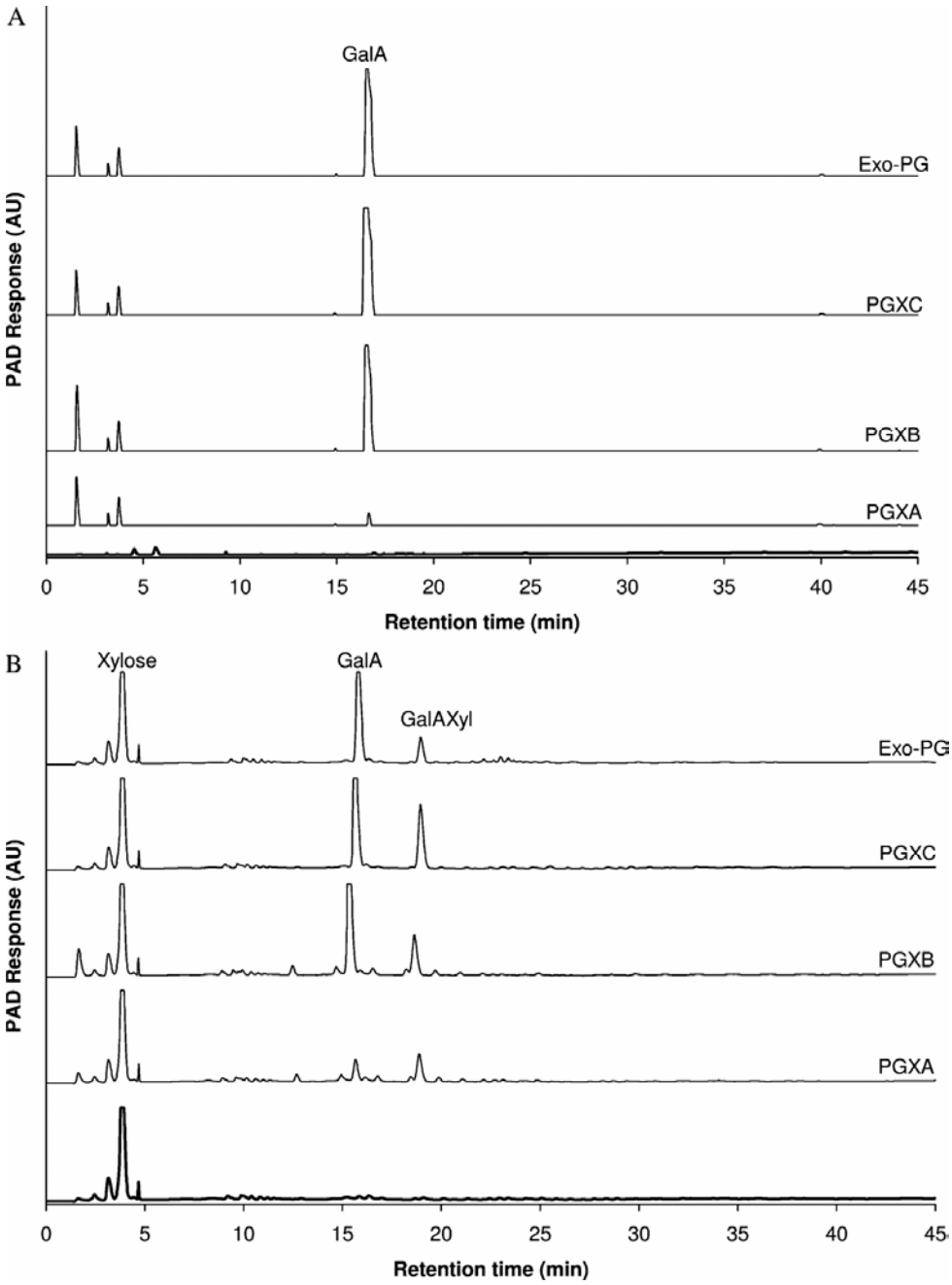

Figure 3. **HPAEC of PGA (A) and XGA (B) hydrolysis after treatment for 1 h with PGXA, PGXB, PGXC and Exo-PG respectively**. Spectra of untreated PGA and XGA are in bold.

Summary and conclusions

This paper presents for the first time an overview of the entire set of pectinolytic glycoside hydrolases encoded in the genome of *Aspergillus niger*.

In addition to the already well-studied gene group of seven endo-polygalacturonan hydrolases, we were able to extend the number of endo-rhamnogalacturonan hydrolases to six. Furthermore, a new gene group comprising seven genes encoding for exo-acting pectinolytic glycoside hydrolases was identified. In addition one gene encoding for a pectinolytic glycoside hydrolase was identified to be a xylogalacturonan hydrolase.

Subsequent sequence analysis of the new gene group in combination with transcriptional data allowed for the prediction of two subgroups of enzyme activities. Four genes were assessed as encoding the exo-polygalacturonan hydrolases – PGAX, PGXA, PGXB, PGXC and three genes as encoding the exo-rhamnogalacturonan hydrolases RGXA, RGXB and RGXC. PGAX encoded by the *A. niger* gene *pga*X is almost identical with the earlier described ExoPG of *A. tubingensis.*

Biochemical characterization of the three proposed exo-polygalacturonan hydrolases PGXA, PGXB and PGXC demonstrated that indeed all three enzymes were active towards homogalacturonan in an exo-fashion. Yet, while the biochemical properties of PGXB do not differ much from those of the ExoPG of *A. tubingensis*, the data obtained for PGXA strongly indicate that this enzyme is a novel exo-xylogalacturonan hydrolase. Interestingly, PGXC appears to be an enzyme with reduced substrate specificity and remains unaffected in its activity when GalA residues of the substrate are substituted with xylose.

Additionally, the MS analysis of a tryptic digest of the RG galacturono-hydrolase produced by *A. aculeatus*, a protein for which no gene sequence is known, allowed the identification of its *A. niger* homolog RGXC.

Although RGXB showed some activity towards pnp-rhamnose we were not able to identify the natural substrate of this enzyme. Additional analysis is required to investigate the possible function of RGXA and RGXB. Yet, both enzymes posses the catalytic residues and the proper spacing required for the inverting mechanism characteristic for enzymes from family 28 glycoside hydrolases. The expression of the genes encoding these two

enzymes on rhamnose and sugar beet pectin suggests their involvement in the degradation of pectin regions that contain rhamnose, most probably rhamnogalacturonan I.

The generally higher expression levels observed for genes encoding exo–acting glycoside hydrolases compared to those of genes encoding endo-activities, the strong initial rates of the reactions catalysed by them and the fact that *A. niger* is able to utilise galacturonic acid in its monomeric form suggest that exo-activities play a much more important physiological role than expected previously during pectin degradation.

Acknowledgements

We would like to thank Esther Tichon for cloning the *pgx*C gene. We also thank Margaret Bosveld for the preparation of linear rhamnogalacturonan oligosaccharides.

Chapter 4

Genome Wide Analysis and Transcriptional Profiling of the Pectinolytic System Of *Aspergillus niger*

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Submitted

The saprobic fungus *Aspergillus niger* is an efficient producer of a wide range of extracellular enzymes involved in carbohydrate modification and degradation. Based on the recently elucidated genomic sequence of *A. niger*, it was estimated that only a fraction of the potentially secreted enzymes by *A. niger* has been currently characterized. Database mining has resulted in the prediction of at least thirty-eight genes encoding enzymes involved in the depolymerisation of the backbone of the complex polysaccharide pectin. Additional genes, encoding enzymatic activities, required for the degradation of the arabinan and arabinogalactan side chains attached to the backbone of the pectin polymer were detected as well. DNA microarray analysis was used to study the expression of the genes involved in pectin degradation, their regulation, and to generate insight in the interaction between the individual members of the pectinolytic network of *A. niger*. For this purpose *A. niger* was grown on sugar beet pectin and on the main monomeric sugar constituents of pectin, *viz.* galacturonic acid, rhamnose and xylose. An analysis of the corresponding transcriptomes revealed the induction of 46 different genes encoding pectinolytic enzymes. Their transcriptional profiles are discussed in detail and a cascade model of pectin degradation is proposed.

Abbreviations

GRAS, Generally Regarded As Safe; PIR, Protein Information Resources; CDD, conserved domain database; PFAM, Protein family; EST, expressed sequence tag; SBP, sugar beet pectin; AP, apple pectin; PGA, polygalacturonic acid; ORF, open reading frame

Introduction

Pectin is a heterogeneous, heavily branched plant carbohydrate composed of a range of different (poly)saccharides [26, 172]. These (poly)saccharides can be linked by various chemical bonds and modified by substituents like methyl-, acetyl- and feruloylesters. The actual chemical composition of pectin varies between plant species in terms of the nature of the neutral sugars present, the length, branching and distribution of individual subunits, as well as by the degree and distribution of esterification [28]. The complete decomposition of pectin requires a vast array of enzymatic activities from microorganisms that use this complex polymer as carbon source. The saprobic fungus *Aspergillus niger* is well known for its ability to produce a broad range of pectin modifying enzymes.

So far, more than twenty *A. niger* genes encoding pectinases and accessory enzymes involved in pectin degradation have been cloned and their products have been characterized. Among them are a family of seven endopolygalacturonases [75], a family of six pectin lyases [37], a pectate lyase [39], a pectin methylesterase [41], two rhamnogalacturonase genes [35], a rhamnogalacturonan lyase [24] and a rhamnogalacturonan acetyl esterase [40]. Of these enzymes, all polygalacturonases and several pectin lyases have been biochemically characterized using simple model substrates, but little is known about their *in vivo* substrate specificity [45]. Recently we have reported the presence of a new group of exo-acting glycoside hydrolases with activity towards homogalacturonan, xylogalacturonan and rhamnogalacturonan, that are encoded in the genome of *A. niger* [116]. Moreover, several of the known *A. niger* enzymes with activities toward specific linkages and/or saccharide motif's within the pectin molecule have been isolated from commercially available pectinase preparations, but the genes encoding these activities have been not yet identified [42-44].

At present, little is known about the expression regulation of genes encoding the pectinolytic enzymes produced by *A. niger* during cultivation on complex polysaccharides.

Initial studies on the transcriptional regulation of the genes encoding pectin-active enzymes have shown that the regulatory system is induced by galacturonic acid or one of its metabolites [24]. However, a separate regulatory system involving rhamnose and galacturonic acid was also suggested [75, 173].

The recent elucidation of the genomic sequence of *A. niger* CBS 513.88 [17] and the subsequent design of Affymetrix GeneChip microarrays provided the opportunity to study the enzymatic network of this organism in a comprehensive manner by pinpointing all the genes encoding carbohydrate modifying activities and by studying their coordinated expression. In this study, we provide an inventory of the genes predicted to encode enzymes degrading the pectin backbone and side chain that are present in the genome of *A. niger*. Moreover, we used microarray profiling to determine the pectin-induced transcriptomes and to gain insight in the effects of pectin and its main constituents on the expression of pectin-related genes.

Materials and Methods

Screening of the *A. niger* genome

A list of accession numbers for available protein sequences of carbohydrate active enzymes was obtained from the CAZy web-server [144] at http://afmb.cnrs-mrs.fr/CAZY/. The corresponding protein sequences were retrieved from the SWISS-PROT database [145] at http://www.expasy.org/sprot/ and used to build hidden Markov model profiles using the HMMER package [146] from http://hmmer.wustl.edu/. The genome sequence of *A. niger* CBS 513.88 (deposited at NCBI under accession numbers NT_166518 to NT_166533 and NT_166537 to NT_166539 by Pel *et* al. in 2007) was screened for DNA sequences putatively encoding these pectinolytic activates with the obtained profiles using the WISE 2 package from http://www.ebi.ac.uk/Wise2/ [147]. The identified loci were compared to previously predicted protein-coding genes [17] and examined further using the provided information about PIR superfamilies, CDD, and conserved PFAM domains. Locus tags of genes cited in this study refer to the NCBI database.

Strains and growth conditions

Wild type strain *Aspergillus niger* N400 (CBS 120.49) was used in all transcriptional profiling experiments. 300 ml minimal medium [128], pH6.0, containing 0.1% (w/v) yeast extract and Vishniac trace elements [129] with 2% (w/v) D-fructose as a sole carbon source was inoculated with 10^6 spores/ml and cultivated at 30°C at 250 rpm in an orbital shaker. After 18 h of incubation, mycelium was harvested on a Büchner funnel with nylon gauze, washed once with sterile 0.9% (w/v) NaCl, and aliquots of 1.5 g (wet weight) mycelium were transferred to 50 ml minimal medium, pH6.0, 0.1% (w/v) yeast extract, Vishniac trace element solution and 1% (w/v) of the various sole carbon sources: D-fructose (Merck), D-glucose (Merck), D-galacturonic acid (Fluka Chemica), L- rhamnose (ACROS organics), D-xylose (Merck), D-sorbitol (Merck), polygalacturonic acid (United States, Biochemical Corp.) and sugar beet pectin (GENU, Copenhagen pectin). The sugar and uronic acid content of the polymeric substrates polygalacturonic acid, and sugar beet pectin used in this study are given in supplementary table 1. At 2, 4, 8 and 24 h after transfer, mycelium was harvested on a Büchner funnel with nylon gauze and immediately stored at -70°C. The amount of monomeric sugars remaining in the culture fluid was assessed using standard HPLC techniques [174].

RNA isolation and microarray processing

Total RNA was isolated from frozen ground mycelia using TRIZOL® Reagent (Invitrogen™) according to the instructions of the supplier. RNA concentrations were estimated using a NanoDrop® ND-1000 Spectrophotometer. Total RNA was amplified, labeled and hybridized strictly following the Affymetrix protocols for “Eukaryotic Target Preparation” and “Eukaryotic Target Hybridization”. Before and during microarray processing RNA and cDNA quality were verified by analyzing aliquots on 1% TAE agarose gel electrophoresis and Agilent Bioanalyzer “Lab on chip” system (Agilent Technologies, Palo Alto, CA). Messenger RNA levels were assessed using custom made “dsmM_ANIGERa_coll” Affymetrix GeneChip® Microarrays kindly provided by DSM

Food Specialties (Delft, The Netherlands).

For probe array washing and staining the "Antibody Amplification Washing and Staining Protocol" was used. Arrays were scanned with Agillent technologies G2500A Gene Array Scanner at pixel value 3 µm and wavelength 570 nm. Raw intensity measurements and present/absent calls were derived in Microarray Suite Software version MAS5 (Affymetrix, UK Ltd) after applying the "Mask all outliers" algorithm. The detection p-value was used to assign whether a measured transcript was detected as: present (P) at significant level, absent (A) i.e. below detection level, or marginal (M) i.e. in-between P and A. All chip data were scaled to an arbitrary target gene intensity of 500 and further visualized in the software package "GeneSpring" to facilitate analysis.

Results

Inventory of pectinolytic enzymes encoded in the genome of *Aspergillus niger*

Polygalacturonases, rhamnogalacturonan and xylogalacturonan hydrolases, pectin, pectate and rhamnogalacturonan lyases, pectin methylesterases and pectin acetylesterases are the enzymes involved in the depolymerisation of the backbone of pectin, and are commonly referred to as pectinases. An additional group of enzymes (also called accessory pectinolytic enzymes) is required for the depolymerisation of the arabinan and arabinogalactan side chains of pectin, and includes different enzymatic activities such as arabinases, arabinofuranosidases, β-galactosidases, galactanases, and feruloyl esterases. To identify the *A. niger* genes that may play a role in pectin degradation, the genomic sequence of *A. niger* CBS 513.88 was screened for the presence of ORFs encoding known and predicted pectinolytic activities. This led to the identification of at least a total of 58 sequences that encode partly-putative pectinolytic activities, from which almost half (25) were not previously described (Fig. 1). Within the group of pectinases no additional sequences encoding endopolygalacturonases, pectin or pectate lyases were detected. In

contrast, in addition to the strongly conserved ortholog of *pga*X, the gene encoding the previously characterized exopolygalacturonase of *Aspergillus tubingensis*, four more exopolygalacturonases, and three additional exorhamnogalacturonan hydrolases were identified [116]. Besides the two genes encoding the known endorhamnogalacturonan hydrolases, *rhg*A and *rhg*B, and the rhamnogalacturonan lyase gene *rgl*A, four additional ORFs encoding putative endorhamnogalacturonan hydrolases and an additional putative rhamnogalacturonan lyase were detected in the genome of *A. niger*. Only one gene was identified that coded for a putative endoxylogalacturonan hydrolase, which is highly similar to the previously described *xgh*A from *A. tubingensis*. Three ORFs predicted to encode pectin methylesterase were found, from which only one (*pme*A) was previously known. In addition to the known *rgae*A gene, a second ORF encoding a putative rhamnogalacturonan acetyl esterase was detected. Despite the fact that a pectin acetyl esterase activity was previously isolated from *A. niger* [42], so far, no gene encoding an enzyme with this function has been allocated. However, in the genome of *A. niger* there are two ORFs encoding putative acetylesterases that may be involved in the deacetylation of homogalacturonan. Additionally, *A. niger* has two ORFs possibly encoding unsaturated rhamnogalacturonyl hydrolases from the newly discovered glycoside hydrolase family 105 [175]. Several new sequences predicted to encode accessory enzymes were also identified along with the already known genes. These included putative ORFs for two new arabinofuranosidases, three endoarabinases and two feruloyl esterases. In addition to the three already known α-galactosidases, four additional sequences potentially coding for this hydrolase activity were identified. Also, four more candidate β-galactosidases were found to be encoded by the genome of *A. niger* along with a hitherto unknown arabinogalactan endogalactosidase and a 1,3-β-galactanase activity, previously described for *A. niger* [44].
Altogether, the group of 26 known genes encoding pectinases was enriched with 13 additional candidate sequences. Moreover, 12 additional ORFs coding for accessory enzymes were predicted, in addition to the 8 known genes for this function. We were not able to find the *pel*E gene during this survey. However, the corresponding sequence was recently detected on chromosome VI, in a region that was not sequenced prior to the release of the *A. niger* CBS 513.88 genome (P. Schaap, unpublished results).

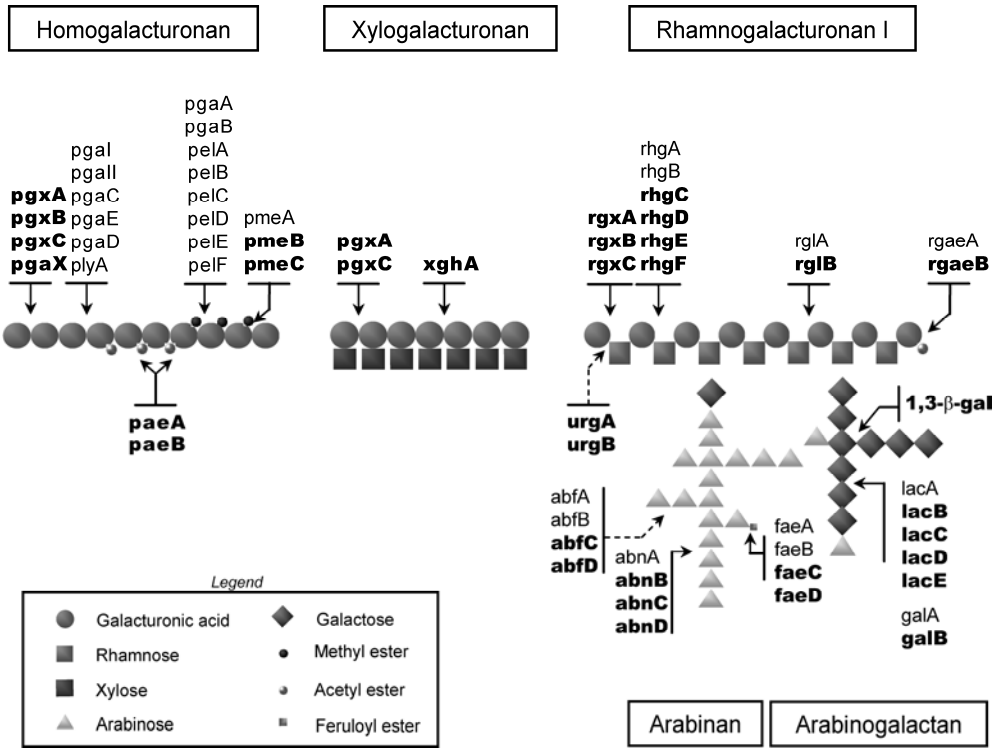

Figure 1. Inventory of *A. niger* genes encoding pectinolytic activities with a schematic representation of the points of attack of the corresponding enzymes on the pectin main- and side chains. Previously known genes are represented in regular font. Newly identified genes are given in bold black. Dashed arrows represent enzymes that act on oligomeric sugar units released from the polymeric blocks of pectin. Please note that the shown here polymers are only an illustration of the major pectin components and do not depict definite molecular structures. A detailed list of the genes with the corresponding locus tag numbers, (putatively) encoded enzymatic activities, assigned CAZy family numbers and assigned EC numbers is given in Supplementary table 3.

Transcriptional profiling of the pectinolytic system of *A. niger* by microarray analysis

To study the induction of the genes encoding known and putative enzymes from the pectinolytic system of *A. niger*, the transcriptional profiles of the selected 58 known or putative pectinolytic genes identified in its genome (Supplementary Table 3) were monitored by microarray experiments. For this purpose, *A. niger* was cultivated on the

complex substrates sugar beet pectin and polygalacturonic acid as primary carbon sources. Galacturonic acid, rhamnose and xylose were used to assess the effects on gene expression caused by simple well-defined carbon sources, representing the most abundant sugar residues present in the backbone of pectin. As control substrates, we selected fructose, as a strong repressor of the expression of genes that are under carbon catabolite regulation, and sorbitol, as a non-inducing sugar-like alcohol, which does not affect the carbon catabolite regulation mechanisms. Mycelia of *A. niger* were pregrown for 18 h on 2% fructose, transferred to medium containing the different pectic and control substrates, and sampled at four time points during 24 h of incubation. Culture filtrates of all sampled conditions were used for HPLC analysis of the sugar and acid content while their pH was determined as well (Supplementary Table 4).

Under the tested conditions, the expression of four genes, namely *abf*C (encoding a putative α-L-arabinofuranosidase) and *abn*B, *abn*C and *abn*D (encoding a putative endoarabinases) was detected on all carbon sources and at all time points tested. While *abn*B and *abn*D were expressed at constant low levels and did not show strong induction, *abn*C revealed elevated expression levels 24 hours after transfer to monomeric carbon sources, coinciding with the depletion of substrate. In contrast, *abf*C was induced early during incubation on galacturonic acid, polygalacturonic acid and sugar beet pectin.

For a set of another twelve genes, no mRNA levels could be detected under any of the tested conditions. Surprisingly, this group included the genes coding for three well characterized pectin lyases of *A. niger* – *pel*B, *pel*C and *pel*D. The remaining unexpressed genes were *rhg*E, *rhg*D and *rhg*F (encoding three of the newly identified putative endorhamnogalacturonases); *urg*B putatively coding for a unsaturated rhamnogalacturonyl hydrolase; *fae*C and *fae*D (encoding putative feruloylesterases); *gal*B (encoding a putative endo-β-1,4-galactanase); *abf*D (encoding a putative arabinofuranosidase), and *lac*D (encoding a putative β-galactosidase).

Induction of homogalacturonan and xylogalacturonan degrading enzymes

During growth on non-pectic carbon sources, *viz.* fructose and sorbitol, except for *pga*A and *pga*B, none of the 7 members from the gene family encoding endopolygalacturonases was transcribed. Unexpectedly, galacturonic acid and polygalacturonic acid did not induce

the remaining endopolygalacturonases except for *pga*I and *pga*C, which were also moderately expressed on sugar beet pectin [116]. A similar expression pattern was observed for *xgh*A, the only gene encoding a putative endoxylogalacturonan hydrolase. In contrast, galacturonic acid and polygalacturonic acid caused strong induction of *pga*X together with the induction of the *pgx*B and *pgx*C - all coding for exo-acting polygalacturonases*. Low expression levels also were observed for the genes encoding pectin and pectate lyases. For these genes, we detected the expression of only *pel*A, *pel*F and *ply*A. While *pel*A was well induced on pectic substrates, the expression of *pel*F and *ply*A was close to background levels (Fig. 2A). Among the most unexpected observations was the absence of *pme*A transcripts, as for a long time it was thought that this is the only gene encoding a pectin methylesterase in the genome of *A. niger*. Instead, another gene, *pme*B possibly encoding a second pectin methylesterase, was well induced on galacturonic acid, polygalacturonic acid and sugar beet pectin. A moderate expression of the genes encoding the two putative pectin acetyl esterases, *pae*A and *pae*B was observed after transfer to galacturonic acid and polygalacturonic acid. On sugar beet pectin, the expression of both genes was slightly delayed, and reached maximum levels at eight hours after transfer, similar to the pattern observed for exopolygalacturonases (Fig. 2B).

Induction of rhamnogalacturonan I degrading enzymes

Within the group of genes encoding rhamnogalacturonan-degrading enzymes, basal levels of expression were detected for the two known genes coding for rhamnogalacturonases, *rhg*A and *rhg*B only during long (24 hours) incubation on polygalacturonic acid and sugar beet pectin. Similar low expression levels were observed for *rhg*C, but in contrast to the genes for the other two endorhamnogalacturonases, this gene was constitutively expressed on all carbon sources (data not shown).

We have previously observed high expression levels on rhamnose for the three genes thought to encode exo-acting activities – *rgx*A, *rgx*B and *rgx*C and a strong induction of *rgx*B on sugar beet pectin [116]. Other genes with similar high expression on rhamnose included *urg*A, encoding the putative unsaturated rhamnogalacturonyl hydrolase assigned

* A detailed description of family GH28 enzymes encoded in the genome of *A. niger*, together with their biochemical identification and an assessment of their transcriptional profiles is given in Chapter 3 [116].

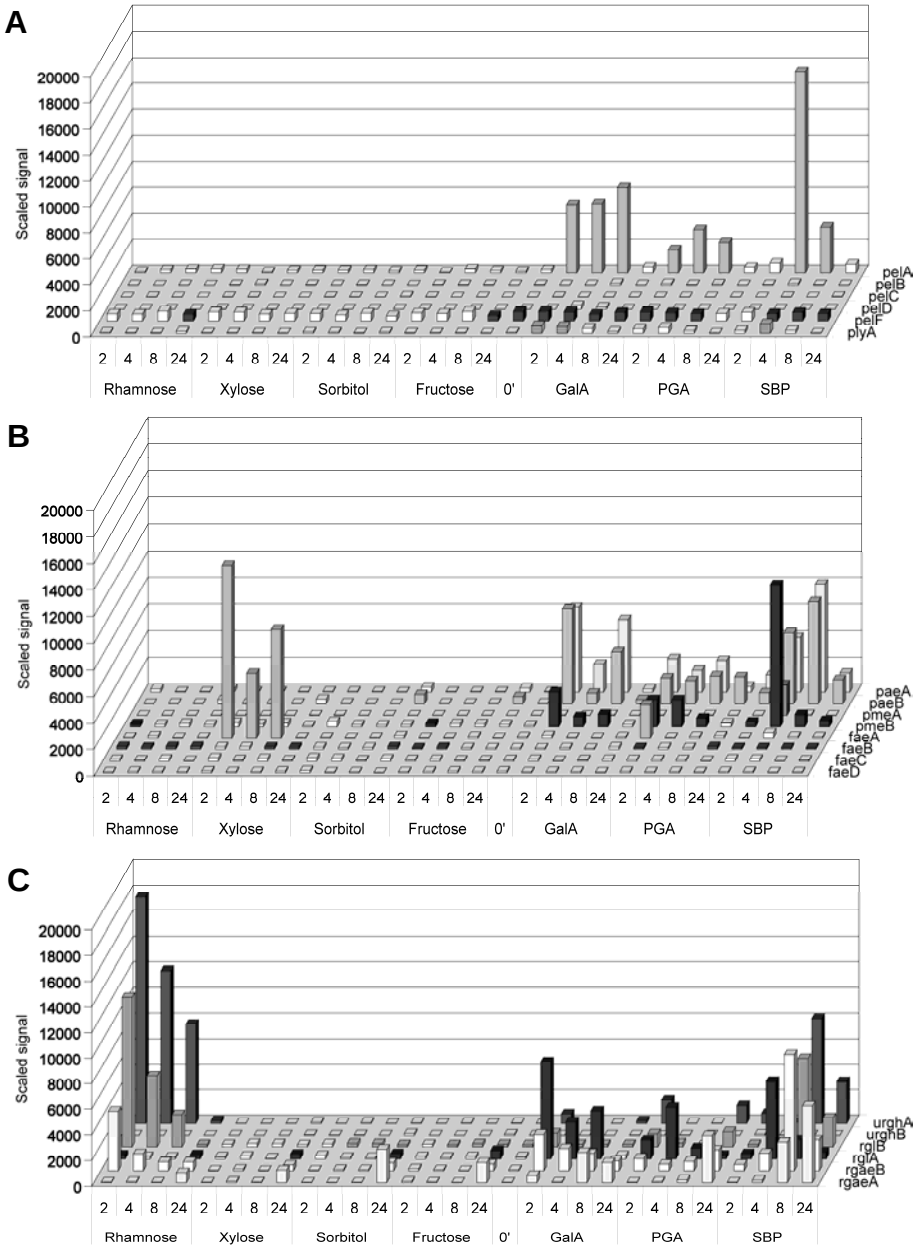

Figure 2. Transcriptional levels of *A. niger* pectinolytic enzymes: 24 h time course on different carbon sources. (A) Pectin lyases and pectate lyase. (B) Carbohydrate esterases. (C) Rhamnogalacturonan lyases, esterases and unsaturated rhamnogalacturonyl hydrolases. Minor axis intervals, time points of harvesting after transfer from fructose (in hours); 0 indicates 18 h preculture on 2% (w/v) fructose; White color indicates an absent call.

to family GH105. This gene was strongly induced after transfer to rhamnose and its expression levels gradually decreased in time, coinciding with depletion of the substrate. Slight induction of *urg*A was also observed two hours after transfer to galacturonic acid and polygalacturonic acid. On sugar beet pectin, *urg*A was moderately induced in the later time points – 8 and 24 hours after transfer. A similar induction pattern was observed for the hitherto unknown genes *rgl*B encoding a putative rhamnogalacturonan lyase, and *rgae*B encoding a putative rhamnogalacturonan acetylesterase. Low amounts of rhamnogalacturonan lyase *rgl*A transcripts were detected 24 hours after incubation on all monomeric carbon sources coinciding with the depletion of substrate (Supplementary Table 4). This gene did not respond strongly to rhamnose, but it was moderately induced immediately after exposure of the mycelium to galacturonic acid, polygalacturonic acid and sugar beet pectin (Figure 2C).

Induction of pectin side chain degrading enzymes

In the group of pectin side chain degrading enzymes, besides the expected transcription of the known genes encoding arabinofuranosidases *abf*A and *abf*B on xylose, galacturonic acid, polygalacturonic acid, and sugar beet pectin, we also detected the *abn*A gene encoding an endoarabinase (Figure 4A). The *fae*A gene, encoding the known ferulic acid esterase, was as expected, well induced by xylose [24]. In addition, we observed basal levels of expression on rhamnose and sugar beet pectin of a second gene encoding ferulic acid esterase *fae*B [176]. The β-galactosidase encoding *lac*A was induced after transfer to all pectic carbon sources, but not on fructose or sorbitol. From the remaining expressed β-galactosidases, *lac*C was induced by rhamnose, galacturonic acid, polygalacturonic acid and pectin, *lac*B was detected during growth on galacturonic acid, polygalacturonic acid and pectin, and *lac*E was expressed only in the late 24 h after transfer from fructose. The gene encoding the putative galactan 1,3-β-galactosidase was expressed on all carbon sources tested, at 4 and 8 h after transfer except on sugar beet pectin, where its transcripts could be detected during the entire time course. Interestingly, the *gal*A gene for the arabinogalactan endogalactosidase was specifically induced by galacturonic acid, polygalacturonic acid and sugar beet pectin (Fig. 3B).

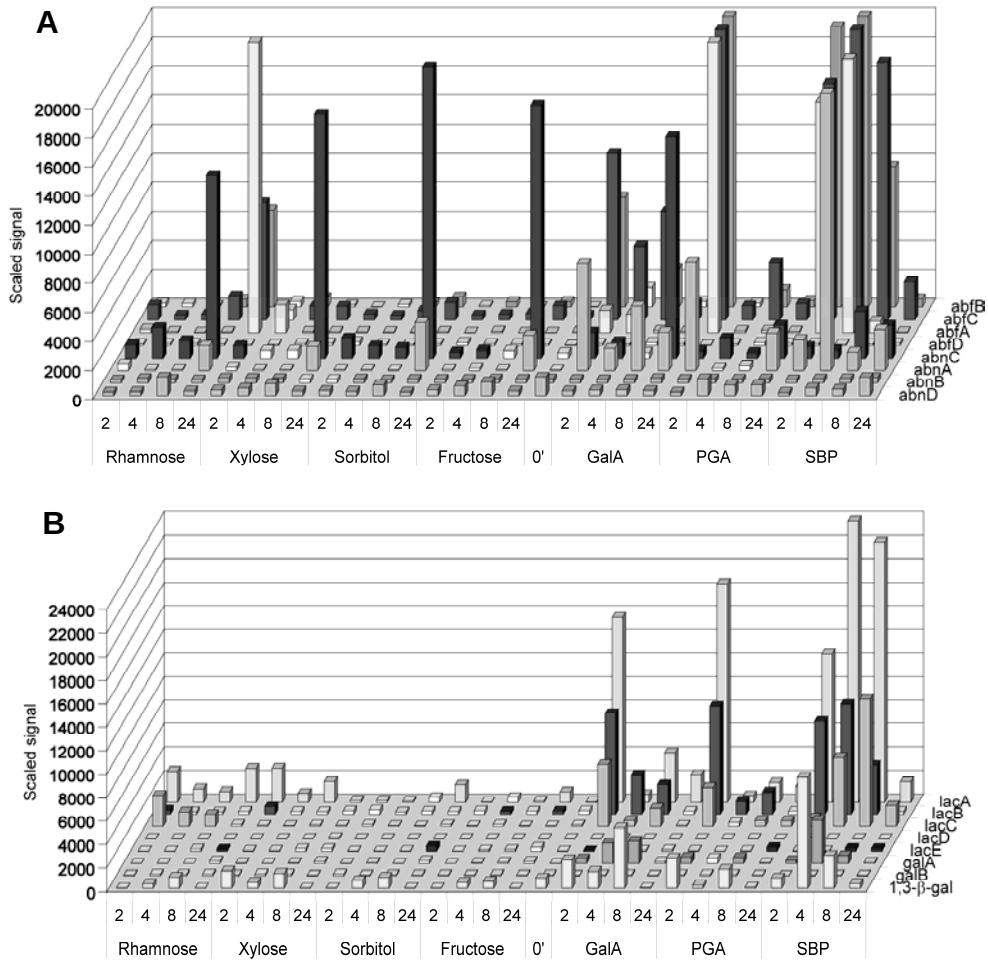

Figure 3. Transcriptional levels of *A. niger* accessory enzymes: 24 h time course on different carbon sources. (A) Arabinases and arabinofuranosidases. (B) β-galactosidases and galactanases. Minor axis intervals, time points of harvesting after transfer from fructose (in hours); 0, 18 h preculture on 2% (w/v) fructose; White color indicates an absent call. Note the different scale of the y-axis of (B)

Discussion

In the present study, we surveyed the genomic sequence of *A. niger* CBS 513.88 for the presence of carbohydrate degrading enzymes involved in the depolymerisation of the backbone and the side chains of the complex plant polysaccharide pectin. This resulted in

the identification of at least 58 sequences comprising already known and newly discovered genes. This vast number and the variety of different (partly) putative enzyme activities are required for the efficient and complete degradation of the complex pectin structure. The largest set of identified enzymes (21) was assigned to family 28 glycoside hydrolases. In addition to the seven endopolygalacturonases, seven exo-acting glycoside hydrolases are encoded in the genome of this fungus. GH family 28 is completed by six endorhamnogalacturonan hydrolases (4 of which putative) and one endoxylogalacturonan hydrolase with high similarity to the xylogalacturonan hydrolase from *A. tubingensis*. Furthermore, six pectin lyases, a single pectate lyase and two rhamnogalacturonan lyases together with three pectin methylesterases, two candidate pectin acetylesterases and two rhamnogalacturonan esterases complete the collection of *A. niger* enzymes needed for the degradation of pectin backbone.

In addition, this fungus has a large set of genes encoding accessory enzymes involved in the degradation of the side chains of pectin. For the degradation of arabinan, four endoarabinases, four α-L-arabinofuranosidases, and four ferulic acid esterases are encoded in its genome. For the degradation of arabinogalactan there are five β-galactosidases, one exo 1,3-β-galactosidase and two b-1,4-endogalactanases.

To study the induction of all the genes encoding these enzymes in a simultaneous fashion we analyzed the transcriptome of *A. niger* by microarray experiments. The acquired data demonstrated that at least 16 of the 25 predicted pectinolytic ORFs correspond to functional genes (Supplementary table 3). Furthermore, based on the obtained transcriptional profiles of 58 pectinolytic genes described in this study, we can hypothesize that the pectinolytic network of *A. niger* acts in the following manner:

i) In the absence of an easily metabolized carbon source, a small set of genes encoding various enzymatic activities targeting the different blocks of pectin is derepressed. This set comprises a polygalacturonase (encoded by *pga*D), that targets homogalacturonan; a rhamnogalacturonan lyase (*rgl*A) which assisted by a rhamnogalacturonan acetylesterase (the *rgae*A product) attacks rhamnogalacturonan, and several side chain degrading enzymes (encoded by *abn*A, *abn*C, *abf*B, *fae*B and *lac*E). Most probably, the function of these enzymes, together with additional constitutively expressed activities (encoded by *pga*A,

*pga*B, *rhg*C, *abn*B and *abn*D, *abf*C, exo-1,3-β-galactosidase) is to act as sensors by generating low molecular weight inducers. This role has been previously suggested for the endopolygalacturonases encoded by *pga*A, *pga*B and *pga*D [34, 45, 132].

ii) If pectin is present, monomeric sugars are released by the action of these enzymes. These monomeric sugars act synergistically in the induction and modulation of expression of a broad range of pectinolytic enzymes. For instance, galacturonic acid induces the exo- and endopolygalacturonase encoding genes *pgx*A, pgxB, pgxC, *pga*X, *pga*I (see also chapter 3, [116]), together with the lyase genes *pel*A, *ply*A, and esterase genes *pme*B, *pae*A, *pae*B. The enzymes encoded by these genes complement each other in the degradation of homogalacturonan. Furthermore, the gene products of *pgx*A and *pgx*C can also degrade xylogalacturonan [116].

iii) The simultaneous presence of galacturonic acid together with the released rhamnose, arabinose, and/or galactose results in the expression of *pga*I, *pga*C, *xga*A, rgxC, *rgx*B, *rgae*B, *rgl*A *abn*A *abf*B, *abf*C, *lac*A, *lac*B, *lac*C and *gal*A which leads to further degradation of rhamnogalacturonan I, and the arabinan and arabinogalactan side chains.

For the complete degradation of pectin, a synergistic action of all the enzymes is required and has been observed for several of them in previous studies (for a review see [126]). In addition, for the maximum efficiency of its pectinolytic network, *A. niger* should have a regulatory system that can coordinate the expression of several types of enzymes in response to the variable structure and composition of pectin. The here reported cascade induction of expression in response to the different (amounts of) pectin constituents, starting with galacturonic acid, can explain the flexibility needed by the pectinolytic network of *A. niger*. Such a cascade of induction events, strongly suggests multiple levels of regulation of the pectinolytic system of *A. niger* involving the action of several transcriptional regulators.

Although we have inspected the transcriptional profiles of over 300 *A. niger* genes predicted to code for regulatory proteins, we were not able to identify any new ones, that display pronounced up or down regulation on galacturonic acid, polygalacturonic acid and/or pectin, or that are co-expressed with genes coding for pectinolytic activities. The reason for that could be the relatively lower expression levels of regulatory genes, or the

modulation of transcription factor activity on a posttranscriptional level. More appropriate techniques for the detection and validation of low expressed genes (such as quantitative PCR) should be applied in combination with functional screening methods and promoter binding assays, in order to identify the transcriptional factors regulating the expression of the pectinolytic system.

Chapter 5

An Evolutionarily Conserved D-Galacturonic Acid Metabolic Pathway Operates Across Filamentous Fungi Capable Of Plant Cell Wall Degradation

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Submitted

Transcriptome analysis of *Aspergillus niger* cultures cultivated on pectin and on its constituents galacturonic acid, rhamnose and xylose *vs.* fructose and sorbitol enabled the identification of three proteins - GAAA, GAAB, and GAAC, that are involved in the catabolism of galacturonic acid in this fungus. Functional annotation by conserved domain search revealed that the GAAA, GAAB and GAAC possibly code for the reductase, dehydratase, and aldolase activities necessary for the conversion of galacturonic acid to pyruvate and glyceraldehyde via L-galactonate and 2-keto-3-deoxy-L-galactonate. Biochemical analysis of the recombinant GAAA and GAAB proteins confirmed that GAAA is a D-galacturonic acid reductase and that GAAB is an L-galactonate dehydratase, and demonstrated that GAAA can use both NADPH and NADH as co-factors. *Aspergillus nidulans* strains in which the orthologs of *gaa*A and *gaa*B genes were mutated were defective in galacturonic acid degradation. In *A. niger* the *gaaA* and *gaaC* genes share a common bidirectional promoter region. This specific genomic arrangement is strictly conserved in the genomes of plant cell wall degrading fungi from subphylum

Pezizomycotina, but not in sequenced zoopathogenic species. This feature combined with the presence of orthologs of *gaa*B in the same cell wall degrading fungi strongly suggests that these fungi use a common D-galacturonic acid utilization pathway.

Introduction

D-galacturonic acid is the major constituent of pectin and as such represents an important carbon source for bacteria and fungi living on decaying plant material. In bacteria, galacturonic acid is degraded in a five step pathway via D-tagaturonate, D-altronate, 2-keto-3-deoxy-gluconate and 2-keto-3-deoxy-6-phospho-gluconate, resulting in the formation of pyruvate and glyceraldehyde-3-phosphate [57, 58, 60].

In eukaryotes, the degradation of D-galacturonic acid takes place via a different metabolic route. In the filamentous fungus *Aspergillus nidulans* two mutants with strongly reduced growth on D-galacturonic acid were described [177] and an alternative non-phosphorolytic pathway leading to glyceraldehyde and pyruvate was proposed. It has been suggested that the glyceraldehyde produced in this way is further reduced to glycerol [178].

Enzymatic activities corresponding to D-galactonate dehydratase and 2-keto-3-deoxy-D-galactonate aldolase have been described in *Aspergillus terreus* [63] and *Aspergillus niger* [64]. In *A. nidulans* an NADPH dependent glycerol dehydrogenase activity has been detected that is specifically induced by galacturonic acid [66]. Recently, an enzyme converting D-galacturonic acid to L-galactonate and the encoding gene have been discovered in strawberry plants [179]. However, this reaction takes place in an alternative biosynthetic route leading to the production of vitamin C and it is not a part of a catabolic pathway.

In the fungus *Trichoderma reesei*, a pathway for the degradation of galacturonic acid was recently postulated. This pathway begins with the conversion of D-galacturonic acid to L-galactonate by a NADPH dependent D-galacturonic acid reductase [67]. An L-galactonate dehydratase further converts L-galactonate to 2-keto-3-deoxy-L-galactonate [68], demonstrating that indeed the degradation of galacturonic acid occurs via L-compounds. The 2-keto-3-deoxy-L-galactonate aldolase was also recently characterised [69]. Furthermore, a specific NADPH dependent glycerol dehydrogenase has been proposed to be involved in the further conversion of L-glyceraldehyde to glycerol [70].

Here we describe the identification of a set of genes involved in the metabolism of

galacturonic acid in the filamentous fungus *Aspergillus niger*, the biochemical identification of the encoded enzymes that perform the first 2 steps of galacturonic acid degradation, as well as the identification of the molecular basis of the *gaa* mutations in *A. nidulans*. Furthermore, evidence is provided, that this pathway is strictly conserved in plant cell wall degrading fungi from subphylum Pezizomycotina.

Experimental Procedures

Fungal strains and growth conditions

Aspergillus niger

Wilde type strain N400 (CBS 120.49) was used for transcriptional profiling experiments as previously described [116]. For growth, 300 ml of minimal medium [128] (pH 6.0) containing 0.1% (w/v) yeast extract and Vishniac trace elements [129] with 2% (w/v) D-fructose as a sole carbon source was inoculated with 10^6 spores/ml and cultivated at 30°C at 250 rpm in an orbital shaker. After 18 h of incubation, mycelium were harvested on a Büchner funnel with nylon gauze, washed once with sterile 0.9% (w/v) NaCl, and aliquots of 1.5 g (wet weight) of mycelium was transferred to 50 ml of minimal medium (pH 6.0) containing 0.1% (w/v) yeast extract, Vishniac trace element solution [129],and 1% (w/v) of the various sole carbon sources: D-fructose (Merck), D-glucose (Merck), D-galacturonic acid (Fluka Chemica), L-rhamnose (ACROS Organics), D-xylose (Merck), D-sorbitol (Merck), PGA (polygalacturonic acid; United States Biochemical Corporation) and SBP (sugar beet pectin; GENU, Copenhagen). At 2, 4, 8, and 24 h after transfer, mycelium was harvested on a Büchner funnel with nylon gauze and immediately stored at -70°C. The amount of monomeric sugars remaining in the culture fluid was assessed by standard HPLC techniques [153].

Aspergillus nidulans

Strains WG145 (*w*A3; *pyro*A4), WG222 (*w*A3; pyroA4, *gaa*A) and WG262 (*w*A3; *pyro*A4; *gaa*B) were used for the analysis of mutant loci affecting D-galacturonic acid degradation.

Growth tests were performed on minimal medium [128] supplemented with 1 mg/L pyridoxine HCl. Medium was solidified with 1.5 % (w/v) agar. D-galacturonic acid (Fluka Chemica) or D-fructose (Merck) were used as sole carbon sources at a final concentration of 1% (w/v). pH of the medium was set at 6.0.

Trichoderma reesei

Strain CBS 383.78 (QM6a) was grown on complete medium, containing from minimal medium supplemented with 0.2 % (m/v) meat peptone, 0.1 % (m/v) vitamin free Casamino acids and 0.03 % (m/v) g yeast ribonucleic acids. The medium was solidified with 1.5 % (m/v) agar and 2 % (w/v) D-fructose was used as a carbon source.

Microarray processing, normalization and analysis

To search for genes specifically induced by galacturonic acid we analyzed a microarray data set derived from samples from an 18 hours pre-culture on fructose and from 2, 4, 8, and 24 hours time point samples on sorbitol, fructose, rhamnose, xylose, galacturonic acid, polygalacturonic acid, and sugar beet pectin. Total RNA samples were prepared using TRIZOL® Reagent (Invitrogen™) according to the instructions of the supplier. RNA integrity before and during sample processing was verified by analyzing aliquots with the Agilent Bioanalyzer 'Lab-on-chip' system (Agilent Technologies). mRNA levels were assessed using custom made 'dsmM_ANIGERa_coll' Affymetrix GeneChip® Microarrays kindly provided by DSM Food Specialties (Delft, The Netherlands). Total RNA samples were amplified, labeled, hybridized, washed, and stained strictly following the Affymetrix protocols. Probe arrays were scanned with an Agilent Technologies G2500A Gene Array Scanner at a pixel value of 3 mm and a wavelength of 570 nm. Signal intensities and detection p-values were derived using Microarray Suite Software version MAS5 (Affymetrix) after applying the 'Mask all outliers' algorithm. The detection p-value was used to assign whether a measured transcript was detected as: present (P) at significant level, absent (A) i.e. below detection level, or marginal (M) i.e. in-between P and A. [116].
Data was processed using the GeneSpring software package version 7.2 (Silicon Genetics, Redwood, CA) using the following settings: prior normalization all raw gene measurements

with a signal intensity below 0.01 were set to 0.01; data was further normalized per chip to the 50th percentile of the measurements for all genes taken from that chip; and per gene to the median of all measurements for that gene across all array samples (default settings). Analysis was performed in "Log of Ratio" mode with the "Cross Gene Error Model" active, and based on deviation from 1, where 1 indicates no change relative to a gene median across all samples. Genes were filtered on "Confidence" by performing a t-test based on deviation from 1. The global error model was used to run the t-test analysis, where the t-test p-value cut off was 0.1. The minimum number of samples in which genes should show differential expression was set to 9. Search for genes with similar expression profiles was performed on a subset of 5 samples consisting of a fructose pre-culture (time 0), and a galacturonic acid time course experiment (time: 2 hours, 4 hours, 8 hours, and 24 hours). "Standard Correlation" algorithm that measures the angular separation of expression vectors between genes around zero (GeneSpring 7.2) was used as similarity measure, where the correlation coefficient cut off was set to 0.95.

Sequencing of genomic regions in *A. nidulans* and *T. reesei*

Genomic DNA from *A. nidulans* strains WG222, WG262, and WG145 and from *Trichoderma reesei* strain CBS 383.78 (QM6a) was isolated as described [152] and used for the amplification of targeted gene regions with Pfu Turbo Polymerase (Stratagene) according to the instructions of the manufacturer. *Escherichia coli* DH5α was used as a host during cloning procedures.

800 bp PCR fragment containing the 5'end of AN6035.2 (*gaa*B) gene was amplified from genomic DNA originating from WG145 and WG262, respectively, using primer pair 5'-CAGACAGCACAATGGTGAAG-3' and 5'-CTCCTGTCATCCTCCAAGTT-3'. Overlapping 801 bp PCR fragment, containing the 3'end of AN6035.2 gene was amplified using primer pair 5'-AACTTGGAGGATGACAGGAG-3' and 5'-GTTGTACGAAGAAC-CAGCCT-3'. Genomic DNA containing the gene tandem AN2858.2 (*gaa*A) and AN2859.2 (*gaa*C) was amplified from genomic DNA originating from strains WG145 and WG222, respectively, using primer pair 5'- TACTCCAACAGGTCATCTGC -3' and 5'- TGTCTA-GAATCCAGAGGTGC -3', resulting in 3231bp PCR fragments. All PCR products were cloned in pGEM-T-easy vectors.

The *T. reesei* genomic region carrying the genes orthologous to *gaa*A and *gaa*B was amplified with primer pair 5'-AAGTCGCTGTCGCACTATGG-3'and 5'-CTG-GTGGTGAGTGTGAGATT-3', resulting in PCR product of 4719 bp. The PCR product was gel purified with QIAquick® gel extraction kit (Qiagen).

Obtained plasmids and PCR products were outsourced to BaseClear Labservices (BaseClear B.V., Leiden, The Netherlands) for full length sequence determination of both strands.

Cloning, expression of *A. niger gaa*A and *gaa*B genes, and purification of the encoded proteins

The open reading frames (ORF) of the *A. niger gaa*A and *gaa*B genes were amplified from total RNA isolated from 2 hour transfer culture grown on D-galacturonic acid using reverse transcription PCR as described above, using primers: A-NdeI 5'-ATCACATATGGC-TCCCCCAGCTGTGTTG-3' and A-NotI 5'- ATAAGCGGCCGCACAGCCATACAT-AAC-3' for the *gaaA* gene and B-NdeI 5'- CCGACATATGGTCAAGATCACAAG-3' and B-NotI 5'- ATTTGCGGCCGCCCCACTATACTATCC-3' for the *gaaB* gene. PCR products were cloned in pGEM-T easy vector to insure proper restriction digest in subsequent cloning procedures. The NdeI and Not I restriction sites were used for the cloning of both open reading frames into expression vector pET28a+ (Novagen). For cloning procedures and plasmids propagation, the non-expression host *E. coli* NovaBlue (Novagen) was used. Prior to expression, plasmids were transferred to *E. coli* strain ROSETA™(DE3)pLysS (Novagen). Transformed cells were grown overnight at 25^{o}C; 200 rpm in a LB medium containing 30 µg/ml kanamycin. After 17 hours of growth (OD_{600} ~ 2) cell cultures were diluted to final volume 100 ml (final OD_{600} ~ 0.05) and grown further for 5 hours under the same conditions. When OD_{600} reached ~ 1.0, cultures were split into 2 x 50 ml. IPTG to a final concentration of 0.5 mM was added to one of the 50 ml cultures, while the other one served as a non-induced control. Cultures were further incubated overnight in an orbital shaker at 16^{o}C and 200 rpm and harvested by centrifugation (5000 x *g*) for 15 min at 4°C, in a yield of 0.8 g per 50 ml culture media. The cell pellet was resuspended in 3 ml of cold binding buffer (20 mM sodium phosphate buffer, pH 7.4, 500

mM NaCl, 30 mM imidasole, 1 mM DTT, and EDTA free Complete protease inhibitor, (Roche). The suspension was passed twice through a French Press at 10 000 p.s.i., and then centrifuged at 4°C for 60 min at 5500 g.

The cleared soluble fraction was applied to a HisGraviTrap Ni column (GE Healthcare) equilibrated with 10 ml binding buffer. Bound protein was washed twice with 5 ml binding buffer and eluted with 2 x 5 ml elution buffer (20 mM sodium phosphate buffer, pH 7.4, 500 mM NaCl, 500 mM imidasole, 1 mM DTT, and EDTA free Complete protease inhibitor). The protein concentration of the first elution fraction was ~ 1 mg/ml and of the second elution fraction ~ 0.2 mg/ml (A280, Nanodrop® Spectrophotometer). Elution fractions containing recombinant protein were dialyzed overnight against 20 mM sodium phosphate buffer, pH 7.2. All extraction and purification procedures were performed at 4^{o}C.

Enzymatic assays

D-galacturonic acid reductase

Activity of the recombinant histidine tagged galacturonic acid reductase was measured by monitoring the change in NADPH or NADH absorbance at 340 nm in 10 mM sodium phosphate buffer pH 7.2 at room temperature. Final reaction volume was 600 µl. 10 µg of protein was added to the reaction mixture. Michaelis-Menten constants with D-galacturonic acid as a substrate were determined at a cofactor concentration of 160 µM. Michaelis-Menten constants with NAD(P)H as a substrate were determined at final concentration of D-galacturonic acid of 100 mM.

L-galactonate dehydratase

Activity of histidine tagged recombinant L-galactonate dehydratase was tested towards L-galactonic acid as a substrate by measuring the formation of 2-keto-3-deoxy-L-galactonate from L-galactonic acid sodium salt as described [180]. For the determination of the Michaelis-Menten constants, the concentration of L-galactonic acid was varied between 2.5 and 10 mM. The assays were performed at room temperature in 10 mM phosphate buffer pH 7.2. Absorbance was monitored at 549 nm, where the absorbance coefficient was taken to be 67.8 x 10^{3} M^{-1} cm^{-1}. L-galactonic acid sodium salt was obtained by dissolving L-

galactonic acid γ–lactone (Sigma) in 2 ml water to final concentration of 0.5 M, heating to 55^{o}C, stirring and slowly adding 1 M NaOH (total volume of added 1 M NaOH was 1 ml) until pH stabilized at 6.0 at room temperature.

Bioinformatics

Function prediction of hypothetical proteins

Annotation of protein sequences encoded by hypothetical proteins described in this study was done by performing an RPSBLAST search in NCBI Conserved Domain Database and Search Service, v2.11 [181].

A. nidulans orthologous genes/proteins were identified by selecting the best bidirectional best hit gene pair [182] in BLASTP searches in the *A. nidulans* sequence database at Broad Institute http://www.broad.mit.edu/tools/data/seq.html.

Phylogenetic analysis

Syntenic orthologs of both *gaa*A (An02g07710) and *gaa*C (An02g07720) were identified by a search query against an "in house" Conserved Orthologous Groups database comprising 21 fungal species i. e. *Saccharomyces cerevisiae, Kluyveromyces lactis, Ashbya gossypii, Debaryomyces hansenii, Candida albicans, Yarrowia lipolytica, Schizo-saccharomyces pombe, Neurospora crassa, Magnaporthe grisea, Gibberella zeae, Trichoderma reesei, Aspergillus nidulans, Aspergillus fumigatus, Aspergillus niger, Aspergillus oryzae, Aspergillus terreus, Aspergillus fisheri, Penicillium chrysogenum, Stagonospora nodorum, Ustilago maydis, Cryptococcus neoformans, Phanerochaete chrysosporium* and against the 57 finished fungal genomes present in the NCBI fungal genomes database.

Protein sequences encoded by the identified orthologous genes were aligned using CLUSTAL-W [183] program with default settings. Phylogenetic analysis was performed with the Mega 3.1 software package [149]. Phylogenetic trees were reconstructed using the Neighbor-Joining Method. To assess the reliability of the phylogenetic trees, bootstrap re-sampling was carried out 500 times. The cut-off value for the consensus trees was set at 75%. Gaps/Missing Data were calculated by the "Complete Deletion" algorithm. The used

substitution model was “Number of differences” with all substitutions included. Homogeneous pattern among lineages and uniform rates among sites were applied.

Phylogenetic footprinting

Phylogenetically conserved promoter elements in the promoter regions of the syntenic D-galacturonic acid reductase and 2-keto-3-deoxy-L-galactonate aldolase from the 18 fungal species were identified with the FootPrinter program version 2.1 [184] at http://bio.cs.washington.edu/software.html. The following search parameters were used: the motif size was set to six base pares with at most two mutations, where the maximum allowed number of mutations per branch was one. The subregion size was set to 100 bp with a subregion change cost of one mutation. Motif losses were allowed, at the cost of one mutation. The spanned tree significance level was set to “significant”.

Accession numbers

A. niger genomic region carrying the putative 2-keto-3-deoxy-L-galactonate aldolase (*gaa*C) and D-galacturonic acid reductase (*gaa*A) genes and *T. reesei* genomic region carrying the 2-keto-3-deoxy-L-galactonate aldolase (*kdg1*) and putative D-galacturonic acid reductase (*gar2*) genes were submitted to the NCBI database under accession numbers EF563988 and EF563987, respectively.

Results

Identification of *A. niger* genes encoding enzymes involved in the D-galacturonic acid degradation pathway by transcriptional profiling

To identify *A. niger* genes potentially involved in galacturonic acid catabolism, we analysed a microarray data set of 29 samples, obtained from mycelium pre-grown on fructose and transferred to the carbon sources fructose, sorbitol, rhamnose, xylose, galacturonic acid, polygalacturonic acid, and sugar beet pectin and sampled at four time points, namely 2, 4, 8 and 24 hours after transfer [116]. Obtained RNA was used for

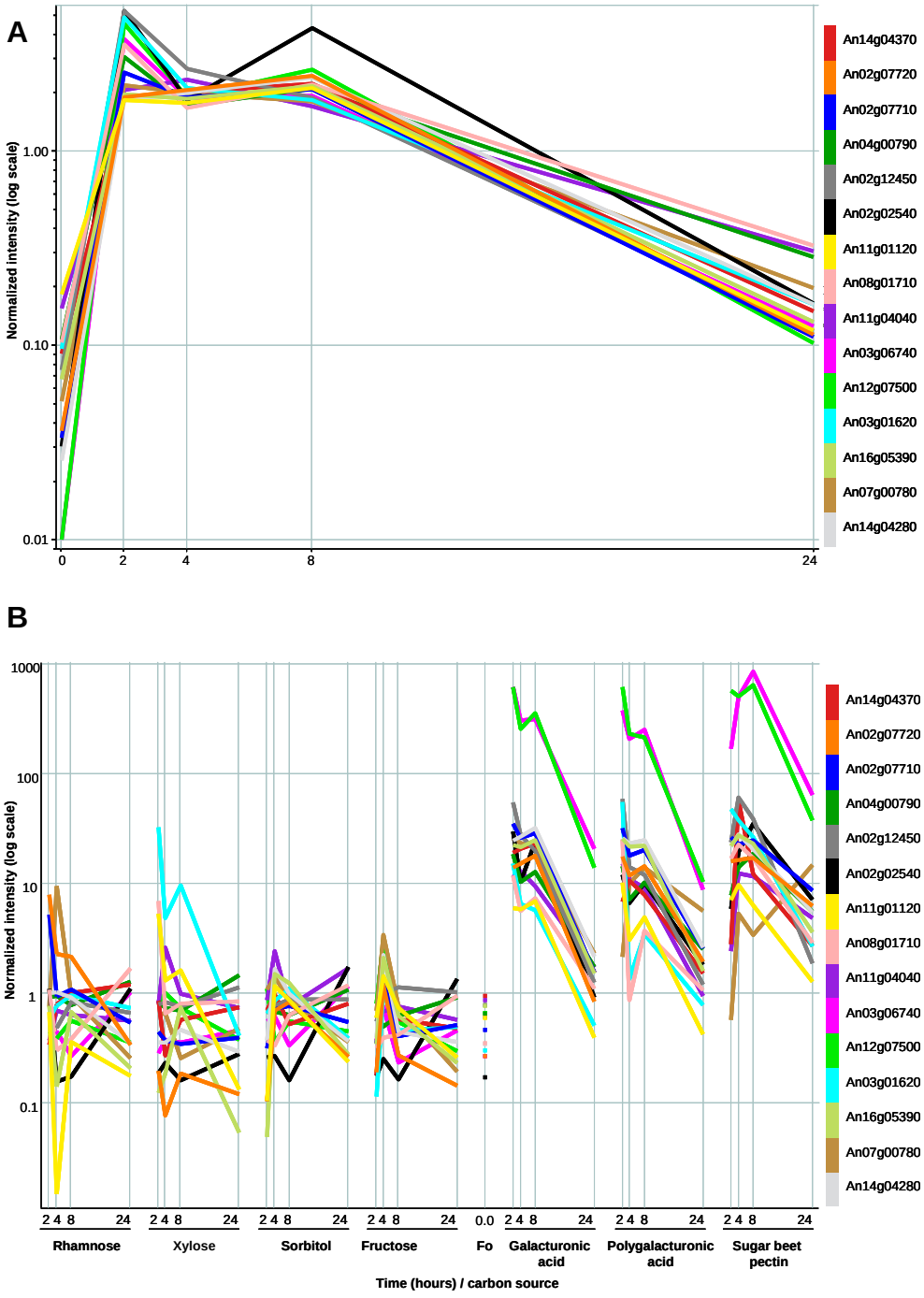
hybridisation of *A. niger* Affymetrix chips as described under “Materials and Methods”.
Ranking the 14,005 *A. niger* protein encoding genes included on the chip by scaled signal intensity after transfer to galacturonic acid, identified the hypothetical gene An16g05390 as the gene with the highest signal intensity of 16,359 from the maximum attainable intensity of 20,000, two hours after transfer from fructose to galacturonic acid. The expression levels of An16g05390 on galacturonic acid, polygalacturonic acid or on sugar beet pectin remained high for the next 2 to 6 hours, and decreased to background levels 24 hours after transfer coinciding with the depletion of galacturonic acid from the culture medium as determined by HPLC (Supplementary table 4). No pronounced signal corresponding to An16g05390 transcript was detected on any of the other tested substrates.
Since a putative galactonate dehydratase function could be assigned to the protein encoded by An16g05390 (see below), we investigated if there were other genes with a transcriptional profile similar to that of An16g05390. To identify genes that are significantly changing in response to the presence galacturonic acid, a t-test assessing the signal deviation of each gene transcript in each sample from its median value across the entire data set was performed. The minimum number of samples, in which genes should be differentially expressed was set to 9 since 24 hours after transfer to the different carbon sources, no galacturonic acid could be detected in cultures grown on eihter galacturonic acid, polygalacturonic acid or sugar beet pectin (Supplementary table 4). With the specified t-test parameters, 278 genes were identified as differentially expressed across the entire data set (Supplementary table 5). This gene list was further inspected for genes that display a transcriptional profile similar to that of An16g05390 during growth on galacturonic acid, using “Standard correlation” as a similarity measure. 14 genes were identified to exhibit an expression pattern highly correlating to that of An16g05390, when correlation coefficient of 0.95 was used as a cut off value (Figure 1A).
In addition to An16g05390, the cluster contained three more hypothetical proteins (An11g01120, An02g07710, and An02g07720), three putative transporters, and there was an overrepresentation of genes encoding pectinolytic enzymes (Table 1). To ensure that the induction of these 15 genes is solely due to galacturonic acid and not to other carbon sources, their transcriptional profiles on other substrates were examined as well. All genes

in this cluster were well induced on galacturonic acid, polygalacturonic acid and sugar beet pectin, but not on fructose, sorbitol, rhamnose or xylose (Figure 1B), supplementary table 6), except for a putative hexose transporter encoded by An03g01620 that was relatively highly expressed after 2 hours of cultivation on xylose as well. Further inspection of the genomic localization of the 15 genes within this cluster, revealed that An02g07710 and An02g07720 share a bidirectional promoter region.

Table 1. Genes with equivalent transcriptional profiles on galacturonic acid time course

Locus Tag	Standard correlation coefficient	Functional Annotation	Gene name	Reference
		Galacturonic acid degradation		
An02g07710	0.996	Galacturonic acid reductase	*gaaA*	This study
An16g05390	1.000	L-galactonate dehydratase	*gaaB*	This study
An02g07720	0.998	Putative 2-keto-3deoxy-galactonate aldolase	*gaaC*	This study
An11g01120	0.971	Putative L- glyceraldehyde reductase	*gaaD*	[70]
		Pectin degrading enzymes		
An14g04370	0.999	Pectin lyase A	*pelA*	[154]
An12g07500	0.979	Exopolygalacturonase X	*pgaX*	[116]
An11g04040	0.978	Exopolygalacturonase A	*pgaA*	[116]
An03g06740	0.971	Exopolygalacturonase B	*pgxB*	[116]
An02g12450	0.964	Exopolygalacturonase / Exoxylogalacturonan hydrolase	*pgxC*	[116]
An08g01710	0.953	Putative arabinofuranosidase		[185]
An02g02540	0.963	Putative pectin acetylesterase		[185]
		Transporters		
An07g00780	0.989	Major Facilitator family monocarboxylate/oxalate transporter		[185]
An14g04280	0.984	Major Facilitator family hexose transporter		[185]
An03g01620	0.958	Major Facilitator family hexose transporter		[185]
		Other processes		
An04g00790	0.971	Arom pentafunctional enzyme		[185]

Figure 1. **A graphical representation of the genes identified to have a transcriptional profile correlating to the one of L-galactonate dehydratase, *gaa*B (An16g05390). (A)** Galacturonic acid time course: 0, 18 hours pre-culture on fructose; 2, 2 h after transfer to galacturonic acid; 4, 4 h after transfer to galacturonic acid; 8, 8 h after transfer to galacturonic acid; 24, 24 h after transfer to galacturonic acid. **(B)** Time courses on all carbon sources used in this study: 0, 18 h pre-culture on fructose; 2, 2 h after transfer; 4, 4 h after transfer; 8, 8 h after transfer; 24, 24 h after transfer. Legend key: An02g07710, galacturonic acid reductase *gaa*A; An16g05390, L-galactonate dehydratase *gaa*B; An02g07720, putative 2-keto-3deoxy-galactonate aldolase *gaa*C; An11g01120, L-glyceral-dehyde reductase *gaa*D; An14g04370, pectin lyase A *pel*A; An12g07500, exopolygalacturonase X *pga*X; An11g04040, exopolygalacturonase A *pga*A; An03g06740, exopolygalacturonase B *pgxB*; An02g12450, exo-polygalacturonase C *pgx*C; An08g01710, putative arabinofuranosidase; An07g00780, monocarboxylate/oxalate transporter; An14g04280, hexose transporter; An03g01620, hexose transporter; An04g00790, Arom penta-functional enzyme; An02g02540, putative acetyl-esterase.


A
Normalized intensity (log scale)
1.00
0.10
0.01
0
2
4
8
24
An14g04370
An02g07720
An02g07710
An04g00790
An02g12450
An02g02540
An11g01120
An08g01710
An11g04040
An03g06740
An12g07500
An03g01620
An16g05390
An07g00780
An14g04280
B
Normalized intensity (log scale)
1000
100
10
1
0.1
2 4 8 24
Rhamnose
Xylose
Sorbitol
Fructose
0.0
Fo
Galacturonic acid
Polygalacturonic acid
Sugar beet pectin
Time (hours) / carbon source

Function prediction of hypothetical proteins by RPSBLAST

To predict the possible functions of the proteins encoded by the four hypothetical genes within the identified transcriptional cluster, the corresponding protein sequences were used in a RPSBLAST search against NCBI Conserved Domain Database (CDD) to identify conserved domain regions. The obtained results (Table 2) suggested that the 4 hypothetical proteins possess the putative activities needed for the conversion of galacturonic acid to pyruvate and glycerol. The protein encoded by An02g07710 (here assigned *gaa*A) was identified as a putative member of the MviM group of predicted dehydrogenases, and an NAD/NADP binding Rossmann fold characteristic for the GFO_IDH_MocA oxidoreductase family was detected in its sequence. In An16g05390 (assigned *gaa*B) a conserved "rTS beta_L-fuconate_dehydratase"domain was found. rTS beta is a member of the mandalate racemase (MR)-like subfamily of the enolase superfamily. Some other hierarchically related members of the MR subfamily are mandelate racemase, D-glucarate/L-idarate dehydratase, D-galactonate dehydratase, D-gluconate dehydratase, and L-rhamnonate dehydratase. An02g07720 (assigned *gaa*C) was identified as a putative member of the "Dihydrodipicolinate synthase" (DHDPS)-like family, which is a branch of class I aldolases family CD00945.

The DHDPS-like family includes several pyruvate-dependent class I aldolases that use the same catalytic step to catalyze various reactions in different pathways and includes activities such as 5-keto-4-deoxy-glucarate dehydratase, and 2-keto-3-deoxy- gluconate aldolase. Therefore, an aldolase function was assigned to the encoded protein. Finally, An11g01120 (assigned *gaa*D) appeared to encode a putative aldehyde reductase from the

Table 2. Conserved domains in the four A. niger genes encoding hypothetical proteins strongly induced on galacturonic acid and their orthologs in A. nidulans

Gene name	*A. niger* locus tag	*A. nidulans* locus tag	NCBI CDD ID	NCBI CDD description	E- value
*gaa*A	An02g07710	AN2858.2	COG0673	MviM; Predicted dehydrogenases and related proteins	4e-21
			PFAM01408	GFO_IDH_MocA, Oxidoreductase family, NAD-binding Rossmann fold	6e-07
*gaa*B	An16g05390	AN6035.2	CD03324	rTS beta_L- fuconate_dehydratase	0.00
*gaa*C	An02g07720	AN2859.2	CD00408	DHDPS-like; Dihydrodipicolinate synthase family	3e-53
*gaa*D	An11g01120	AN7193.2	COG0656	ARA1, Aldo/keto reductases related to di-keto-gulonate reductase	2e-62

ARA1 aldo/keto reductases group, related to di-keto-gulonate reductase (COG0656). The newly assigned gene names, locus tags, and CDD descriptions of *gaa*A, *gaa*B, *gaa*C, and *gaa*D are summarised in Table 2.

Biochemical identification of recombinant galacturonic acid reductase (GAAA) and L-galactonate dehydratase (GAAB)

To investigate if the proteins encoded by the *A. niger gaa*A and *gaa*B genes, indeed posses the predicted biochemical activities, the corresponding open reading frames were cloned as 6xHisTag N-terminal fusions and expressed in *E. coli* under the strong inducible T7*lac* promoter. The apparent molecular mass of the purified tagged proteins upon SDS-PAGE was in agreement with the predicted molecular masses of 44 kDa for GAAA and 50 kDa for GAAB (data not shown). The activity of the recombinant L-galactonate dehydratase towards L-galactonate was measured by the thiobarbituric acid assay. The determined specific activity was 5.6 nkat/mg at L-galactonate concentration of 7.5 mM and pH 7.0, where Km for L-galactonate was 3.4 mM. L-galactonate concentration of 10 mM had an inhibiting effect on the enzyme activity.

In contrast to the previously described galacturonic acid reductase GAR1 from *T. reesei*, the *A. niger* D-galacturonic acid reductase GAAA was active towards D-galacturonic acid and D-glucuronic acid with both NADH and NADPH as a cofactor. The enzyme also catalysed the reverse reaction with L-galactonate as a substrate. The kinetic properties of

Table 3. Kinetic properties of the recombinant D-galacturonic acid reductase of *A. niger*. Activities were measured in 10 mM sodium phosphate buffer (pH7.2) at room temperature. Cofactor concentration was 160 μM. 1100 mM galacturonic acid was used as a substrate. N. d., mot determined.

	Specific activity		Km	Kcat/Km
	nkat.mg^{-1}	Kcat	mM	s^{-1}.M^{-1}
NADH[1]	105	4.98	0.326	15 273
NADPH[1]	150	7.11	0.036	197 222
D-galacturonic acid/NADH	115	5.45	7.110	767
D-galacturonic acid/NADPH	145	6.88	0.175	39 289
D-glucuronic acid/NADH	2.8	0.13	n.d.	n.d.
D-glucuronic acid/NADPH	65.6	3.11	n.d.	n.d.
L-galactonate/NAD+	1.7	0.08	n.d.	n.d.
L-galactonate/NADP+	3.5	0.16	n.d.	n.d.

the recombinant GAAA are listed in Table 3.D-galacturonic acid reductase was also tested with xylose, rhamnose, fructose, D,L-glyceraldehyde, D,L-glyceraldehyde phosphate, dihydroxyacetone and dihydroxyacetone phosphate as substrates. No activity with these compounds was detected.

Identification of mutant loci of *A. nidulans* strains affected in galacturonic acid catabolism

WG222 and WG262 are mutant *A. nidulans* strains affected in galacturonic acid utilisation. The mutant locus *gaa*B present in WG262 is assigned to linkage group I, while strain WG222 carries the mutant locus *gaa*A, assigned to linkage group VI. Yet, no molecular functions linked to the mutant phenotype of these strains nor the affected genes were identified [61].

We examined if orthologs of the identified *A. niger gaa* genes are responsible for the mutant phenotype of the WG222 and WG262 mutant *A. nidulans* strains, and hence involved in galacturonic acid degradation in this fungus.

The *A. nidulans* genes orthologous to the *A. niger gaa*A, *gaa*B, *gaa*C, and *gaa*D were identified by bidirectional BLAST at protein level against the *A. nidulans* sequence database at Broad Institute (Table 2). Their genomic locations are as follows: AN7193.2 is localized at chromosome IV (equivalent to linkage group IV) and AN6035.2 at chromosome I (equivalent to linkage group I). AN2858.2 and AN2859.2 are positioned next to each other on chromosome VI and share a common promoter as previously observed in *A. niger*. Since the mutant *gaa*A and *gaa*B loci are located at chromosomes VI and I, respectively, AN6035.2 and gene pair AN2858.2/AN2859.2 were further screened for possible mutations. Genomic DNA from the *A. nidulans* wild type WG145, and the two mutant strains WG262 and WG222 was used as a template for the amplification of AN6035.2, and the gene pair AN2858.2 and AN2859.2, respectively. Comparison of the sequences obtained from the wild type strain, with those from the two mutant strains resulted in the identification of a single point mutation in each of the corresponding mutant strains. Within the gene pair AN2858.2/AN2859.2 no mutations were detected in the

sequence of gene AN2859.2, however the sequence of AN2858.2 contained a single point mutation, leading to a stop codon at the position of Gly61. This mutation most likely causes the mutant phenotype of the *gaa*A locus. In the sequence of AN6035.2, a single point mutation leading to a Pro102Thr amino acid substitution in the corresponding protein was found. Inspection of Pro102 in a multiple alignment of closely related protein sequences from different species (data not shown) suggested that Pro102 is positioned at the beginning of a loop, specifically conserved within sequences from fungal origin. Such a mutation could be critical for the stability of the protein, and thus could cause the mutant phenotype of the *gaa*B locus. To test this hypothesis, *A. nidulans* mutant strains WG262 and WG222, and the wild type strain WG145 were grown on agar plates containing 1% GalA as a sole carbon source at 37^{o}C (optimal temperature) and 16^{o}C. At 37^{o}C, the growth of both mutant strains was severely reduced to equal extent compared to that of the wild type. Incubation at 16^{o}C resulted in a growth recovery of up to 60% for strain WG262, carrying the Pro102Thr mutation in the putative dehydratase encoded by AN6035.2, possibly due to stabilisation of the protein at lower temperature. WG222 retained its mutant phenotype at lower temperature.

Galacturonic acid degradation in other fungi

Since a non-phosphorolytic galacturonic acid degradation pathway was previously described for the fungus *T. reesei*, we also investigated if orthologs of the genes encoding the catalytic steps of D-galacturonic acid degradation in *A. niger* are present in *T. reesei* and other filamentous fungi. For this we used the genomic region carrying the *gaa*A and the *gaa*C genes from *A. niger* as a search query against an in house Conserved Orthologous Groups database comprising 21 fungal species, and against the 57 finished fungal genomes present to date in the NCBI database. Equivalently organized syntenic orthologs of *gaa*A (An02g07710) and *gaa*C (An02g07720) were present in all ascomycete species from subphylum *Pezizomycotina*, comprising plant pathogen and plant saprobic species, with the exception of the four completely sequenced fungi that belong to the order *Onygenales viz. Ascosphaera apis*, *Ajellomyces capsulatus*, *Coccidioides immitis*, and *Uncinocarpus reesii*

(Figure 2).

A. apis, *A. capsulatus*, and *C. immitis*, are well known zoopathogens while *U. reesii* is a soil saprobe, most closely related to the *Coccidioides* species [186]. Considering the habitat of *U. reesii*, we inspected the genome database of *U. reesii* at Broad Institute for the presence of genes with PFAM domains corresponding to different glycoside hydrolase (GH) families. No genes encoding GH28 (homogalacturonan, rhamnogalacturonan, and xylogalacturonan hydrolases), or GH17 (pectin lyases) were found the genome of *U. reesii or* in the genomes of *A. apis*, *A. capsulatus*, and *C. immitis*. This result strongly suggests that these four fungi are not capable of pectin degradation, and thus cannot recruit galacturonic acid as a carbon source.

In the provisional assembly of the genome of *T. reesei*, available at DOE Joint Genome Institute, both genes are located on neighboring contigs with the 5' end of the D-galacturonic acid reductase ortholog positioned 1770 bp upstream of the 5' end of the *lga*1 gene, encoding the 2-keto-3-deoxy-L-galactonate aldolase at contig 281; and its 3' end positioned at contig 280. The sequence encoding the missing 90 amino acids from the *T. reesei* ortholog of GAAA is located in a contig gap of 271 nucleotides, which we were able to verify by sequencing of the complete locus. Interestingly, the previously identified D-galacturonic acid reductase of *T. reesei* (GAR1) is not a part of this specific genomic arrangement, but is located elsewhere on the genome. The sequence of the *T. reesei* ortholog of the *gaa*A gene, for which we propose the name *gar*2 was deposited in NCBI GenBank with accession number EF563987. In genomes of basidiomycete species, genes with a lower sequence similarity to *gaa*A and *gaa*C were found, however these genes did not share a common promoter. No homologues were found in the genomes of any members of the yeast taxa or in the genome of the zygomycete *Rhizopus oryzae*.

Equivalent results were obtained for the *gaa*B gene encoding L-galactonate dehydratase. Orthologous sequences were present in all sequenced Pezizomycotina species, except for the order *Onygenales*. More distantly related sequences similar to L-galactonate dehydratase were detected in the genomes of the non-pathogenic yeast *Pichia guilliermondii*, the zygomycete *R. oryzae*, and in the genomes of the basidiomycetes *U. maydis*, *P. chrysosporium*, *Coprinopsis cinerea*, and *C. neoformans*.

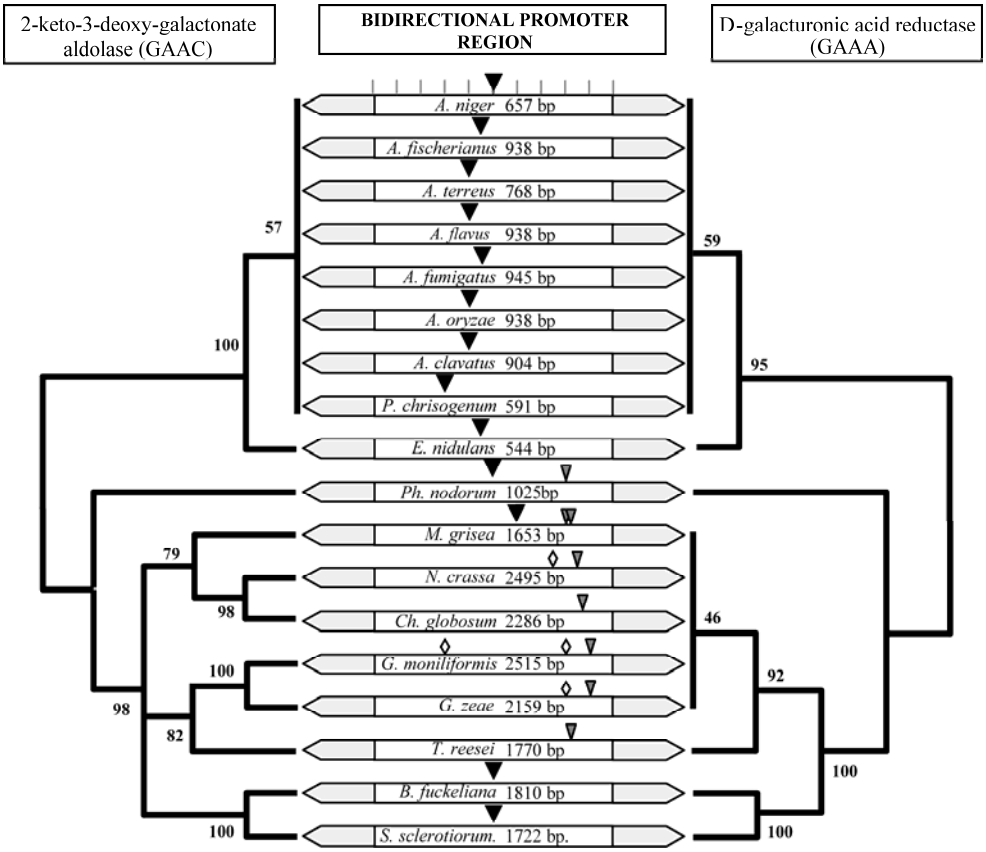


Figure 2. Conservation of the genomic organization of the gene pair *gaa*A/*gaa*C encoding D-galacturonic acid reductase and putative 2-keto-3-deoxy-L-galactonate aldolase in *Pezizomycotina* species. Left and right - Neighbor-Joining phylogeny reconstruction of GAAC and GAAA, respectively. Bootstrap scores are displayed at the nodes. Centre – Schematic representation of the genomic organisation of *gaa*C/*gaa*A locus. Shared promoter regions are in white. Coding regions are in gray. Species name and promoter size (in bp) are indicated. The positions of the detected motifs relative to the start codon are indicated: ↓ TCCACCAAT; ◊ CCACCAA; ▼ CCNCCAA.

*A. niger's gaa*D gene appeared to be orthologous to the previously described *gld*1 gene from *T. reesei*, encoding glycerol dehydrogenase GLD1 (EC 1.1.1.72) [70]. In that study, the authors demonstrated that GLD1 functions as an aldehyde reductase, which converts D-glyceraldehyde and L-glyceraldehyde to glycerol, but cannot perform the reverse reaction, and hypothesized that this enzyme is active in pathways for sugar acid catabolism such as D-galacturonate degradation. Conserved homologues of *gaa*D were identified in all 18 fungal species carrying sequences orthologous to *gaa*A *gaa*B and *gaa*C.

Table 4. Identified promoter elements in the upstream regions of genes with equivalent transcriptional profiles on galacturonic acid time course. The phylogenetically conserved core motive is presented in bold, variant nucleotides are underlined. [1]Motive was found in the complementary strand; [*]An02g07710 and An02g07720 share the same promoter region.

Locus Tag	Functional Annotation	Gene name	Distance from ATG start codon (in bp)	Identified promoter element
An02g07710	Galacturonic acid reductase	*gaaA*	-415	T**CCACCAA**T[*]
An16g05390	L-galactonate dehydratase	*gaaB*	-327	T**CC**<u>**G**</u>**CCAA**T
An02g07720	Putative 2-keto-3deoxy-galactonate aldolase	*gaaC*	-293	T**CCACCAA**T[1,*]
An11g01120	L- glyceraldehyde reductase	*gaaD*	-584	T**CCACCAA**T[1]
An14g04370	Pectin lyase A	*pelA*	-530	T**CCACC**<u>**T**</u>**A**T
An12g07500	Exopolygalacturonase X	*pgaX*	-389	T**CC**<u>**C**</u>**CCAA**T
An11g04040	Exopolygalacturonase A	*pgaA*	-595	T**CCACCAA**T[1]
An03g06740	Exopolygalacturonase B	*pgxB*	-299	<u>**C**</u>**CCACCAA**T[1]
An02g12450	Exopolygalacturonase / exoxylogalacturonan hydrolase	*pgxC*	-269	T**CCACCAA**T
An08g01710	Putative arabinofuranosidase		-240	T**CCA**<u>**T**</u>**CAA**T
An02g02540	Putative pectin acetylesterase		-1237	<u>**C**</u>**CC**<u>**C**</u>**CCAA**<u>**G**</u>
An07g00780	Major Facilitator family monocarboxylate/oxalate transporter		-995	<u>**C**</u>**CCACCAA**T[1]
An03g01620	Major Facilitator family hexose transporter		-674	<u>**A**</u>**CC**<u>**T**</u>**CCAA**T
An14g04280	Major Facilitator family hexose transporter; Putative quinate transporter		-334	T**CCA**<u>**G**</u>**CAA**T[1]
An04g00790	Arom pentafunctional enzyme		-315	<u>**C**</u>**CCA**<u>**G**</u>**CAA**<u>**G**</u>[1]

Conserved promoter elements

The promoter regions shared between D-galacturonic acid reductase and 2-keto-3-deoxy-L-galactonate aldolase from the 18 identified fungal species were analyzed for evolutionary conserved elements using the FootPrinter program. The motif identified with the highest significance score of 5.20 was CCNCCAA (Figure 2), which could be extended to TCCACCAAT in *Aspergillus* species. This extended motif with one allowed mismatch at either position one or four (YCCNCCAAT) was also present in the 1 kb upstream regions of seven of the remaining 13 genes from the identified transcriptional cluster (Table 4). This motif was found in less then 10% of the 1 kb upstream regions when the total search space was extended to all annotated *A. niger* genes. Interestingly, a systematic analysis of the promoter region of the polygalacturonase II gene of *A. niger* revealed that the same

motif is essential for high expression levels of the corresponding protein [75], and it was later identified in the promoter sequences of *pga*I and *pga*C genes [75]. Bussink *et al*. also reported the presence of an additional upstream *cis*-acting repressor region present in the promoter of PGII.

Discussion

While fungal extra-cellular enzymes that are involved in the degradation of pectin, have been studied in detail in the past decades, until recently, little was known about the metabolic processes by which fungi utilise galacturonic acid, the major constituent of this abundant plant polymer.

Microarray analysis of *A. niger* cultures grown on sugar beet pectin, polygalacturonic acid and different pectic sugars allowed us to identify the hitherto unknown genes involved in the degradation of galacturonic acid in this fungus. Biochemical verification of the enzymatic activities encoded by the *gaa*A and *gaa*B genes confirmed this finding. Interestingly, the here described galacturonic acid reductase of *A. niger* GAAA, is different from the previously reported enzyme GAR1 from *T. reesei*. While GAR1 can function only with NADPH as a co-factor, GAAA can utilise both NADH and NADPH. In addition, the kinetic properties and the substrate preferences of both enzymes differ, as GAAA has a much lower Km value towards galacturonic acid with NADPH as a co-factor, and it is not capable to utilise glyceraldehyde as a substrate. It is very well possible that two different enzymes operate in *T. reesei*, as the *gaa*A ortholog of this fungus – the *gar*2 gene is strictly conserved and found in the same genomic arrangement as in *A. niger* and other fungi reported in this study. The ortholog of *gar*1 in *A. niger*, the An16g04770 gene, is strongly induced by rhamnose, where its maximum intensity levels reach 4391, compared to 664 on galacturonic acid, 748 on polygalacturonic acid, and 1281 on sugar beet pectin. These differences in expression levels suggest that it is more likely that the gene product of An16g04770 is involved in processes related to the degradation of rhamnose, rather than galacturonic acid. The L-galactonate dehydratase of *A. niger* GAAB is orthologous to L-

galactonate dehydratase LGD1 of *T. reesei*, but the recombinant tagged GAAB has slightly lower Km value of 3.4 mM and higher specific activity towards L-galactonate compared to LGD1 measured in cell free extracts of *T. reesei*.

Inspection of the *A nidulans* genes orthologous to *gaa*A, *gaa*B, *gaa*C, and *gaa*D from *A. niger* allowed the identification of the two mutant loci of the *A nidulans* strains affected in galacturonic acid catabolism and the assignment of a molecular function to them. Further search for orthologous sequences in the genomes of other fungi provided evidence that a specific galacturonic acid catabolic pathway, different from that in bacteria, operates in several fungal species. The genes encoding the enzymatic activities needed for the non-phosphorolytic degradation of galacturonic acid via L-galactonate and 2-keto-3-deoxy-L-galactonate to pyruvate and L-glyceraldehyde are strictly conserved in the genomes of all 18 sequenced plant pathogenic and plant saprobic ascomycete species from subphylum *Pezizomycotina*. In contrast, these genes could not be detected in genomes of the sequenced typically zoopathogenic members of this subphylum, demonstrating that a galacturonic acid degradation pathway is ubiquitously present in pectin degrading fungi. The particular genomic organization of the D-galacturonic acid reductase and the 2-keto-3-deoxy-L-galactonate aldolase encoding genes seems to be an important feature of this catabolic system since it has been conserved over hundreds of millions of years of evolution.

The results of this study demonstrate that, at least in *A. niger*, the genes encoding the galacturonic acid catabolic pathway are co-expressed with a subset of the available pectinolytic genes encoded in the genome. This subset of highly expressed extracellular activities includes the three exopolygalacturonases, the single exopolygalacturonase/ exoxylogalacturonan hydrolase and one of the pectin lyases of *A. niger* and forms a core group of activities that is capable of initial degradation of pectin. The identification of a common promoter element that is phylogenetically conserved and present in the promoters of several co-expressed pectinases of *A. niger* suggests the presence of pectinolytic regulatory system that may share common components across different fungal species.

Chapter 6

Comparative Transcriptome Analysis of the Molecular Response to Galacturonic Acid Induction in *Aspergillus niger* and *Aspergillus nidulans*

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Manuscript in preparation

Galacturonic acid *vs.* fructose induced changes in the transcriptomes of two filamentous fungi, *Aspergillus niger* and *Aspergillus nidulans*, were assessed using Affymetrix and NimbleGen microarrays. Orthology assignment of the two genomes, combined with comparison of the transcriptional data identified a shared group of 29 functional orthologs that demonstrated a comparable transcriptional profile in response to carbon source induced changes. Galacturonic acid induced 22 of these shared genes. The highest up-regulated transcripts included the genes coding for the enzymatic activities needed for the non-phosphorolytic degradation of galacturonic acid to glyceraldehyde and pyruvate. The induction of several specific genes involved in central carbon metabolism suggests that the formed pyruvate and glyceraldehyde are further catabolized via oxaloacetate and glycerol, respectively. Only two, of the identified 21 pectinase encoding orthologous gene pairs, showed equivalent induction profiles on galacturonic acid, suggesting that the regulatory mechanisms controlling the pectinolytic systems of *A niger* and *A. nidulans* differ from each other and that, in addition to galacturonic acid, other factors are involved in their induction.

Introduction

The genome sequences of several *Aspergillus* species have become available during the last few years, which make it feasible to do all-against-all sequence compressions of the proteins encoded by these genomes, to establish groups of orthologous proteins, and to distinguish these orthologs from closely related paralogs through conservation of synteny. Orthologs are sequentially equivalent proteins that arise from vertical evolution and are thought to play similar developmental or physiological roles in different species, whereas paralogs are the result of duplication events and their function may have diverted from the original ancestor [187].

Comparative genome approaches have been used for *Aspergillus* species to transfer knowledge from one organism to another in order to provide useful information about their physiology and to indicate functional similarities or differences between them [17, 79, 88]. However, information transferred through orthology assignment only provides a static model of the potential encoded by the respective genomes, and does not reveal differences in phenotype and plasticity in response to external signals. To better understand these evolutionary processes, the data, obtained from sequencing and comparison of individual genomes, should be complemented with results obtained from transcriptome and proteome profiling experiments, as they can reveal which of the many genetic elements present in the cell are functional under determined conditions and how they may interact with each other. In addition, comparison of genome, transcriptome, and proteome data from different organisms can reveal evolutionarily conserved mechanisms and pathways of expression control [188], and can disclose to what extent gene conservation can be linked to functional conservation.

Conservation of transcriptional response during estrogen dependant growth of uterine

fibroids (leiomyoma) between human and rat, using DNA microarray data integration has identified 12 orthologous genes involved in the underlying molecular mechanisms [189]. A significant, but surprisingly small overlap of ~40 orthologous genes with conserved cell cycle regulated expression was found between *S. cerevisiae* and *S. pombe* [123]. This finding was later confirmed by comparative transcriptional analysis between these two yeast species and human. [124]. Comparative transcriptomics studies of metal homeostasis in *Arabidopsis thaliana* and *A. halleri* have also been performed, revealing again only a small number of orthologous genes with an equivalent transcriptional response [190].

The results of these studies indicate that, although biological systems can be evolutionarily conserved on genome level in the form of orthologous structural proteins and transcription factors, the transcriptional regulation of the majority of them is not, and the relation between them cannot be inferred by comparative genomics. Therefore, evolutional conservation of transcriptional control, that arised from a selective advantage, is a powerful criterion, when the aim is to distinguish the core set of functionally important for a biological process genes from the greater part of co-expressed genes [191].

Here we compare transcriptional responses to galacturonic acid, through microarray analysis of two filamentous fungi belonging to the genus *Aspergillus*, the industrial enzyme and acid producer *Aspergillus niger* and the model organism *Aspergillus nidulans*. Both fungi are soil plant saprobes that can degrade plant cell wall material and they posses a comparable number of genes encoding carbohydrate-degrading enzymes [17]. Previously, we have shown that *A. niger* and *A. nidulans* share an evolutionary conserved three-step pathway for the degradation of galacturonic acid to pyruvate and glyceraldehyde and that the genes encoding the corresponding enzymatic activities are orthologous in both fungi [134]. At least in *A. niger* this pathway is strongly induced on galacturonic acid and the expression profiles of the genes encoding this pathway are tightly clustered with three genes putatively encoding transporter proteins and a defined subset of the available pectinolytic genes. Together, the cluster encodes a core group of activities, capable of degradation and further catabolism of homogalacturonan, an important component of pectin [134]. In this study, our goal was to determine to what extent transcriptional control of the pectinolytic system is conserved between these species.

Materials and methods

Identification of *A. niger* and *A. nidulans* orthologs and pectinase inventory.

A. niger CBS 513.88 [17] and *A. nidulans* FGSC A4 [88] genome and protein sequences were obtained from GenBank http://www.ncbi.nlm.nih.gov/Genbank/. Orthologous genes were identified by bidirectional BLAST searches [182]. Gene and protein identifiers, corresponding to different pectinase activities from *A. niger* and *A. nidulans* were derived from the Carbohydrate Active Enzymes database (CAZy) at http://www.cazy.org/ [144].

Strains and growth conditions

Wilde type strain *A. niger* N400 (CBS 120.49) was pregrown in 300 ml minimal medium [128] (pH 6.0) containing 0.1% (w/v) yeast extract and Vishniac trace elements [129] with 2% (w/v) D-fructose as a sole carbon source, inoculated with 10^6 spores/ml and cultivated at 30°C at 250 rpm in an orbital shaker. After 18 h of incubation, mycelium were harvested on a Büchner funnel with nylon gauze, washed once with sterile 0.9% (w/v) NaCl, and aliquots of 1.5 g (wet weight) of mycelium were transferred to 50 ml of minimal medium (pH 6.0) containing 0.1% (w/v) yeast extract, Vishniac trace element solution and 1% (w/v) D-fructose (Merck), or D-galacturonic acid (Fluka Chemica). Four hours after transfer, mycelium was harvested on a Büchner funnel with nylon gauze and immediately stored at -70°C.

A. nidulans strain WG145 (*w*A3; *pyro*A4), was cultured using the same medium, supplemented with 1 mg/L pyridoxine HCl. Cultures were inoculated with 10^7 spores/ml and cultivated at 37°C at 250 rpm in an orbital shaker for 18 hours, before transfer to minimal media supplemented with 1 mg/L pyridoxine HCl and containing either D-fructose (Merck), or D-galacturonic acid as a sole carbon source. Further cultivation and harvesting was performed as described above for A. *niger*.

RNA isolation and Affymetrix microarray sample processing

Total RNA samples were prepared using TRIZOL® Reagent (Invitrogen™) according to the instructions of the supplier. *A. niger* biotin-labeled cRNA was prepared from approximately 10 μg of total RNA, fragmented and hybridized strictly following standard Affymetrix protocols [192]. RNA quality and integrity before and during sample processing was verified by analyzing aliquots with the Agilent Bioanalyzer 'Lab-on-chip' system (Agilent Technologies). Probe arrays were washed using fluidics protocol EukGE-WS2v5-450 and scanned with an Agilent Technologies G2500A Gene Array Scanner at a pixel value of 3 mm and a wavelength of 570 nm. *A. nidulans* total RNA samples were outsourced to NimbleGen Systems, Iceland for further processing and array hybridization.

Microarray platforms and data analysis

A. niger transcript levels were assessed using custom made 'dsmM_ANIGERa_coll' Affymetrix GeneChip® Microarrays kindly provided by DSM Food Specialties (Delft, The Netherlands). *A. nidulans* transcript levels were assessed by NimbleGen gene expression microarrays 2005-12-09_A_nidulans_60mer_expr, array design 2855.0 based on Release 3 of the completed genome of *A. nidulans* FGSC A4 [88]**.** A comparison of both microarray types is given in Table 1.

Data from both systems was RMA preprocessed and further normalized to the median of all genes using GeneSpring software package version 7.2. Genes with raw signal intensity below background levels of 100 for *A. niger* and 150 for *A. nidulans*, respectively, and genes with fold change ≤ 2fold, were filtered out. Genes that were significantly changing in response to the carbon source were identified by One-way ANOVA analysis using parametric Student's t-test with p-value cutoff of 0.05 and a subsequent multiple testing correction by the False Discovery Rate method of Benjamini and Hochberg [193]. Differentially expressed genes, with equivalent transcriptional response to carbon source induction, were identified by (i) querying the lists obtained from ANOVA analysis from each of the two fungi against the list of all identified orthologous proteins and (ii) by

examining the significance of transcriptional response of the genes identified in (i) in the other fungus (figure 1).

Table 1 Features comparison between *A. niger* Affymetrix and *A. nidulans* NimbleGen microarrays

	Aspergillus niger	***Aspergillus nidulans***
Platform	Affymetrix	NimbleGen
Strain used in array design	*A. niger* CBS 513.88	*A. nidulans* FGSC A4
Sequence origin and year of release	DSM, 2007	Broad Institute, 2005
Number of protein encoding genes represented on chip	14006	9457
Oligo size	25mer	60mer
Number of probes per gene	12	13
Mismatch probes	yes	no
Probe replicate	No replicated probes	Triplicate
Probe placement	random	random
Control probes	included	included
Intensity range	0-15 500	0-60 000

Results and Discussion

Determination of orthologs and an inventory of pectinases encoded in the genomes of *A. niger* and *A. nidulans*

To identify orthologous protein encoding genes shared between *A. niger* and *A. nidulans*, an all-against-all bidirectional search was performed using the encoded proteins as input. Only bidirectional best hit pairs were considered as orthologous. Between the 14162 *A. niger* genes [17] and 10701 *A. nidulans* genes (Broad Institute, Release 4[*]) present in the genomes of each of both species, respectively, 7328 orthologous pairs were identified.

A list of *A. nidulans* and *A. niger* locus tag numbers for genes (putatively) encoding pectinase activities was obtained from the Carbohydrate Active Enzymes database (CAZy) [15]. This list was further inspected for the presence of orthologs by comparing each entry to members of the 7328 orthologous pairs, and to the recently published recombinant carbohydrate degrading enzymes from *A. nidulans* [194]. Both species were found to have a

[*] http://www.broad.mit.edu/annotation/genome/aspergillus_group/News.html; March 7, 2006

set of 38 potential pectinase encoding genes, however only 21 of them were identified as orthologous pairs (table 2). Furthermore, despite the equal number in total, a different distribution across the functional groups of pectinases was observed. While in the genome of *A. niger* 21 genes encode glycoside hydrolases from CAZy family GH28, in *A. nidulans* only nine genes putatively encoding proteins from that family were detected, eight of which are orthologous to one of the *A. niger* genes. In contrast, the group of polysaccharide lyases from *A. niger* comprises of eight genes compared to 18 from *A. nidulans*. Although, both fungi have five genes encoding for pectin lyases from CAZy family PL1, only two of those genes are orthologous. Compared to the single pectate lyase gene from *A. niger*, an impressive number of nine pectate lyases from three different CAZy families - PL1, PL3, and PL9 are encoded by the genome of *A. nidulans*. In addition to the two genes orthologous to *A .niger* proteins, *A nidulans* has three more genes putatively encoding for rhamnogalacturonan lyases. Together with the sequences, orthologous to the two putative unsaturated rhamnogalacturonyl hydrolases from *A. niger*, two additional putative enzymes assigned to GH Family 105 were identified in the genome of *A. nidulans.* Interestingly, such diversity was not observed in the group of carbohydrate esterases. Both fungi posses an equal number of orthologous genes encoding pectin methyl esterases, pectin acetyl esterases, and rhamnogalacturonan acetyl esterases. The preference for either hydrolase (in the case of *A. niger*) or lyase activities (in the case of *A. nidulans*) may be explained by the differences in their physiology. While *A. niger* is well known for its ability to produce organic acids and readily acidifies its direct environment during growth, *A. nidulans* does not produce organic acids but accumulates and eventually secretes polyols [195]. Subsequently, the pectinolytic system of *A. niger* is enriched with glycoside hydrolases, with an pH optimum between 3 and 5 [45], while in *A. nidulans* mainly pectic lyases with a pH optimum between 6 and 8.5 (pectin lyases) or 8.5 to 9.5 (pectate lyases) are found [196]. This also may explain the doublenumber of candidate unsaturated rhamnogalacturonyl hydrolases encoded in the genome of *A. nidulans*, as this type of enzymes acts on the products formed by rhamnogalacturonan lyases [175].

Previously, we have identified in *A. niger* a tightly regulated transcriptional cluster including the genes encoding the enzymatic activities needed for the catabolism of

galacturonic acid, a subset of the available pectinolytic genes, as well as with one putative monocarboxylate/oxalate transporter, and two putative hexose transporters [134]. We also demonstrated that the metabolic genes needed for the utilisation of galacturonic acid, the galacturonic acid reductase *gaa*A (An02g07710/ AN2858), the L-galactonate dehydratase *gaa*B (An16g05390/AN6035), the 2-keto-3-deoxy-L-galactonate aldolase *gaa*C (An02g07720/AN2859) and the L-glyceraldehyde reductase *gaa*D (An11g01120/ AN7193), have conserved orthologs in *A. nidulans*. From the remaining genes, both hexose transporters included in this cluster are orthologous, forming the gene pairs An14g04280/AN7667 and An03g0162/AN9173, while the monocarboxylate/oxalate transporter (An07g00780) was not detected in the genome of *A. nidulans*. The subset of co-regulated pectinolytic genes includes the three exopolygalacturonases *pgx*A, *pgx*B, and *pga*X, the single exopolygalacturonase/exoxylogalacturonan hydrolase *pgx*C, the pectin lyase *pel*A, the putative arabinofuranosidase *abf*C, and the putative pectin acetyl esterase *pae*A. Five of these genes, namely *pgx*B (An03g06740/AN8891.3), *pga*X (An12g07500/AN8761.3), *pel*A (An14g04370/AN2331.3), *abf*C (An08g01710/AN1277.3), and *pae*A (An02g02540/AN5930), were dentified to have orthologs represented in the genome of *A. nidulans*.

Identification of differentially expressed orthologous genes with equivalent response to carbon source induction

Genome wide transcriptional changes of orthologous genes in response to galacturonic acid *vs.* fructose were assessed by microarray analysis of the transcriptomes of both fungi. For that, three independent biological replicates for each carbon sources and each species were performed. *A. niger* N400 (CBS 120.49), and *A nidulans* WG145 (R153) where cultivated under identical conditions – both species were pre-grown over night in minimal medium with two percent D-fructose. After 18 hours of growth, mycelium was transferred to minimal medium, pH6.0, containing either one percent D-galacturonic acid or one percent D-fructose as a sole carbon source and incubated further for four hours. At the time point of harvesting the pH of the *A. nidulans* cultures was determined to be 7.0 independently of the

Table 1 Pectinases present in the genomes of *A. niger* and *A. nidulans*.

Functional description	Specie	Gene name	A. niger locus tag	A. nidulans locus tag	CaZy family	EC number
Endopolygalacturonase						
Endopolygalacturonase I	*A. niger*	*pgaI*	An01g11520		GH28	3.2.1.15
Endopolygalacturonase II	*A. niger*	*pgaII*	An15g05370		GH28	3.2.1.15
Endopolygalacturonase A	*A. niger*	*pgaA*	An16g06990		GH28	3.2.1.15
Endopolygalacturonase B	*A. niger; A. nidulans*	*pgaB*	An02g04900	AN4372.3	GH28	3.2.1.15
Endopolygalacturonase III	*A. niger; A. nidulans*	*pgaC*	An05g02440	AN8327.3	GH28	3.2.1.15
Endopolygalacturonase D	*A. niger; A. nidulans*	*pgaD*	An09g03260	AN6656.3	GH28	3.2.1.15
Endopolygalacturonase E	*A. niger*	*pgaE*	An01g14670		GH28	3.2.1.15
Endoxylogalacturonan hydrolase						
Endoxylogalacturonan hydrolase	*A. niger; A. nidulans*	*xghA*	An04g09700	AN3389.3	GH28	3.2.1.-
Endorhamnogalacturonase						
Rhamnogalacturonase	*A. niger; A. nidulans*	*rhgA*	An12g00950	AN9134.3	GH28	3.2.1.15
Rhamnogalacturonase B	*A. niger*	*rhgB*	An14g04200		GH28	3.2.1.-
Putative rhamnogalacturonase	*A. niger*	*rhgC*	An06g02070		GH28	3.2.1.15
Putative rhamnogalacturonase	*A. niger*	*rhgD*	An11g06320		GH28	3.2.1.-
Putative rhamnogalacturonase	*A. niger*	*rhgE*	An11g08700		GH28	3.2.1.-
Putative rhamnogalacturonase	*A. niger*	*rhgF*	An07g01000		GH28	3.2.1.-
Exopolygalacturonase						
Exopolygalacturonase X	*A. niger; A. nidulans*	*pgaX*	An12g07500	AN8761.3	GH28	3.2.1.67
Exopolygalacturonase A	*A. niger*	*pgxA*	An11g04040		GH28	3.2.1.67
Exopolygalacturonase B	*A. niger; A. nidulans*	*pgxB*	An03g06740	AN8891.3	GH28	3.2.1.67
Exopolygalacturonase/exoxylogalacturonase C	*A. niger*	*pgxC*	An02g12450		GH28	3.2.1.67
Exopolygalacturonase	*A. nidulans*			AN9045.3	GH28	3.2.1.67
Exo-rhamnogalacturonan hydrolase						
Putative exorhamnogalacturonase A	*A. niger*	*rgxA*	An01g14650		GH28	3.2.1.67
Putative exorhamnogalacturonase B	*A. niger; A. nidulans*	*rgxB*	An03g02080	AN10274.3	GH28	3.2.1.67
Putative exorhamnogalacturonase C	*A. niger*	*rgxC*	An18g04810		GH28	3.2.1.67
Pectin lyase						
pectin lyase A	*A. niger; A. nidulans*	*pelA*	An14g04370	AN2331.3	PL1	4.2.2.10
pectin lyase B	*A. niger*	*pelB*	An03g00190		PL1	4.2.2.10
pectin lyase C	*A. niger*	*pelC*	An11g04030		PL1	4.2.2.10
pectin lyase D	*A. niger*	*pelD*	An19g00270		PL1	4.2.2.10

pectin lyase F	*A. niger; A. nidulans*	*pelF*	An15g07160	AN4882.3	PL1	4.2.2.10
pectin lyase	*A. nidulans*			AN2569.2	PL1	n.d.
Putative pectin lyase	*A. nidulans*			AN10147.3	PL1	n.d.
Putative pectin lyase	*A. nidulans*			AN9439.2	PL1	n.d.
Pectate lyase						
pectate lyase A	*A. niger; A. nidulans*	*plyA*	An10g00870	AN7646.3	PL1	4.2.2.2
pectate lyase	*A. nidulans*	*pelA*		AN0741.3	PL1	4.2.2.2
Putative pectate lyase	*A. nidulans*			AN5333.3	PL1	n.d.
Pectate lyase	*A. nidulans*			AN8453.3	PL3	4.2.2.2
Pectate lyase	*A. nidulans*			AN3337.3	PL3	4.2.2.2
Putative pectate lyase	*A. nidulans*			AN6106.3	PL3	n.d.
Putative pectate lyase	*A. nidulans*			AN2542.3	PL3	n.d.
Putative pectate lyase	*A. nidulans*			AN6748.3	PL3	n.d.
Putative pectate lyase	*A. nidulans*			AN2537.2	PL9	n.d
Rhamnogalacturonan lyase						
Rhamnogalacturonan lyase A	*A. niger; A. nidulans*	*rglA*	An14g01130	AN7135.3	PL4	4.2.2.-
Rhamnogalacturonan lyase B	*A. niger; A. nidulans*	*rglB*	An11g00390	AN6395.3	PL4	4.2.2.-
putative rhamnogalacturonan lyase	*A. nidulans*			AN3950.3	PL4	n.d.
putative rhamnogalacturonan lyase	*A. nidulans*			AN4139.3	PL4	n.d.
putative rhamnogalacturonan lyase	*A. nidulans*			AN2543.3	PL11	n.d.
Carbohydrate esterase						
Pectin methylesterase fragment	*A. nidulans*			AN7966.3	CE8	n.d.
Pectin methylesterase A	*A. niger*	*pmeA*	An03g06310		CE8	3.1.1.11
Putative pectin methylesterase	*A. niger; A. nidulans*	*pmeB*	An04g09690	AN3390.3	CE8	3.1.1.11
Putative pectin methylesterase	*A. niger; A. nidulans*	*pmeC*	An02g12505	AN4860.3	CE8	3.1.1.-
Putative pectin acetyl esterase	*A. niger; A. nidulans*	*paeA*	An02g02540	AN5930.3	n.d.	n.d.
Putative pectin acetyl esterase	*A. niger; A. nidulans*	*paeB*	An07g08940	AN9442.3	CE12	n.d.
Rhamnogalacturonan acetyl esterase						
Rhamnogalacturonan acetyl esterase	*A. niger; A. nidulans*	*rgaeA*	An09g02160	AN2528.3	CE12	3.1.-.-
Putative rhamnogalacturonan acetyl esterase	*A. niger; A. nidulans*	*rgaeB*	An04g09360	AN2834.3	CE12	n.d.
Rhamnogalacturonyl hydrolase						
Putative rhamnogalacturonyl hydrolase	*A. niger; A. nidulans*	*urgA*	An14g02920	AN3196.3	GH105	n.d.
Putative rhamnogalacturonyl hydrolase	*A. niger; A. nidulans*	*urgB*	An14g05340	AN10505.3	GH105	n.d.
Putative rhamnogalacturonyl hydrolase	*A. nidulans*			AN7828.3	GH105	n.d.
Putative rhamnogalacturonyl hydrolase	*A. nidulans*			AN9383.3	GH105	n.d.

carbon source used. The *A. niger* transfer cultures grown on fructose had a pH of 5.3, while those grown on galacturonic acid had a pH of 4.2. The differences in pH between the fructose and galacturonic acid grown *A. niger* cultures are most probably due to the higher amounts of oxalate formed during growth on galacturonic acid (supplementary table 4).

To obtain global expressional profiles of both fungi, total RNA extracted from the transfer cultures to galacturonic acid and fructose was used for the hybridization of Affymetrix GeneChip expression microarrays for *A. niger* and NimbleGen gene expression microarrays for *A. nidulans*. One-way ANOVA analysis identified 489 *A. niger* genes as differentially expressed and between galacturonic acid and fructose (supplementary table 7). In *A. nidulans* 181 genes were differentially expressed in response to the carbon source (supplementary table 8). The larger number of changing *A. niger* genes could be explained by its larger genome and by the simultaneous regulatory effects of pH and carbon source, since *A. niger* acidified the medium faster on galacturonic acid then on fructose.

To identify orthologous genes with evolutionary conserved transcriptional profiles, we followed the scheme represented in figure 1. Comparison of the transcriptional response to galacturonic acid *vs.* fructose between both fungi revealed that the majority of significantly changing orthologous genes in *A. niger* is not influenced by the difference in carbon source in *A. nidulans* and *vise versa* (figure 2). Only 29 genes reacted in an equivalent manner in both fungi (table 3). Unexpectedly, only seven from these 29 genes were up regulated on fructose. Between them, four genes are thought to encode putative hexose and fructose transporters from the Major Facilitator family; one was identified as a multidrug efflux transporter, one as an oligopeptide transporter, and one as a putative phosphatase with sequence similarity to the survival protein SurE from *Escherichia coli*.

The remaining 22 genes were up regulated on galacturonic acid. From them, seven were previously identified as co-regulated in *A. niger* [134], namely the genes encoding the enzymatic activities needed for the metabolic conversion of galacturonic acid to pyruvate and glyceraldehyde *viz. gaa*A, *gaa*B, and *gaa*C, the putative hexose transporter (An14g04280/AN7667) with a similarity to the known quinic acid transporter *qut*D (AN1138) from *A. nidulans* [135], two pectinase encoding genes, the exopolygalacturonase *pga*X (An12g07500/AN8761), and the pectin lyase *pel*A (An14g04370/AN2331), as well

as one of the accessory pectinolytic genes, the putative arabinofuranosidase C (An08g01710/AN1277). Among the other genes that responded equivalently to galacturonic acid were two genes possibly involved in lipid, fatty acid, and isoprenoid metabolism (An09g01210/AN2402; An09g01240/AN779.3), three genes putatively participating in the intracellular conversions of acetate (AN07g00760/AN0058.3; An06g00600/AN5331.3; An12g04640/AN6064.3), and *neoformans.*

the isocitrate lyase *acu*D (An01g09270/AN5634) [197], a key enzyme of the glyoxylate bypass. Interactions between the fatty acid metabolism and the peroxisomal functions via acetate and acetyl-CoA pathways have been recently demonstrated in *A. nidulans.* ([198] and references there in).

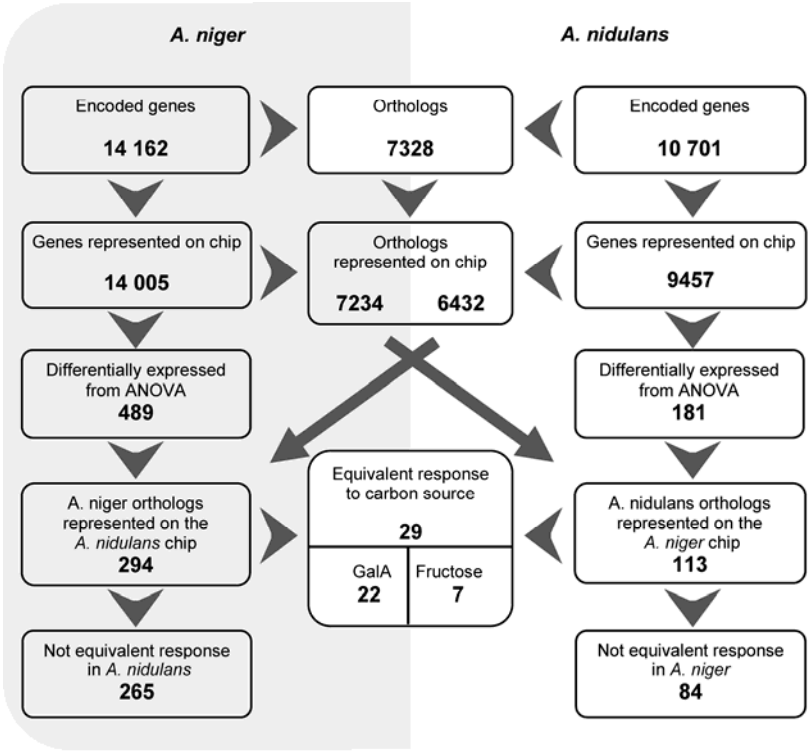


Figure 1. Comparison of *A. niger* and *A. nidulans* gene numbers, their representation on DNA microarrays, their detected gene activity, and functional orthology.

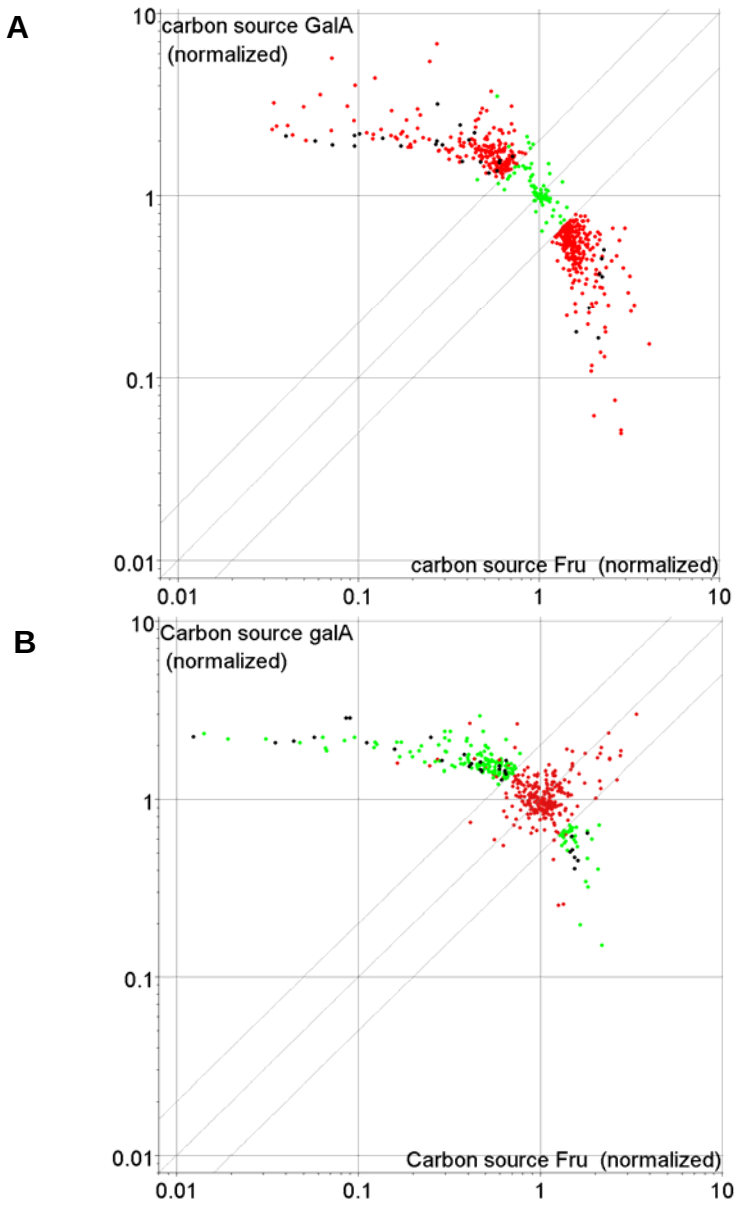


Figure 2. Comparison of the transcriptional response of *A. niger* (panel A) and *A. nidulans* (panel B). In black – orthologous genes with equivalent response in both fungi. In red genes that passed ANOVA testing only in *A. niger*. In green genes that passed ANOVA testing only in *A. nidulans*. Gal A –galacturonic acid; Fru – fructose. The central diagonal line represents no change in gene expression. The external diagonal lines represent 2 fold change on either of the carbon sources.

Table 3 Deferentially expressed orthologous genes with equivalent response to carbon source induction in A. niger and *A. nidulans*. In bold – genes that were identified as co-regulated by galacturonic acid (Chapter 5)

	A. niger				*A. nidulans*			
	locus tag	Fold Change	ANOVA P value	Description	locus tag	Fold Change	ANOVA P value	Reference
Up regulated on galacturonic acid	**An12g07500**	**54**	3.00E-04	**exopolygalacturonase *pga*X**	**AN8761.3**	**181**	6.90E-05	[134]
	An02g07710	**35**	6.00E-04	**Galacturonic acid reductase, *gaa*A**	**AN2858.3**	**19**	5.20E-04	[134]
	An02g07720	**27**	1.20E-03	**2-keto-3-deoxy-L-galactonate reductase, *gaa*C**	**AN2859.3**	**12**	1.30E-02	[134]
	An08g01710	**23**	6.00E-04	**arabinofuranosidase *abf*C** (putative)	**AN1277.3**	**3**	2.70E-02	[17]
	An16g05390	**20**	1.30E-03	**L-galactonate dehydratase, *gaa*B**	**AN6035.3**	**48**	6.90E-05	[134]
	An08g04040	22	8.00E-04	Major facilitator family hexose transporter, (putative quinate transporter)	AN1109.3	39	2.70E-03	[17]
	An14g04280	**15**	1.60E-03	**Major facilitator family, hexose transporter** (putative quinate transporter)	**AN7667.3**	**59**	2.30E-03	[17]
	An11g02200	12	2.50E-02	4-hydroxyphenylpyruvate dioxygenase (putative)	AN1899.3	34	2.60E-02	[17]
	An02g00140	11	6.00E-04	b-xylosidase, family GH43 (putative)	AN6751.3	32	1.30E-02	[17]
	An01g12220	3	9.00E-04	nucleoside-diphosphate-sugar epimerase (cell envelope biosynthesis)	AN0746.3	2	7.40E-03	[17]
	An14g04370	**7**	8.00E-04	**pectin lyase A, *pel*A**	**AN2331.3**	**9**	2.10E-02	[37]
	An09g01240	7	7.00E-04	Lysophospholipase (putative)	AN7792.3	3	2.90E-02	[199]
	An14g06950	7	4.40E-03	Major facilitator family fuconasol transporter protein	AN8089.3	3	1.10E-02	[17]
	An16g02760	7	2.10E-03	alpha-fucosidase (putative)	AN8149.3	3	3.40E-03	[194]
	An08g01720	5	1.90E-02	Major facilitator family hexose transporter (putative quinate transporter)	AN1276.3	4	2.90E-02	[17]
	An09g01210	5	4.00E-02	3-ketoacyl-(acyl-carrier-protein) reductase (putative)	AN2402.3	5	4.00E-03	[17]
	An08g00210	4	6.90E-03	Glycerol 3-phosphate dehydrogenase, FAD dependent (putative)	AN1396.3	2	3.80E-03	[200]
	An07g00760	3	7.10E-03	CoA transferase /carnitine dehydratase (putative)	AN0058.3	6	4.40E-02	[201]
	An06g00600	3	1.20E-02	short chain dehydrogenase (putative)	AN5331.3	2	2.70E-02	[17]
	An01g09270	3	3.10E-02	isocitrate lyase, *acu*D	AN5634.3	4	1.70E-02	[202]
	An12g04640	2	3.50E-03	isoamyl-acetate hydrolyzing esterase (putative)	AN6064.3	2	1.10E-03	[203]
	An01g07300	2	2.05E-02	gluconokinase (putative)	AN3641.3	3	6.70E-03	[17]
Up regulated on fructose	An15g03940	13	1.10E-03	Major facilitator family, hexose transporter (putative)	AN1797.3	4	1.60E-02	[17]
	An02g03540	9	2.40E-02	Major facilitator family, hexose transporter (putative)	AN5860.3	3	1.50E-02	[17]
	An02g01480	8	8.90E-03	Major facilitator family, transporter, multidrug efflux (putative)	AN6277.3	3	2.60E-02	[17]
	An15g01500	6	1.70E-02	Major facilitator family, fructose transporter (putative)	AN2794.3	2	2.60E-03	[17]
	An05g01290	6	3.20E-03	Major facilitator family, hexose transporter (putative)	AN3357.3	4	4.50E-02	[17]
	An18g04040	5	1.20E-02	Predicted phosphatase	AN4025.3	3	2.60E-02	[17]
	An15g07510	4	4.10E-02	Proton-dependent Oligopeptide Transporter Family (putative)	AN8915.3	3	1.00E-02	[17]

The observed here induction of isocitrate lyase on galacturonic acid, together with putative fatty acid and acetate metabolic genes could be explained by the further conversion of the pyruvate formed in the last step of galacturonic acid degradation, to oxaloacetate by the allosterically regulated enzyme, pyruvate carboxylase.

The pyruvate carboxylase encoding, orthologous gene pair (An04g02090/AN4462) was highly expressed in both *A. niger* and *A. nidulans.* In *A. niger*, oxaloacetate can be further converted to oxalate and acetate by oxaloacetate hydrolase (OAH) encoded by An10g00820 [204], [205]. The expression levels of OAH were elevated by approximately 30% on galacturonic acid compared to fructose, which is consistent with the previously observed by us increased levels of extracellular oxalate, when *A. niger* cultures are grown on galacturonic acid and polygalacturonic acid (supplementary table 4). The remaining acetate is possibly recovered by an acetate-CoA ligase [204] and further metabolized via the glyoxylate cycle [206]. *A. nidulans* does not encode an OAH activity [207] and as a result cannot cleave oxaloacetate to oxalate and acetate. However, we observed significant induction (p-value 1.0E-04, fold change 2.3) of the glyconeogenic gene *acu*F (AN1918), encoding the phosphoenolpyruvate carboxykinase PEPCK, which is in agreement with the previously proposed induction of PEPCK by oxaloacetate or malate [208].

Another interesting gene pair that was simultaneously induced in both fungi after transfer to galacturonic acid, was the one encoding mitochondrial FAD dependant glycerol-3-phosphate dehydrogenase (An08g00210/AN1396), which, together with the glycerol kinase, *glc*A (An04g04890; AN5589) forms the major route of glycerol catabolism in *A. nidulans* [200]. It has been suggested several times that the glyceraldehyde formed in the galacturonic acid catabolic route is further metabolized to glycerol by a glyceraldehyde reductase in *A. nidulans*, *A. niger* and *T. reseei* [177], [62], [70]. In this study, the corresponding gene *gaa*D (An11g01120) was identified as differentially expressed on galacturonic acid in *A. niger*. Its *A. nidulans* ortholog AN7193 was highly expressed on both fructose and galacturonic acid, however its expression signal values did not vary significantly between both conditions. Instead, another possible *A. nidulans* candidate (AN2860) was strongly upregulated on galacturonic acid (p-value 3.0E-03; fold change

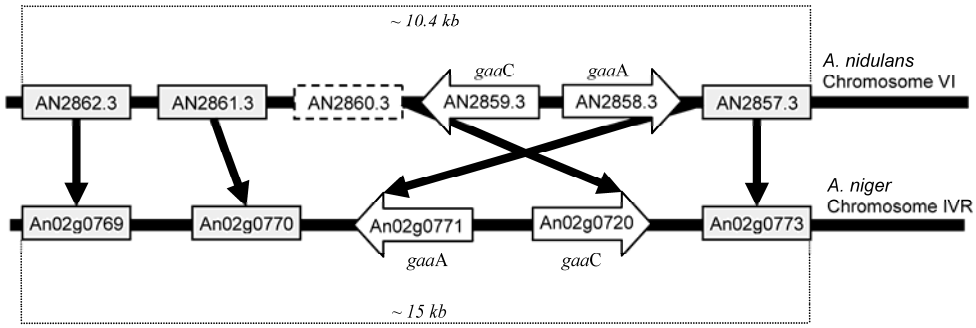


Figure 3. Genomic rearrangement in the *gaa* loci of *A. niger* and *A. nidulans.* *gaa*A (An02g07710/AN2858.3), *gaa*C (An02g027720/AN2859.3) and the lost by *A. niger* AN2860 (dashed line) are colored in white. Neighboring genes are in gray.

44). AN2860 encodes a putative $NADP^+$ alcohol dehydrogenase with proposed EC number 1.1.1.2 (KEGG database), hence it is possible that this enzyme may be able to perform the conversion of glyceraldehyde to glycerol. AN2860 is a neighboring gene to *gaa*A (AN2858) and *gaa*C (AN2859) in *A.nidulans*, but it lacks an ortholog in *A. niger*. Inspection of the *A. niger* genomic region carrying the *gaa*A and *gaa*C genes revealed that the loss of AN2860 from the genome of *A. niger* is probably due to a local micro-inversion that has affected the *gaa* locus in *A. niger* (figure 3).

Differentially expressed pectinases

Within the list of orthologous genes with equivalent response to carbon source induction that passed ANOVA testing in both *A. niger* and *A. nidulans*, fell only two pectinase encoding genes (see above). Since 21 of the 38 pectinases in each of the two fungi are orthologous, we searched for pectinases in the full lists of significantly changing orthologs for *A. niger* (489 genes) and *A. nidulans* (181 genes). As expected, galacturonic acid induced several different pectinase activities in *A. niger* (table 4). Together, they form a minimum set needed for the initial degradation of pectin backbone as the included enzymes modify and cleave the three major pectic polysaccharides – homogalacturonan (HG), xylogalacturonan (XG) and rhamnogalacturonan I (RG I). In *A. niger*, six of these genes (*pga*X, *pgx*A, *pgx*B, *pgx*C, *pel*A, and *pae*A) are co-regulated by galacturonic acid and share

Table 2. *A. niger* and/or *A. nidulans* pectinases significantly induced by galacturonic acid. In bold – *A. niger* genes identified as co-regulated by galacturonic acid in Chapter 4 and their functional orthologs in *A. nidulans*. HG homogalacturonan; aHG acetylated homogalacturonan; mHG methylated homogalacturonan; XG xylogalacturonan; RG I rhamnogalacturonan I.* indicates orthologous pair

Locus tag	Description	Gene name	Fold change	ANOVA p-value	Substrate
Pectinases up regulated in *A. niger*					
An12g07500	**exopolygalacturonase X***	***pga*X**	**54**	**6.9E-05**	**HG**
An02g12450	**exopolygalacturonase C**	***pgx*C**	**40**	**1.0E-03**	**HG/XG**
An03g06740	**Exopolygalacturonase B***	***pgx*B**	**28**	**1.0E-03**	**HG**
An02g02540	**Putative pectin acetyl esterase***	***pae*A**	**10**	**1.0E-03**	**aHG**
An14g01130	Rhamnogalacturonan lyase A*	*rgl*A	10	0.0	RG I
An14g04370	**pectin lyase A***	***pel*A**	**7**	**1.0E-03**	**mHG**
An07g08940	Putative pectin acetyl esterase*	*pae*B	6	1.0E-03	aHG
An04g09360	Putative rhamnogalacturonan acetyl esterase*	*rgae*B	6	2.7E-02	aRG I
An18g04810	Putative exorhamnogalacturonase C	*rgx*C	5	2.0E-03	RG I
An04g09690	Pectin methylesterase B*	*pme*B	3	1.0E-03	mHG
An11g04040	**Exopolygalacturonase A**	***pgx*A**	**3**	**3.0E-03**	**XG/HG**
An10g00870	pectate lyase A*	*ply*A	3	1.0E-03	HG
An01g11520	polygalacturonase I	*pga*I	2	1.0E-03	HG
Pectinases up regulated in *A. nidulans*					
AN8761	**Exopolygalacturonase X***	***pga*X**	**181**	**0.0**	**HG**
AN7828	Putative rhamnogalacturonyl hydrolase		165	0.0	RG I
AN9383	Putative rhamnogalacturonyl hydrolase		35	1.9E-02	RG I
AN4860	Putative pectin methylesterase*	*pme*C	30	0.0	mHG
AN2331	**pectin lyase A***	***pel*A**	**9**	**2.1E-02**	**mHG**
AN3196	Putative rhamnogalacturonyl hydrolase *	*urg*A	7	5.0E-03	RG

similar transcriptional profile with *gaa*A, *gaa*B, *gaa*C, and *gaa*D [134].

Surprisingly, in *A. nidulans* only six pectinase encoding genes were up regulated by galacturonic acid. The list included three homogalacturonan degrading enzymes, and three of the putative unsaturated rhamnogalacturonyl hydrolases (table 4). From these six genes, four have orthologs in *A. niger*, but only two (AN8761 and AN2331) were identified as functional orthologs in terms of equivalent response to galacturonic acid. Nevertheless, the simultaneous expression of the *A nidulans* orthologs of *pga*X (AN8761) and *pel*A (AN2331), together with the orthologous arabinofuranosidase (AN7667), the hexose transporter (AN7667), and the three galacturonic acid catabolic genes *gaa*A (AN2858), *gaa*B (AN6035), and *gaa*C (AN2859), strongly resembles the galacturonic acid inducible

transcriptional cluster identified in *A. niger* and suggests an evolutionary conserved common element involved in the regulation of these genes.

Conclusions

In the presented study, we used a comparative transcriptomics approach to asses the transcriptional response of two filamentous fungi to galacturonic acid. Only a limited number of orthologous genes of the pectinolytic system respond to galacturonic acid induction in a similar manner revealing evolutionary plasticity of transcriptional control. Inspection of these functional orthologs demonstrated the presence of a small subset of intracellular and extracellular proteins involved in the initial steps of galacturonic acid release from homogalacturonan, its subsequent catabolic route, and the possible further metabolic conversions of the formed pyruvate and glyceraldehyde**.** Together these genes form a transcriptional core that remained evolutionary conserved, as it includes elements indispensable for growth on galacturonic acid. In contrast, the apparent discrepancy in the induced pectinase spectra between both fungi indicates that other factors (between which ambient pH) in addition to galacturonic acid became involved in their regulation, possibly as a result of adaptation events.

Regarding the fate of L-glyceraldehyde, it stays unclear whether, in *A. nidulans*, this compound is converted to glycerol by the orthologous *gaa*D (AN7193) gene or by the here identified putative aldehyde reductase AN2860. Further analysis including biochemical characterization and comparison of the properties of the proteins encoded by these two genes could give the answer to this question. Finally, as it appears that the regulation of galacturonic acid release, uptake and metabolism in *A. niger* and *A. nidulans* shares a common element as observed here, we can not conclude that this is valid to the same extent for the extracellular pectinolytic enzymes.

Chapter 7

Summary and concluding remarks

The genus *Aspergillus* consists of a widespread group of filamentous fungi separated by hundreds of millions years of evolution. Among the over 200 different species isolated, there are many that have a significant impact on human society as beneficial organisms employed in the production of medicines, metabolites and industrial enzymes. One of them, the black mold *Aspergillus niger*, a soil saprobe prevalently found on decaying plant debris has a particularly pronounced economical value, because of its application in the biotechnology industry for the production of food ingredients, pharmaceuticals and industrial enzymes.

To adapt to its natural environment, *A. niger* developed a remarkably versatile metabolism, allowing it to utilize many different organic substances for growth. For the efficient release of nutrients from various complex plant biopolymers, *A. niger* uses a large enzymatic network, comprising a vast variety of extracellular hydrolytic enzymes that are specialized in the depolymerisation of structural (cellulose, hemicellulose, pectin) and storage (starch, inulin) plant polycarbohydrates. Among these polymers, pectin is structurally the most complex one.

As many as 17 different monosaccharides are accounted as pectin constituents; including galacturonic acid, rhamnose, xylose, arabinose, galactose, and several other rare sugars like apiose, aceric acid, 2-keto-3-deoxy-D-manno-octulosonic acid, and 2-keto-3-deoxy-D-lyxo-heptulosaric acid. Between these, galacturonic acid residues account for up to half of the total content of pectin. Rather than randomly linked, these monosaccharides are arranged in distinct polymers (homogalacturonan, xylogalacturonan, rhamnogalacturonan I and II, arabinan, arabinogalactan) that can be additionally modified to different extent by methyl or acetyl esters [26].

In plants, the synthesis and modification of pectin involves more than 120 enzymatic activities [209]. Likewise, a large number of different enzymatic activities targeting the specific chemical bonds in each of the distinct pectic polymers are required for the enzymatic degradation and efficient utilization of pectin as a carbon source by microorganisms.

The many pectinolytic enzymes produced by *A. niger* represent a finely tuned, flexible network that comprises over 50 different enzymatic activities, active on the heterogeneous structure of pectin. Because of their industrial importance in textile, paper, pulp, food, and feed industries, the pectinolytic enzymes produced by *A. niger* have been extensively studied for decades and considerable knowledge has been gathered about their structure, function and activities [34-39, 41, 46-49, 74, 132, 133, 154]. Nevertheless, little is known about their synergy and regulation, and the majority of the genes encoding them have been identified in the past by the means of reverse genetics.

The initial aim of the work presented in this thesis was to use a full-scale functional genomics approach to i) identify all the putative activities that possibly play a role in pectin degradation and ii) to gain insight into the dynamics of the entire pectinolytic system of *A. niger*. We approached this by analysis of the genome sequence of *A. niger* [17] in combination with profiling of its transcriptome.

The pectinolytic system of *Aspergillus niger*

In **Chapter 4** we presented the survey of the near-complete genome of *A. niger* CBS 513.88 for the presence of genes putatively encoding pectin backbone and side chain degrading enzymes. In addition, we determined the effect of pectin and its major constituents on the expression of these genes. At least 65 sequences that encode partly-putative pectinolytic activities were identified, from which only 29 were previously described (Table 1). This vast number, and the variety of the found different activities indicate the full potential of the enzymatic network of *A. niger*. However, the possibility exists that some pectinolytic genes have been missed, as the published genome sequence of *A. niger* consists of 500 contigs and has an estimated genome coverage of approximately

95%. For instance, we could not detect the genomic location of *pel*E, one of the six pectin lyases previously described in *A. niger* [37]. Furthermore, very recently, a putative arabinofuranosidase was discovered, located in the 50 kb region that separates supercontig 10 and supercontig 17 (P. Schaap, personal communication). In addition, during sequencing of cDNA library clones, obtained from *A. niger* N400 cultures grown on sugar beet pectin and apple pectin, we identified several ESTs that were not assigned to a protein coding sequences in the genome of *A. niger* CBS 513.88 (**Chapter 2**).

Table 3. Inventory of the pectinolytic system of *A. niger*

	ENZYMATIC ACTIVITY	ENZYME SYMBOL	PREVIOUSLY KNOWN GENES	NEW GENES	TOTAL NUMBER
Pectinases acting on the backbone of pectin	Exopolygalacturonase	exoPG	-	4	4
	Endopolygalacturonase	endoPG	7	-	7
	Endorhamnogalacturonan hydrolase	RG	2	4	6
	Exorhamnogalacturonan hydrolase	RGX	-	3	3
	Endoxylogalacturonan hydrolase	XGA	-	1	1
	Pectate lyase	PLY	1	-	1
	Pectin lyase	PL	6	-	6
	Rhamnogalacturonan lyase	RGL	1	1	2
	Unsaturated rhamnogalacturonyl hydrolase	uRG	-	2	2
	Pectin methylesterase	PME	1	2	3
	Pectin acetylesterase	PAE	-	2	2
	Rhamnogalacturonan acetylesterase	RGAE	1	1	2
Accessory enzymes	Ferulic acid esterase	FAE	2	2	4
	Arabinofuranosidase	ABF	2	2	4
	Endoarabinase	ABN	1	3	4
	β-galactosidase	LAC	1	4	5
	Galactan -1,3-β-galactosidase	1,3-β-GAL	1	-	1
	β-1,4-endogalactanse	GAL	1	1	2
		Total:	**27**	**32**	**59**

A transcriptome assessment **(Chapter 4)** of the induction of the pectinolytic system of *A. niger* by pectin, and some of its main sugar constituents, galacturonic acid, rhamnose, and xylose *vs.* the non-pectic carbon sources fructose, and sorbitol, revealed previously unanticipated low expression levels of the genes encoding several, well studied pectinolytic

enzymes, like the group of endopolygalacturonases and most of the pectin lyases. In contrast, the expression of previously unknown genes coding for different functional classes of pectinases and accessory enzymes, was highly elevated. Striking examples included the high expression levels of hitherto unknown genes predicted to code for proteins with the signature of family 28 glycoside hydrolases (GH28). Therefore, a special focus of attention was given to the entire set of GH28 enzymes - the major class of pectinases encoded in the genome of *A. niger* (**Chapter 3)**. By applying bioinformatics tools, 21 genes encoding enzymes from GH28 were further annotated. From them, 12 were previously unknown genes. Based on sequence similarity, three different groups were observed within the members of GH28, namely: endopolygalacturonases, endorhamnogalacturonases, and a new group of exo-acting glycoside hydrolases. A detailed analysis of the conservation of structural and catalytic residues between the sequences of all the members of GH28 distinguished two different subgroups of exo-activities - four genes were assessed as encoding exo-polygalacturonan hydrolases (PGAX, PGXA, PGXB, PGXC) and three genes as encoding exo-rhamnogalacturonan hydrolases (RGXA, RGXB and RGXC). Biochemical identification using polygalacturonic acid and xylogalacturonan as substrates demonstrated that indeed PGXB and PGXC are exopolygalacturonases, while PGXA is a novel exo-xylogalacturonan hydrolase. PGXC appeared to be an enzyme with reduced substrate specificity that remains unaffected in its activity when GalA residues of the substrate are substituted with xylose [116]. MS analysis of a tryptic digest of the RG galacturono-hydrolase produced by *A. aculeatus*, a protein for which no gene sequence was known, allowed the identification of its *A. niger* homolog exorhamnogalacturonase RGXC [116]. We were not able to identify the natural substrates of RGXA and RGXB. However, the obtained transcriptional profiles of these genes associated with high expression levels on rhamnose and pectin, together with identification of the catalytic residues required for the inverting mechanism characteristic for the GH28 enzymes strongly suggest that these enzymes are involved in the degradation of rhamnogalacturonan I.

The identification of the pectinolytic genes in *A. niger* genome and the studies of their transcriptional control (**Chapter 4**) allowed us to propose a tentative model of the induction of the synergistic enzymatic network of this fungus that is involved in pectin degradation,

represented in Fig. 1. This network comprises of more than 40 different enzymes that are induced in the presence of pectin and can degrade the different building blocks of this structurally complex polymer. A comparison of their transcriptional response on pectin *vs.* galacturonic acid demonstrates that galacturonic acid acts as a common inducer for only some of the activities from each of the major functional classes of pectinolytic enzymes of *A. niger*.

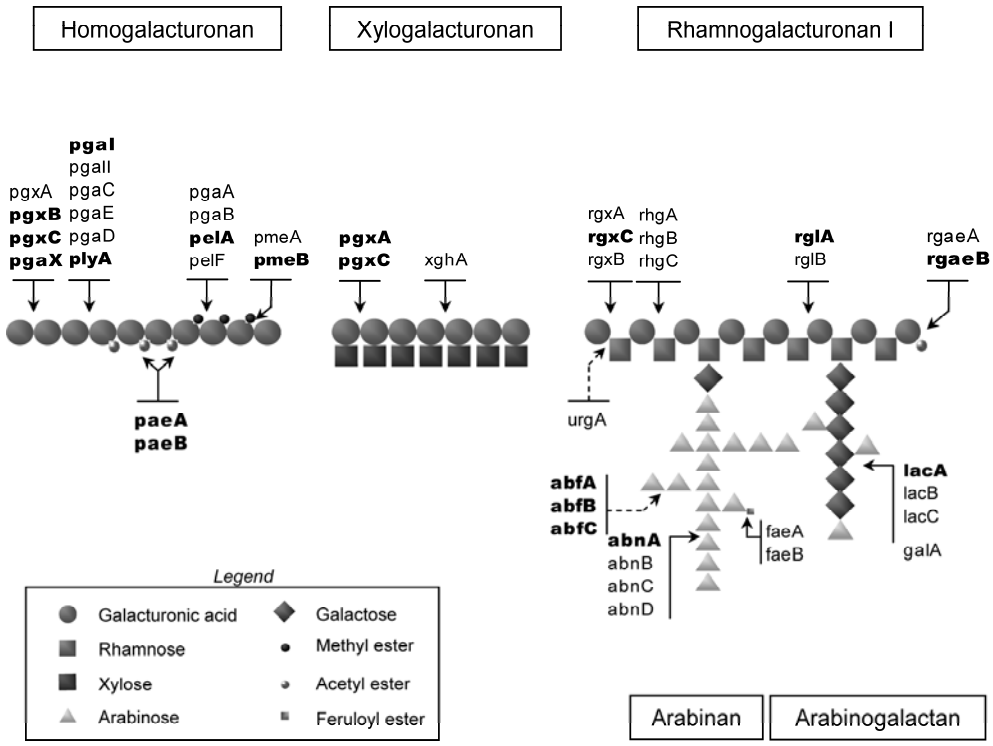


Figure 1. A tentative model of the synergistic enzymatic network of A. niger involved in pectin degradation. In bold are shown enzymatic activities that are induced by galacturonic acid. Arrows represent the (possible) points of attack of different functional classes of enzymatic activities. Dashed arrows represent enzymes that act on oligomeric sugar units released from the polymeric blocks of pectin. Please note that the illustrated here polymers are only a schematic representation of the major pectin components and do not depict definite molecular structures.

An ubiquitous fungal galacturonic acid degradation pathway

Two-way clustering analysis of the data obtained from microarray experiments allowed us to identify a tightly regulated transcriptional cluster (**Chapter 5**), including all four exopolygalacturonases found to be encoded by the genome of *A. niger* together with the endo-acting pectin lyase A, the putative pectin acetyl esterase A, and the putative arabinofuranosidase *Abf*C. Co-expressed are three different putative transporters, i.e. one hexose transporter, one with high similarity to the *A. nidulans* quinic acid transporter protein encoded by *qut*D, and one possible monocarboxylate/oxalate transporter. In addition, this transcriptional cluster contained four genes encoding uncharacterized and hypothetical proteins. Function prediction based on conserved domain similarity with known, publicly available sequences allowed us to assign possible functions to these hypothetical proteins and appoint them to the required reductase (encoded by *gaa*A), dehydratase (encoded by *gaa*B) and aldolase (encoded by *gaa*C) activities, involved in the metabolic conversion of galacturonic acid to pyruvate and glyceraldehyde via L-galactonate and 2-keto-3-deoxy-L-galactonate. Besides these, we were able to point out the additional putative glyceraldehyde reductase (encoded by *gaa*D) involved in the conversion of L-glyceraldehyde to glycerol. Biochemical verification of the enzymatic activities encoded by the *gaa*A and *gaa*B genes confirmed our finding.

A further search for orthologous sequences in the genomes of other fungi led to the identification of previously described mutant loci, in *A nidulans* strains, affected in galacturonic acid catabolism [61] and presented strong evidence that a specific galacturonic acid catabolic pathway, different from that in bacteria, operates in fungal species [134]. The *gaa*A and *gaa*C genes were found to be strictly conserved with the same genomic arrangement across all sequenced genomes of plant pathogenic and plant saprobic ascomycete species from subphylum *Pezizomycotina*. but not in genomes of sequenced zoopathogenic members of this subphylum. This demonstrates that a galacturonic acid degradation pathway is ubiquitously present in pectin-degrading fungi. In addition, the particular genomic organization of *gaa*A and *gaa*C described in **Chapter 5** allowed us to detect a phylogenetically conserved promoter element that was identified also in the

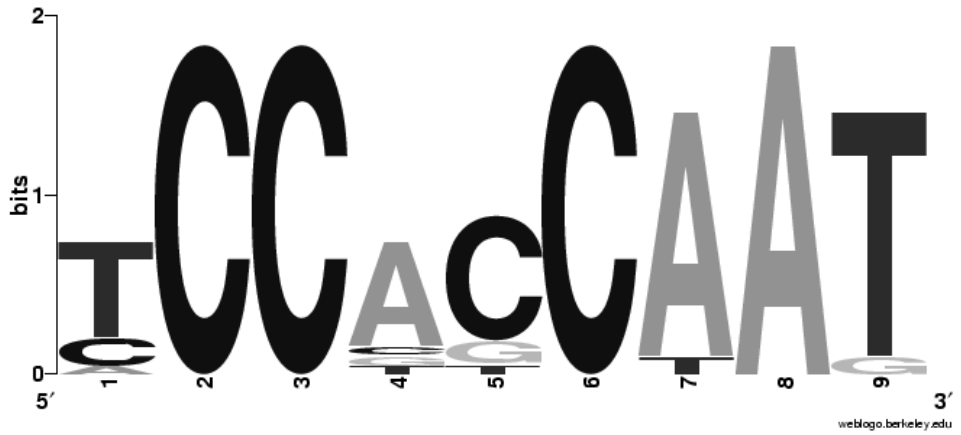


Figure 2. Sequence logo of the conserved promoter element present in the genes from the tightly regulated transcriptional cluster forming a pectinolytic core induced by galacturonic acid.

promoters of several co-expressed pectinases of *A. niger* (Fig. 2). This occurrence of a conserved regulatory sequence in different *Pezizomycotina* species suggested the presence of pectinolytic regulatory system that may share common components across different fungal species.

We were not able to identify the transcriptional regulator(s) involved in the activation of the pectinolytic core of *A. niger* by the methods applied. The sequence presented above is in fact an extended CCAAT box, commonly found in fungal and other eukaryotic promoters that bind HAP-like transcriptional factors, composed of multiple units [210]. The discrepancy in the observed transcriptional patterns of different pectinase encoding genes described in **Chapter 3** and **Chapter 4**, strongly suggests the presence of a multifactorial regulatory system controlling the expression of pectinolytic genes, which is in agreement with previous reports [75, 24]. The detection of a putative regulatory element in the promoters of co-expressed genes can facilitate the identification of the regulators involved in the control of the pectinolytic system of *A. niger*. A combination of promoter binding assay of nuclear protein extracts and mass-spectrometric identification of the isolated bound proteins, followed by molecular analysis of selected targets should lead to the successful identification of the transcriptional factors involved in the regulation of the pectinolytic system of *A. niger*.

Is the transcriptional control of the pectinolytic system evolutionarily conserved?

To find out to what extent the regulation of fungal pectinolytic systems may have been retained during evolution, we used a comparative transcriptomics approach to asses the response to galacturonic acid of two Aspergilli – the industrial enzyme and acid producer *Aspergillus niger* and the model organism *Aspergillus nidulans* (**Chapter 6**). Both fungi are soil plant saprobes that can degrade plant cell wall material and posses a comparable number of genes encoding carbohydrate-degrading enzymes [17]. However, while *A. niger* is well known for its ability to produce organic acids and readily acidifies its direct environment during growth, *A. nidulans* does not produce organic acids but accumulates and eventually secretes polyols [195]. This difference in metabolism is also depicted in the types of enzymes comprising the pectinolytic system of both fungi. While the genome of *A. niger* is enriched with glycoside hydrolases, with a low pH optimum [45], in *A. nidulans* mainly pectic lyases that prefer a neutral or high pH optimum are found [196]. As a result, although both fungi have a full spectrum of pectinases activities, only a limited set consists of orthologous proteins. Similarly, examination of the transcriptome profiles of *A. niger* and *A. nidulans* revealed that only a small number of genes are functional orthologs in terms of regulation. A core group of 22 orthologous genes responsive to galacturonic acid was identified to be controlled in an equivalent manner. This core group contains intracellular and extracellular enzymes involved in the initial steps of galacturonic acid release from homogalacturonan, the subsequent catabolic route of this pectin constituent, and the possible further metabolic conversions of the formed pyruvate and glyceraldehyde. The apparent discrepancy observed between the induced pectinase spectra of both fungi indicates a recruitment of different functions in the pectinolytic systems, possibly because of the differences in their physiology.

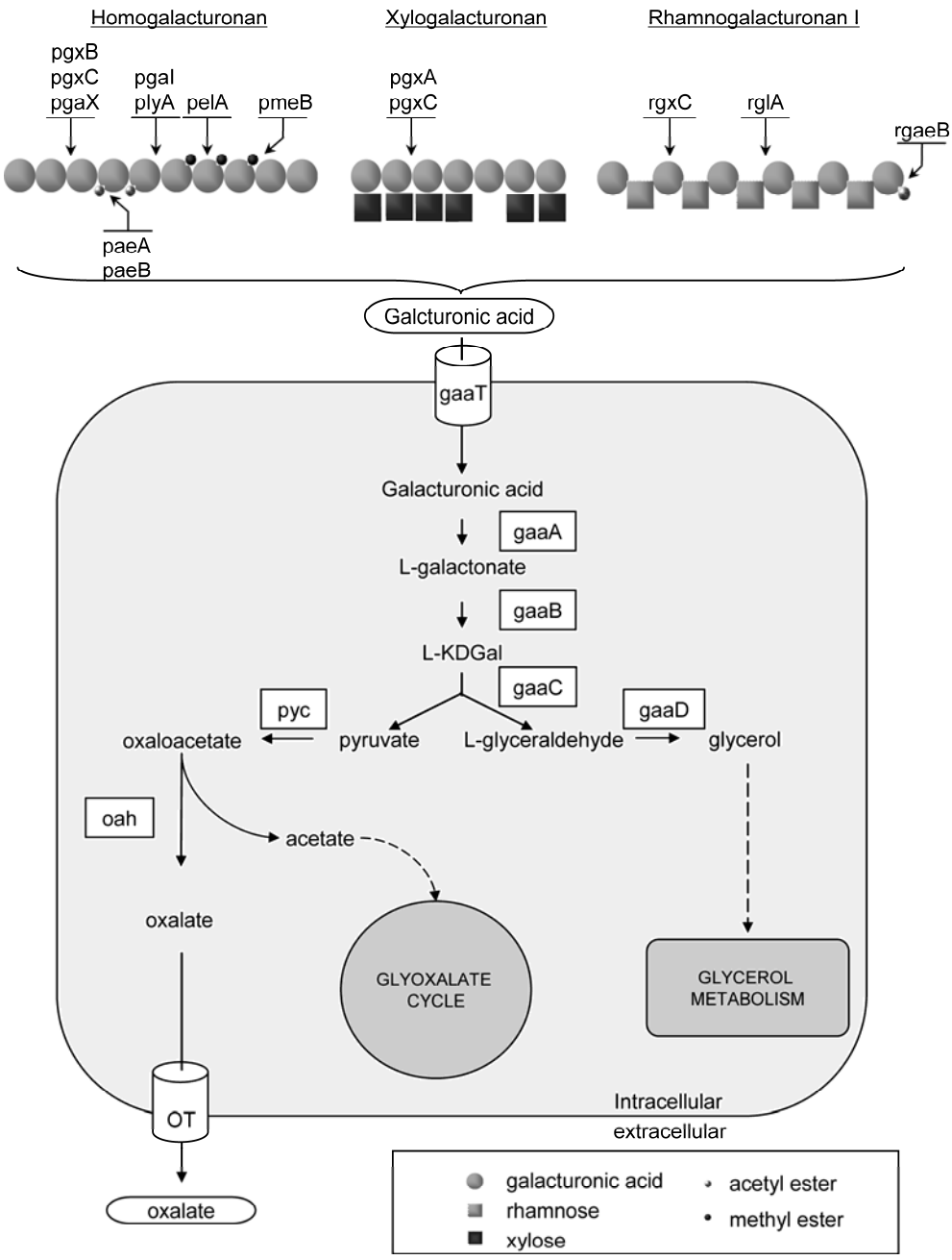
Based on the data presented in this thesis we were able to reconstruct the route of galacturonic acid release, utilization, and catabolism in *A. niger*, and to partially transfer it to *A. nidulans* (Fig. 3). We presume that the presence of galacturonic acid triggers an unknown transcriptional factor that induces the expression of a putative galacturonic acid transporter *gaa*T, orthologous for both fungi. Candidates for such a transporter with high

similarity to the quinic acid transporter encoded by *qut*D of *A. nidulans* [135] have been identified during clustering analysis of the genome wide array data and during the comparison of the transcriptomes of *A. niger* and *A. nidulans*. However, further functional analysis is required in order to confirm their substrate affinity. Intracellularly, galacturonic acid is degraded to pyruvate and L-glyceraldehyde by the products of the evolutionary conserved *gaa*A, *gaa*B and *gaa*C genes. A putative L-glyceraldehyde reductase *gaa*D, which may be encoded by different genes in both fungi, further converts glyceraldehyde to glycerol (**Chapter 6**). In *A. niger* (Fig. 3A), we propose that pyruvate is further converted to oxalate and acetate by pyruvate carboxylase and oxaloacetate hydrolase OAH [207]. Oxalate is excreted from the cell, which is consistent with the observed increased extracellular levels of this acid, when *A. niger* cultures are grown on galacturonic and polygalacturonic acid (**Chapter 4**). A candidate transporter, involved in the secretion of oxalate is the putative monocarboxylate/oxalate transporter (An07g00780) identified in **Chapter 5**. Acetate is most probably metabolized via the glyoxylate cycle as discussed in **Chapter 6**. *A. nidulans* does not encode an OAH activity and as a result can not cleave oxaloacetate to oxalate and acetate. In this fungus oxaloacetate is either directly converted to phosphoenolpyruvate by the glyconeogenic phosphoenolpyruvate carboxykinase PEPCK, or enters the glyoxylate cycle (Fig. 3B).

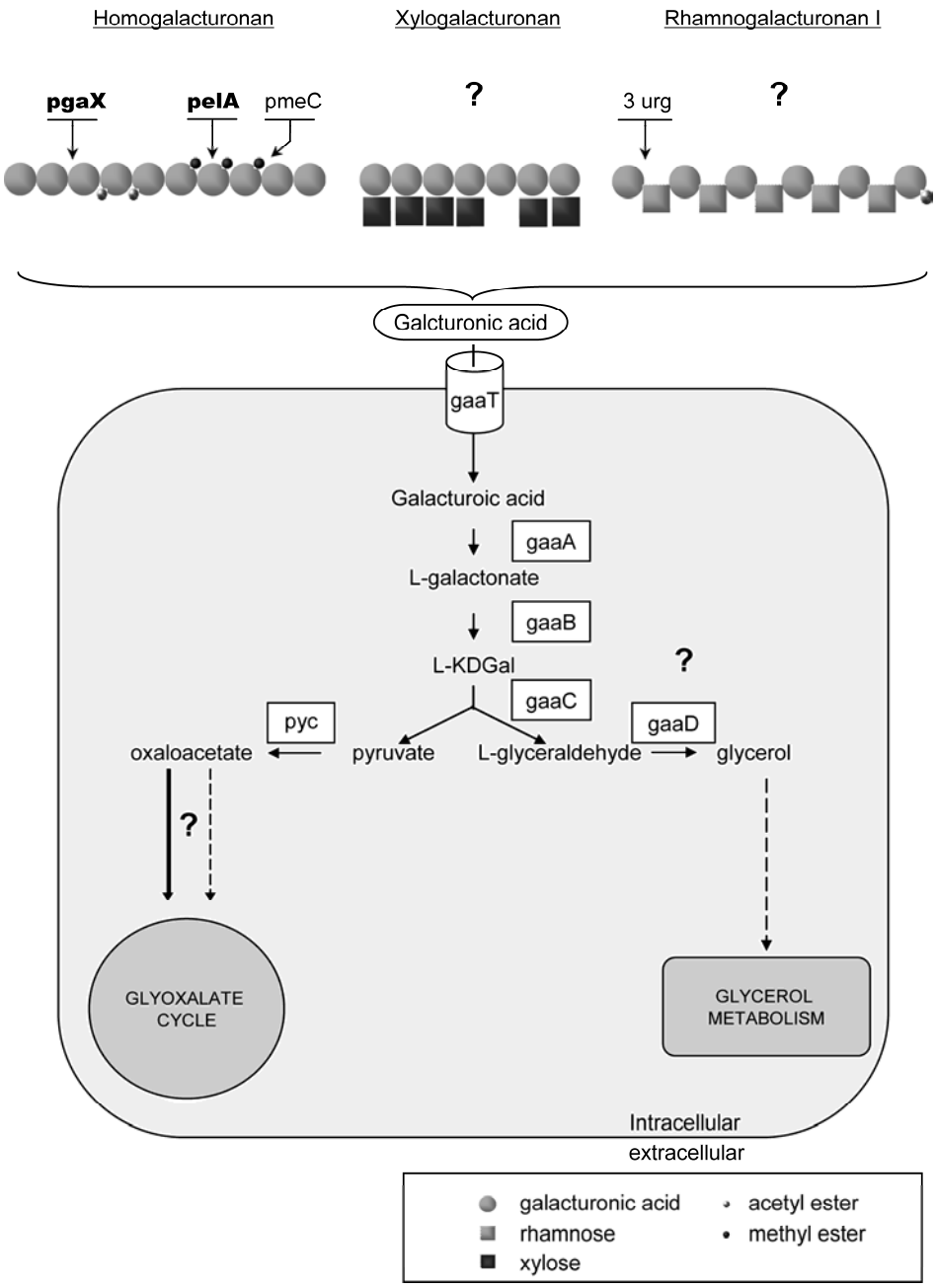
Interestingly, galacturonic acid induces different sets of pectinase activities in *A. niger* and *A. nidulans* (Fig 3A and B). The synergistic action of the enzymes produced by *A. niger* is sufficient for the initial degradation of the homogalacturonan and rhamnogalacturonan I, which leads to the release of free monomeric constituents and probably starts a cascade of induction events that leads to the expression of a vast set op pectinolytic activities (Fig. 1).

Figure 3. Galacturonic acid utilization route in *A. niger* (A) and *A. nidulans* (B).
A *pgx*A exopolygalacturonase A; *pgx*B exopolygalacturonase B; *pgx*C exopolygalacturonase C; *pga*X exopolygalacturonase X; *pga*I polygalacturonase I; *ply*A pectate lyase; *pel*A pectin lyase; *pae*A pectin acetyl esterase A; *pae*B pectin acetyl esterase B; *pme*B pectin methyl esterase B; *rgx*C exorhamnogalacturonase C; *rgl*A rhamnogalacturonan lyase; *rgae*B rhamnogalacturonan acetyl esterase. *gaa*T putative galacturonic acid transporter. OT putative oxalate transporter; *gaa*A galacturonic acid reductase; *gaa*B L-galactonate dehydratase; *gaa*C 2-keto-3-deoxy-L-galactonate aldolase; *gaa*D L-glyceraldehyde reductase; *pyc* pyruvate carboxylase; *oah* oxaloacetate hydrolase. Dashed lines represent multiple enzymatic conversions
B *pga*X exopolygalacturonase X; *pel*A pectin lyase A; *pme*C pectin methyl esterase C; *urg* unsaturated rhamnogalacturonan hydrolase; *gaa*T putative galacturonic acid transporter; *gaa*A galacturonic acid reductase; *gaa*B L-galactonate dehydratase; *gaa*C 2-keto-3-deoxy-L-galactonate aldolase; *gaa*D L-glyceraldehyde reductase; *pyc* pyruvate carboxylase; Dashed lines represent multiple enzymatic conversions. In bold are represented the only two *A. nidulans* pectinases identified as functional orthologs of *A. niger* enzymes.

A ***Aspergillus niger***



B ***Aspergillus nidulans***



In *A. nidulans* galacturonic acid induces a different group of enzymatic activities, from which only two have functional orthologs in *A. niger*. One possible explanation for that would be the observed difference in ambient pH. However, this does not explain the small number of responding genes. Although preliminary, and based on a rather limited data set, these findings suggest that transcription of the pectinolytic system is not conserved between these two Aspergilli and that only a core set of genes, indispensable for growth on galacturonic acid remained universally regulated. Similar evolutionary plasticity of transcriptional control, even over key biological processes like the cell cycle, has been previously observed between yeast and other eukaryotes and it has been explained with evolutionary loss and/or gain of specific gene expression, in order to adjust to the particular needs of each organism [123, 191]. Our comparative study of *A. niger* and *A. nidulans* suggests that the pectinolytic systems of both fungi have undergone a similar process of adaptation to meet the specific requirements of each of these two organisms.

Back to the future

The results presented in this thesis provide the first holistic and genome-wide description of the pectinolytic network of the filamentous fungus *Aspergillus niger*. This study was set off by the sequencing and thorough annotation of the *A. niger* genome, and greatly facilitated by the accessibility of a highly proficient microarray platform. The combination of genomic and expression data permitted the reconstruction of the pectinolytic network of *A. niger* and the better understanding of its dynamics. The solving of the concealed underlying metabolic reactions was part of a process that involved the comparison of several fungal species. This was made possible by the exponential increase in the number of sequenced fungal genomes within the last 5 years and the development of functional approaches, such as transcriptomics.

Today, we are looking towards a new biological science were whole biological systems are being investigated. Fungal comparative genomics studies and the exploitation of comparative fungal genome databases that incorporate the obtained knowledge have already been giving fruitful results [17, 88, 211, 157]. Transcriptomics analysis in fungal

biology is becoming routine, since microarrays for more than 20 different species already exist and over 50 studies have been reported [101]. Fungal transcriptomics are going comparative, as in addition to the already existing GeneChip® Yeast Genome 2.0 Array of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, the first multiplex GeneChip microarray, covering the genomes of three different *Aspergillus* species was made available earlier this year [125]. In addition, recently developed proteomics techniques like SILAC (Stable Isotope Labeling of Amino acids in Cell culture) [212], can be easily adapted for fungi [213] and present an attractive opportunity for comparative and quantitative analysis of the fungal proteomes.

Finally, the systematic approach provided by mathematical modeling, combined with the integration of knowledge obtained from ‘omics systems, promise to bring a deeper understanding of fungal biology and will allow us to appraise the events that shaped the kingdom of Fungi to what it is today.

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Supplementary data

Supplementary table 1. Sugar and-uronic acid composition of polymeric substrates used as carbon source for cDNA library construction and in transcriptional profiling experiments. The amounts of monomeric pectin constituents are given both as mass percentage and as mol percentage.. PGA, polygalacturonic acid; SBP, sugar beet pectin; AP, apple pectin; n.d., not detected.

Polymer		Rhamnose		Fucose		Arabinose		Xylose		Manose		Galactose		Glucose		Uronic acid		Total
Type	**Weight** (mg)	w/w %	mol %	w/w %	mol %	w/w %	mol %	w/w %	mol %	w/w %	Mol %	w/w %	mol %	w/w %	mol %	w/w %	mol %	w/w %
PGA	15.80	n.d.	n.d.	n.d.	n.d.	1	1	< 0.05	< 0.05	2	2	4	5	n.d	n.d.	68	92	74
SBP	16.40	1	2	0.1	< 0.05	4	7	< 0.05	< 0.05	1	1	7	11	< 0.05	1	50	78	63
AP	15.60	1	1	< 0.05	< 0.05	1	2	1	2	1	2	7	11	12	18	48	65	71

Supplementary table 2 Functional annotation of sequenced cDNA clones. Annotations and functional categories were derived by BLAST search against the genome and proteome of *A. niger* CBS 513. 88 (Pel *et al*., 2007) and against the NCBI database

Clone number	Locus tag	Description	Functional category
APL42		bad sequence	
APL71		bad sequence	
SBL07		bad sequence	
APL01		Concordia EST Asn_03899 (conserved hypotetical protein) A. niger CBS513.88 Scafold 1538889-1539410	
APL03		Concordia EST Asn_2236 A. niger CBS513.88 Scafold 8_8R 1010338- 1010910	
APL05		Concordia EST Asn_10279 (putative outer mitochondrial membrane protein porin)	
APL27		Concordia EST Asn_12153 (conserved hypothetical protein)	
SBE76C02		Concordia EST Asn_01434 A. niger CBS513.88 Scafold 18_8L 426815-427343	
APL04		genomic sequence A. niger CBS513.88 Scafold 2_4R 1270055-1270312	
SBE76A05		empty vector	
SBE76C10		empty vector	
SBE76D03		empty vector	
SBE76E05		empty vector	
SBE76E06		empty vector	
SBE76F02		empty vector	
SBL32		empty vector	
SBL33		empty vector	
SBL33		empty vector	
SBL41		empty vector	
SBL41		empty vector	
SBL50		empty vector	
APL75		Escherichia coli sequence	
SBL05		Escherichia coli sequence	
SBL11		Escherichia coli sequence	
SBL16		Escherichia coli sequence	
SBL22		Escherichia coli sequence	
APL07		Mitochondrial ribosomal RNA small subunit	
APL30		Mitochondrial ribosomal RNA small subunit	
APL37		Mitochondrial ribosomal RNA small subunit	
APL80		Mitochondrial ribosomal RNA small subunit	

SBE09		Mitochondrial ribosomal RNA small subunit	
SBE76E02		Mitochondrial ribosomal RNA small subunit	
SBE76E04		Mitochondrial ribosomal RNA small subunit	
SBL01		Mitochondrial ribosomal RNA small subunit	
SBL15		Mitochondrial ribosomal RNA small subunit	
SBL47		Mitochondrial ribosomal RNA small subunit	
APL02	An16g03790	similarity to hypothetical protein SPBC1D7.03c - S. pombe	99 unclassified proteins
APL06	An01g12620	similarity to hypothetical protein 5C2.150 - N. crassa	99 unclassified proteins
APL08	An04g06870	similarity to hypothetical protein CAD21072.1 - N. crassa	99 unclassified proteins
APL09	An12g04030	strong similarity to nuclear distribution protein nudE - A. nidulans	08 cellular transport and transport mechanisms
APL10	An15g01510	strong similarity to P-type ATPase Drs2 - S. cerevisiae	01 metabolism
APL12	An14g06830	strong similarity to hypothetical membrane protein Ptm1 - S. cerevisiae	99 unclassified proteins
APL13	An04g07400	strong similarity to C6 zinc finger transcription factor PRO1 – S. macrospora	04 transcription
APL14	An15g05810	xylanolytic transcriptional activator xlnR - A. niger	01 metabolism
APL15	An11g05680	similarity to cytochrome P450 3A13 - Mus musculus	11 cell rescue, defense and virulence
APL16	An15g06030	strong similarity to hypothetical protein Ecm4 - S. cerevisiae	99 unclassified proteins
APL17	An18g04840	strong similarity to translation elongation factor 1 alpha – P. anserina	99 unclassified proteins
APL18	An12g08000	strong similarity to hypothetical protein SPCC1902.02 - S. pombe	99 unclassified proteins
APL19	An07g06400	strong similarity to copper amine oxidase AO-I - A. niger	01 metabolism
APL20	An07g03880	serine proteinase pepC - A. niger	06 protein fate
APL21	An06g00260	strong similarity to hexose transporter Hxt5 - S. cerevisiae	01 metabolism
APL22	An03g02800	strong similarity to EST an_2890 - A. niger	99 unclassified proteins
APL23	An01g01830	strong similarity to catalase/peroxidase cpeB - S. reticuli	11 cell rescue, defense and virulence
APL24	An18g06400	weak similarity to protein-tyrosine phosphatase ptp - Clostridium perfringens	99 unclassified proteins
APL25	An01g10930	strong similarity to enzyme with sugar transferase activity from patent JP11009276-A	01 metabolism
APL26	An07g07760	strong similarity to DNA damage checkpoint protein rad24p – S.. pombe	03 cell cycle and dna processing
APL28	An08g04490	endoprotease Endo-Pro - A. niger	06 protein fate
APL29	An11g06630	strong similarity to ubiquitin protein ligase Ufd4 - S. cerevisiae	06 protein fate
APL31	An08g09010	strong similarity to spliceosomal protein SAP130 - Homo sapiens	04 transcription
APL32	An15g06110	weak similarity to syntaxin 6 STX6 - Rattus norvegicus	08 cellular transport and transport mechanisms
APL33	An13g01230	weak similarity to colon cancer antigen protein SEQ ID NO:4503 from patent WO200122920	99 unclassified proteins
APL34	An04g05850	strong similarity to cytoplasmic ribosomal protein of the small subunit rps6p - S. pombe	05 protein synthesis
APL35	An16g09020	strong similarity to hypothetical protein SPAC922.05c - S. pombe	99 unclassified proteins
APL36	An16g07410	strong similarity to mitochondrial F1-ATPase alpha-subunit Atp1 - S. cerevisiae	40 subcellular localisation

APL38	An09g03470	strong similarity to hypothetical verprolin related protein encoded by B24P7.40 - N. crassa	99 unclassified proteins
APL39	An16g07795	strong similarity to hypothetical protein CAC18184.2 - N. crassa	100 unclassified proteins
APL40	An11g00510	strong similarity to ATP citrate lyase ACL1 - Sordaria macrospora	01 metabolism
APL41	An08g01720	strong similarity to quinate transport protein qutD - A. nidulans	01 metabolism
APL43	An08g08860	weak similarity to period protein per - Drosophila sucinea	99 unclassified proteins
APL44	An07g03880	serine proteinase pepC - A. niger	06 protein fate
APL45	An04g00410	strong similarity to dipeptidyl peptidase III - Rattus norvegicus	06 protein fate
APL46	An11g05740	weak similarity to hypothetical translation initiation factor SPAC17C9.03 - S. pombe	99 unclassified proteins
APL47	An15g00140	similarity to transcription repressor Tup1 - S. cerevisiae	04 transcription
APL48	An04g05620	strong similarity to acetate--CoA ligase facA - A. nidulans	01 metabolism
APL49	An02g05760	weak similarity to glucan 1,4-alpha-glucosidase Muc1 - S. cerevisiae	99 unclassified proteins
APL50	An04g01410	similarity to protein fragment SEQ ID NO:6371 from patent EP1033405-A2 - A. thaliana	99 unclassified proteins
APL52	An15g07040	strong similarity to phospholipase D Spo14 - S. cerevisiae	01 metabolism
APL53	An18g03310	strong similarity to cytoplasmic ribosomal protein of the small subunit S3 - S. cerevisiae	05 protein synthesis
APL54	An01g02370	strong similarity to siderophore biosynthesis repressor sREA - A. nidulans	04 transcription
APL55	An08g05790	strong similarity to glycogen phosphorylase Gph1 - S. cerevisiae	01 metabolism
APL56	An04g02470	strong similarity to GTP-binding protein ypt5p - S. pombe	08 cellular transport and transport mechanisms
APL57	An19g00300	strong similarity to kinesin light chain KLC - Plectonema boryanum	08 cellular transport and transport mechanisms
APL58	An01g05390	similarity to hypothetical protein YBR007c - S. cerevisiae	99 unclassified proteins
APL59	An02g00590	strong similarity to high-affinity glucose transporter HGT1 - Kluyveromyces lactis	01 metabolism
APL60	An14g06510	hypothetical protein	99 unclassified proteins
APL61	An01g13900	similarity to hypothetical protein dnajp - S. pombe	99 unclassified proteins
APL62	An03g04230	strong similarity to cytoplasmic form of aminopeptidase P - Homo sapiens	06 protein fate
APL63	An15g04270	strong similarity to quinate transport protein qutD - A. nidulans	01 metabolism
APL64	An01g06230	strong similarity to translation initiation factor 3 subunit eIF3 beta - Homo sapiens	05 protein synthesis
APL65	An13g00920	similarity to mandelate racemase - Pseudomonas putida	01 metabolism
APL66	An13g00850	strong similarity to hypothetical Rho GTPase protein - S. pombe	99 unclassified proteins
APL67	An01g07130	strong similarity to salicylate 1-monooxygenase sal1 - Pseudomonas putida	01 metabolism
APL68	An04g00900	weak similarity to beta transducin-like protein het-e1 - Podospora anserina	10 cellular communication/signal transduction
APL69	An14g02100	strong similarity to cell wall antigen MP1 - Penicillium marneffei	99 unclassified proteins
APL70	An01g08240	similarity to transcription regulator Ccr4 - S. cerevisiae	04 transcription
APL72	An14g00040	strong similarity to ATM-related kinase required for DNA damage response uvsB - A. nidulans	03 cell cycle and dna processing
APL73	An02g09560	strong similarity to protein regulating gene expression PRGE-6 from patent WO9964596-	04 transcription
APL74	An18g03570	beta-glucosidase bgl1 - A. niger	01 metabolism

APL76	An16g07410	strong similarity to mitochondrial F1-ATPase alpha-subunit Atp1 - S. cerevisiae	40 subcellular localisation
APL77	An16g04940	strong similarity to cytoplasmic ribosomal protein of the small subunit S12 AS1 – P. anserina	05 protein synthesis
APL78	An01g07890	transcriptional regulator gene cpcA - A. niger	04 transcription
APL79	An08g04310	similarity to hypothetical protein SPAC56E4.07 - S. pombe	99 unclassified proteins
APL81	An16g08820	strong similarity to hypothetical AAA-type ATPase Yta7 - S. cerevisiae	99 unclassified proteins
SBE01	An15g02360	strong similarity to acetylornithine aminotransferase arg8 - Kluyveromyces lactis	01 metabolism
SBE02	An14g00640	strong similarity to hypothetical protein EAA61679.1 - A. nidulans	99 unclassified proteins
SBE03	An09g02470	similarity to protein fragment SEQ ID NO:24319 from patent EP1033405-A2 - A. thaliana	99 unclassified proteins
SBE04	An10g00820	strong similarity to carboxyphosphonoenolpyruvate phosphonomutase bcpA - S. hygroscopicus	01 metabolism
SBE05	An06g01370	strong similarity to Succinyl CoA: 3-oxoacid CoA transferase SCOT - Homo sapiens	01 metabolism
SBE06	An08g04470	strong similarity to mitochondrial elongation factor Tu - A. thaliana	05 protein synthesis
SBE07	An08g03190	strong similarity to tubulin beta chain beta-tubulin - A. flavus	03 cell cycle and dna processing
SBE08	An18g04840	strong similarity to translation elongation factor 1 alpha – P. anserina	99 unclassified proteins
SBE10	An11g07850	hypothetical protein	99 unclassified proteins
SBE11	An18g02680	strong similarity to cytochrome P450 monooxygenase avnA - A. parasiticus	01 metabolism
SBE12	An11g05440	strong similarity to hypothetical protein MUTT - A. thaliana	99 unclassified proteins
SBE13	An07g02540	similarity to carboxylic acid transport protein Jen1 - S. cerevisiae	01 metabolism
SBE14	An02g08920	strong similarity to TCP1-related protein Cct3 - S. cerevisiae	06 protein fate
SBE15	An02g11390	strong similarity to hypothetical protein encoded by DR0708 - Deinococcus radiodurans	99 unclassified proteins
SBE16	An09g04660	strong similarity to meiotic mRNA stability protein kinase Ume5 - S. cerevisiae	06 protein fate
SBE17	An01g10310	hypothetical protein	99 unclassified proteins
SBE18	An08g01710	strong similarity to alpha-L-arabinofuranosidase abfA - Bacillus stearothermophilus	01 metabolism
SBE19	An16g04500	hypothetical protein	
SBE20	An01g04030	weak similarity to hypothetical protein SPCC1259.08 - S. pombe	99 unclassified proteins
SBE76A01	An09g06640	similarity to DNA-directed RNA polymerase III 82 KD subunit Rpc82 - S. cerevisiae	04 transcription
SBE76A02	An08g06440	strong similarity to androgen-inducible aldehyde reductase aiar - Rattus norvegicus	01 metabolism
SBE76A03	An03g06920	similarity to hypothetical protein CG5336 - Drosophila melanogaster	99 unclassified proteins
SBE76A04	An16g03100	strong similarity to TCP-1-containing cytosolic chaperonin zeta subunit CctZ - Mus musculus	06 protein fate
SBE76A06	An12g04170	similarity to Ecm14 - S. cerevisiae	99 unclassified proteins
SBE76A07	An09g05790	weak similarity to BAG-family molecular chaperone regulator-4 - Homo sapiens	06 protein fate
SBE76A08	An08g08740	strong similarity to cytoplasmic ribosomal protein of the small subunit S18 - Homo sapiens	05 protein synthesis
SBE76A09	An01g05780	strong similarity to RuvB-like protein Tih2 - S. cerevisiae	04 transcription
SBE76A10	An16g07410	strong similarity to mitochondrial F1-ATPase alpha-subunit Atp1 - S. cerevisiae	40 subcellular localisation

SBE76A11	An07g07990	hypothetical protein	99 unclassified proteins
SBE76A12	An15g00560	strong similarity to actin gamma - A. nidulans	40 subcellular localisation
SBE76B01	An01g12840	hypothetical protein	99 unclassified proteins
SBE76B02	An03g06140	strong similarity to hypothetical protein encoded by 123A4.300 - N. crassa	99 unclassified proteins
SBE76B03	An18g01700	strong similarity to quinate transport protein qutD - A. nidulans	01 metabolism
SBE76B04	An08g01020	strong similarity to hypothetical protein YJL055w - S. cerevisiae	99 unclassified proteins
SBE76B05	An18g02090	strong similarity to G-protein beta subunit sfaD - A. nidulans	10 cellular communication/signal transduction
SBE76B06	An16g05390	strong similarity to hypothetical protein RTS-beta - Homo sapiens	01 metabolism
SBE76B07	An01g14360	strong similarity to hypothetical protein 68B2.10 - N. crassa (truncated ORF)	99 unclassified proteins
SBE76B08	An11g03970	similarity to hypothetical AAA family ATPase SCD8A.32c - S. coelicolor	02 energy
SBE76B09	An02g11860	strong similarity to hypothetical Ydr124wp-like protein - Pneumocystis carinii	99 unclassified proteins
SBE76B10	An05g00540	strong similarity to cytoplasmic ribosomal protein of the large subunit L19 - S. cerevisiae	05 protein synthesis
SBE76B11	An01g07120	strong similarity to ATP-hydrolyzing 5-oxoprolinase - Rattus norvegicus	01 metabolism
SBE76B12	An14g00190	weak similarity to hypothetical protein At2g17590 - A. thaliana	99 unclassified proteins
SBE76C01	An04g01950	strong similarity to zinc-metalloprotease Ste24 - S. cerevisiae	06 protein fate
SBE76C03	An08g09170	similarity to hypothetical protein SPBC20F10.05 - S. pombe	99 unclassified proteins
SBE76C04	An05g01700	strong similarity to cadmium resistance protein Ycf1 - S. cerevisiae	08 cellular transport and transport mechanisms
SBE76C05	An01g00620	strong similarity to p-cumic aldehyde dehydrogenase cymc - Pseudomonas putida	01 metabolism
SBE76C06	An06g00560	strong similarity to hexose transporter Hxt13 - S. cerevisiae	01 metabolism
SBE76C07	An07g07520	strong similarity to vacuolar alkaline phosphatase Pho8 - S. cerevisiae	01 metabolism
SBE76C08	An16g09190	strong similarity to cytosolic acetyl-CoA C-acetyltransferase Erg10 - S. cerevisiae	01 metabolism
SBE76C09	An02g06660	hypothetical protein	99 unclassified proteins
SBE76C11	An11g07730	similarity to mucin MUC4 - Homo sapiens	06 protein fate
SBE76C12	An11g04980	similarity to RAN-binding protein Kap123 - S. cerevisiae (truncated ORF)	06 protein fate
SBE76D01	An01g07120	strong similarity to ATP-hydrolyzing 5-oxoprolinase - Rattus norvegicus	01 metabolism
SBE76D02	An01g09130	weak similarity to hypothetical photoreceptor membrane-associated protein trp – D. melanogaster	99 unclassified proteins
SBE76D04	An02g07720	similarity to dihydrodipicolinate synthase dapA - Escherichia coli	01 metabolism
SBE76D05	An03g04960	similarity to protein kinase DYRK2 - Homo sapiens	06 protein fate
SBE76D06	An07g05260	hypothetical protein	99 unclassified proteins
SBE76D07	An15g03660	hypothetical protein	99 unclassified proteins
SBE76D08	An09g06590	heat shock protein sspB - A. niger	11 cell rescue, defense and virulence
SBE76E01	An04g04790	strong similarity to capsule-associated protein CAP59 - Cryptococcus neoformans	11 cell rescue, defense and virulence
SBE76E03	An02g13220	strong similarity to lysophospholipase phospholipase B - Penicillium notatum	01 metabolism
SBE76E07	An18g06760	A. niger icdA gene encoding mitochondrial NADP+-specific isocitrate dehydrogenase	01 metabolism

SBE76E08	An14g02910	strong similarity to allantoate permease Dal5 - S. cerevisiae (truncated ORF)	01 metabolism
SBE76F01	An05g01760	similarity to hard surface induced protein 3 chip3 - Glomerella cingulata	99 unclassified proteins
SBE76F03	An14g04710	aspartic proteinase aspergillopepsin I pepA - A. niger	06 protein fate
SBE76F04	An09g05180	strong similarity to cytoplasmic ribosomal protein of the large subunit L4 - S. cerevisiae	05 protein synthesis
SBE76F05	An01g01960	strong similarity to riboflavin biosynthesis protein Rib7 - S. cerevisiae	01 metabolism
SBE76F06	An15g03260	strong similarity to threonine aldolase - Ashbya gossypii	01 metabolism
SBE76F07	An16g05970	similarity to UDP-glucuronosyltransferase UGT2B11 - Homo sapiens	01 metabolism
SBE76F08	An15g00590	weak similarity to NOD2 - Homo sapiens	99 unclassified proteins
SBE76G01	An16g05090	strong similarity to endonuclease SceI 75 kDa subunit Ens1 - S. cerevisiae	06 protein fate
SBE76G02	An08g06560	strong similarity to spermidine synthase Spe3 - S. cerevisiae	01 metabolism
SBE76G03	An17g01410	strong similarity to transcription regulator Snf2 - S. cerevisiae	01 metabolism
SBE76G04	An02g09260	strong similarity to nucleolar protein Nop5 - S. cerevisiae	04 transcription
SBE76G05	An11g02400	strong similarity to cytosolic lysine--tRNA ligase Krs1 - S. cerevisiae	05 protein synthesis
SBE76G06	An11g02430	strong similarity to assembly factor of cytochrome c oxidase Cox15 - S. cerevisiae	02 energy
SBE76G07	An07g08400	strong similarity to allergen rAsp f 4 - A. fumigatus	99 unclassified proteins
SBE76G08	An01g09500	strong similarity to cytosolic threonine--tRNA ligase Ths1 - S. cerevisiae	05 protein synthesis
SBE76H01	An15g05530	strong similarity to fluconazole resistance protein FLU1 - Candida albicans	08 cellular transport and transport mechanisms
SBE76H02	An11g04990	similarity to RAN-binding protein Kap123 - S. cerevisiae (truncated ORF)	06 protein fate
SBE76H03	An03g04580	similarity to hypothetical RNA binding protein CAB53728.1 - S. pombe	99 unclassified proteins
SBE76H04	An08g01600	similarity to histone transcription regulator Hir1 - S. cerevisiae	03 cell cycle and dna processing
SBE76H05	An07g03770	strong similarity to Cu,Zn superoxide dismutase sodC - A. fumigatus	11 cell rescue, defense and virulence
SBE76H06	An14g00800	similarity to hypothetical protein CAC38347.1 - S. pombe	99 unclassified proteins
SBE76H07	An07g02160	strong similarity to mitochondrial malate dehydrogenase Mdh1 - S. cerevisiae	01 metabolism
SBE76H08	An07g10395	strong similarity to hypothetical protein EAA62284.1 - A. nidulans	99 unclassified proteins
SBL02	An16g01830	glyceraldehyde-3-phosphate dehydrogenase gpdA - A. niger	01 metabolism
SBL03	An04g08880	similarity to hypothetical protein CAD60573.1 - Podospora anserina	99 unclassified proteins
SBL04	An04g03850	strong similarity to hypothetical beta transducin-like protein het-e1 - Podospora anserina	99 unclassified proteins
SBL06	An09g06200	strong similarity to transmembrane protein PTH11 - Magnaporthe grisea	40 subcellular localisation
SBL08	An02g05700	strong similarity to translation elongation factor eEF-2 - Cricetulus griseus	05 protein synthesis
SBL09	An16g04220	similarity to 2-haloacid halidohydrolase IVa - Pseudomonas cepacia	01 metabolism
SBL10	An01g10580	strong similarity to ribonuclease T2 precursor rntB - A. oryzae	01 metabolism
SBL12	An06g02120	weak similarity to Tyk2 non-receptor tyrosine - Mus musculus	99 unclassified proteins
SBL13	An04g00420	similarity to MAP kinase homolog WCK-1 - Triticum aestivum	06 protein fate
SBL14	An11g03680	strong similarity to cinnamyl alcohol dehydrogenase CAD - Eucalyptus gunnii	01 metabolism
SBL17	An08g09010	strong similarity to spliceosomal protein SAP130 - Homo sapiens	04 transcription

SBL18	An12g00720	strong similarity to 138 kD subunit of DNA-dependent RNA polymerase II rpb2p – S. pombe	04 transcription
SBL19	An11g01830	similarity to hypothetical fibroin-3 related protein B14D6.620 - N. crassa	99 unclassified proteins
SBL20	An04g01670	similarity to suppressor of kinase protein Sok1 - S. cerevisiae	03 cell cycle and dna processing
SBL21	An11g07780	strong similarity to multidrug resistance-associated protein Mrp2 - Oryctolagus cuniculus	11 cell rescue, defense and virulence
SBL23	An04g03850	strong similarity to hypothetical beta transducin-like protein het-e1 - Podospora anserina	99 unclassified proteins
SBL24	An04g03850	strong similarity to hypothetical beta transducin-like protein het-e1 - Podospora anserina	99 unclassified proteins
SBL25	An12g04670	strong similarity to translation initiation factor Eif-5 - S. cerevisiae	05 protein synthesis
SBL26	An14g01800	strong similarity to alpha-galactosidase - Cyamopsis tetragonoloba	01 metabolism
SBL27	An04g09020	similarity to twinfilin A Twf1 - S. cerevisiae	14 cell fate
SBL28	An08g04010	hypothetical protein [deleted ORF]	99 unclassified proteins
SBL29	An14g01050	similarity to serine/threonine protein kinase Yak1 - S. cerevisiae	03 cell cycle and dna processing
SBL30	An04g02510	similarity to hypothetical membrane protein YOR118w - S. cerevisiae	99 unclassified proteins
SBL31	An16g03520	strong similarity to DNA mismatch repair protein Msh2 - S. cerevisiae	03 cell cycle and dna processing
SBL34	An18g04840	strong similarity to translation elongation factor 1 alpha – P. anserina	99 unclassified proteins
SBL35	An08g03270	strong similarity to beta-COP Sec26 - S. cerevisiae	06 protein fate
SBL36	An13g00130	hypothetical protein	99 unclassified proteins
SBL37	An04g09660	similarity to hypothetical serine/threonine protein kinase AAO52136.1 - D. discoideum	99 unclassified proteins
SBL38	An02g08080	strong similarity to cytoplasmic ribosomal protein of the large subunit L22 - Xenopus laevis	05 protein synthesis
SBL39	An09g06390	strong similarity to lipase LipP - Pseudomonas sp.	01 metabolism
SBL40	An01g00850	similarity to xylose permease xylT - Bacillus megaterium	01 metabolism
SBL42	An09g06800	strong similarity to leucyl aminopeptidase Ape2 - S. cerevisiae	01 metabolism
SBL43	An01g10930	strong similarity to enzyme with sugar transferase activity from patent JP11009276-A	01 metabolism
SBL44	An07g05110	strong similarity to septin aspA - A. nidulans	03 cell cycle and dna processing
SBL45	An11g11160	strong similarity to O-succinylhomoserine (thiol)-lyase met-7 chain - N. crassa	01 metabolism
SBL46	An04g05060	similarity to protein XFIN - Xenopus laevis	04 transcription
SBL48	An08g08980	strong similarity to protein from patent WO200008056-A1 - Homo sapiens	40 subcellular localisation
SBL49	An11g01740	similarity to hypothetical coiled-coil protein SPBP23A10.05 - S. pombe	99 unclassified proteins

Supplementary table 3. Inventory of *A. niger* CBS 513.88 genes, encoding enzymes putatively involved in the degradation of the backbone and side chains of pectin. GH, glycoside hydrolase family; PL, polysaccharide lyase family; CE, carbohydrate esterase family CaZy family, carbohydrate active enzyme family (Henrissat, 1997); n.d. not determined. P (present) indicates detected significant expression in at least one of the tested conditions. A (absent) indicates the lack of expression across the entire data set.

Locus tag	Functional class	Gene symbol	CaZy family	EC number	Functional gene
	Endo-polygalacturonase				
An01g11520	Endo-polygalacturonase I	pgaI	GH28	3.2.1.15	P
An15g05370	Endo-polygalacturonase II	pgaII	GH28	3.2.1.15	P
An16g06990	Endo-polygalacturonase A	pgaA	GH28	3.2.1.15	P
An02g04900	Endo-polygalacturonase B	pgaB	GH28	3.2.1.15	P
An05g02440	Endo-polygalacturonase III	pgaC	GH28	3.2.1.15	P
An09g03260	Endo-polygalacturonase D	pgaD	GH28	3.2.1.15	P
An01g14670	Endo-polygalacturonase E	pgaE	GH28	3.2.1.15	P
	Endo-xylogalacturonan hydrolase				
An04g09700	Endo-xylogalacturonan hydrolase	xghA	GH28	3.2.1.-	P
	Endo-rhamnogalacturonase				
An12g00950	Rhamnogalacturonase A	rhgA	GH28	3.2.1.15	P
An14g04200	Rhamnogalacturonase B	rhgB	GH28	3.2.1.-	P
An06g02070	Putative rhamnogalacturonase	rhgC	GH28	3.2.1.15	P
An11g06320	Putative rhamnogalacturonase	rhgD	GH28	3.2.1.-	A
An11g08700	Putative rhamnogalacturonase	rhgE	GH28	3.2.1.-	A
An07g01000	Putative rhamnogalacturonase	rhgF	GH28	3.2.1.-	A
	Exopolygalacturonase				
An12g07500	Exo-polygalacturonase X	pgaX	GH28	3.2.1.67	P
An11g04040	Exo-polygalacturonase A	pgxA	GH28	3.2.1.67	P
An03g06740	Exo-polygalacturonase B	pgxB	GH28	3.2.1.67	P
An02g12450	Exo-polygalacturonase/exoxylogalacturonase C	pgxC	GH28	3.2.1.67	P
	Exo-rhamnogalacturonan hydrolase				
An01g14650	Putative exo-rhamnogalacturonase A	rgxA	GH28	3.2.1.67	P
An03g02080	Putative exo-rhamnogalacturonase B	rgxB	GH28	3.2.1.67	P
An18g04810	Putative exo-rhamnogalacturonase C	rgxC	GH28	3.2.1.67	P
	Pectin and pectate lyase				
An14g04370	pectin lyase A	pelA	PL1	4.2.2.10	P
An03g00190	pectin lyase B	pelB	PL1	4.2.2.10	A
An11g04030	pectin lyase C	pelC	PL1	4.2.2.10	A
An19g00270	pectin lyase D	pelD	PL1	4.2.2.10	A
An15g07160	pectin lyase F	pelF	PL1	4.2.2.10	P
An10g00870	pectate lyase A	plyA	PL1	4.2.2.2	P
	Rhamnogalacturonan lyase				
An14g01130	Rhamnogalacturonan lyase A	rglA	PL4	4.2.2.-	P
An11g00390	Putative rhamnogalacturonan lyase B	rglB	PL4	4.2.2.-	P
	Carbohydrate esterase				
An03g06310	Pectin methylesterase A	pmeA	CE8	3.1.1.11	P
An04g09690	Putative pectin methylesterase B	pmeB	CE8	3.1.1.11	P
An02g12505	Putative pectin methylesterase C	pmeC	CE8	3.1.1.-	P
An09g00120	Ferulic acid esterase A	faeA	CE1	3.1.1.73	P
An12g10390	Ferulic acid esterase B	faeB	CE1	3.1.1.73	P
An16g05690	Putative feruloyl esterase C	faeC	n.d.		A
An12g02550	Putative feruloyl esterase D	faeD	CE1		A
An02g02540	Putative pectin acetyl esterase	paeA	n.d		P

Locus tag	Functional class	Gene symbol	CaZy family	EC number	Functional gene
An07g08940	Putative pectin acetyl esterase	paeB	CE12		P
	Rhamnogalacturonan acetyl esterase				
An09g02160	Rhamnogalacturonan acetyl esterase	rgaeA	CE12	3.1.-.-	P
An04g09360	Putative rhamnogalacturonan acetyl esterase	rgaeB	CE12		P
	Unsaturated rhamnogalacturonyl hydrolase				
An14g02920	Putative rhamnogalacturonyl hydrolase	urhgA	GH105		P
An14g05340	Putative rhamnogalacturonyl hydrolase	urhgB	GH105		A
	Arabinofuranosidase				
An01g00330	-L-arabinofuranosidase A	abfA	GH51	3.2.1.55	P
An15g02300	-L-arabinofuranosidase B	abfB	GH54		P
An08g01710	Putative -L-arabinofuranosidase C	abfC	GH51		P
An09g00880	Putative -L-arabinofuranosidase D	abfD	GH51		A
	Endoarabinase				
An09g01190	arabinan endo-1,5- -L-arabinosidase A	abnA	GH43	3.2.1.99	P
An02g01400	Putative endoarabinanase B	abnB	GH43		P
An02g10550	Putative endoarabinanase C	abnC	GH43		P
An16g02730	Putative endoarabinanase D	abnD	GH43		P
	-Galactosidase				
An01g12150	-galactosidase	lacA	GH35	3.2.1.23	P
An01g10350	Putative -galactosidase	lacB	GH35		P
An06g00290	Putative -galactosidase	lacC	GH35		P
An07g04420	Putative -galactosidase	lacD	GH35		A
An14g05820	Putative -galactosidase	lacE	GH35		P
	Galactan 1,3- -galactosidase				
An08g10780	Putative Galactan 1,3- -galactosidase	1,3- -gal	GH43		P
	-1,4,-endogalactanase				
An18g05940	-1,4,-endogalactanase A	galA	GH53	3.5.1.89	P
An16g06590	Putative endo- -1,4-galactanase	galB	GH53		A

Supplementary table 4. Monomeric sugar concentrations (in w/v percent) and acid concentrations (mM) in the media fluid of the *A. niger* cultures used for microarray experiments. The starting concentration of the pre-culture on fructose was 2% (w/v). The starting concentration of the carbon source in all transfer samples was 1 % (w/v). The uronic acid content of polygalacturonic acid and sugar beet pectin used in this study was 0.65 % (w/w) and 0.5 % (w/w), respectively.

Array sample	Sorbitol %	Fructose %	Xylose %	Rhamnose %	Galacturonic acid %	Oxalate mM	Citrate mM	Gluconate mM	Lactate mM	Acetate mM	pH
Fructose pre-culture	-	1.38	-	-	-	4.9	0.1	0.1	0.3	0.3	4.7
Sorbitol, 2 hours	0.98	-	-	-	-	-	-	-	0.1	-	5.8
Sorbitol, 4 hours	0.83	-	-	-	-	2.1	-	-	0.1	-	4.5
Sorbitol, 8 hours	0.45	-	-	-	-	6.6	-	-	-	-	4.2
Sorbitol, 24 hours	0.00	-	-	-	-	17.5	-	-	-	-	4.0
Fructose, 2 hours	-	0.85	-	-	-	0.4	-	-	-	-	5.8
Fructose, 4 hours	-	0.75	-	-	-	2.8	-	-	-	-	4.0
Fructose, 8 hours	-	0.27	-	-	-	7.5	-	-	-	-	4.4
Fructose, 24 hours	-	0.00	-	-	-	16.4	-	-	-	-	4.4
Xylose, 2 hours	-	-	0.94	-	-	-	-	-	-	-	5.8
Xylose, 4 hours	-	-	0.84	-	-	1.3	-	-	-	-	5.0
Xylose, 8 hours	-	-	0.50	-	-	5.2	-	-	-	-	4.5
Xylose, 24 hours	-	-	0.00	-	-	14.9	-	-	-	-	4.5
Rhamnose, 2 hours	-	-	-	0.96	-	-	-	-	-	-	5.8
Rhamnose, 4 hours	-	-	-	0.88	-	1.9	-	-	-	-	4.0
Rhamnose, 8 hours	-	-	-	0.69	-	8.0	-	-	-	-	2.7
Rhamnose, 24 hours	-	-	-	0.00	-	12.7	-	-	-	-	4.2
Galacturonic acid, 2 hours	-	-	-	-	0.89	0.4	-	-	-	-	5.8
Galacturonic acid, 4 hours	-	-	-	-	0.83	2.1	-	-	-	-	5.5
Galacturonic acid, 8 hours	-	-	-	-	0.62	9.0	-	-	0.1	-	4.5
Galacturonic acid, 24 hours	-	-	-	-	0.00	33.0	-	-	-	-	3.6
Polygalacturonic acid, 2 hours	-	-	-	-	0.00	0.2	-	-	-	-	5.8
Polygalacturonic acid, 4 hours	-	-	-	-	0.02	1.6	-	-	-	-	5.3
Polygalacturonic acid, 8 hours	-	-	-	-	0.14	8.3	-	-	-	-	4.5
Polygalacturonic acid, 24 hours	-	-	-	-	0.00	30.3	-	-	-	2.1	3.6
Sugar beet pectin, 2 hours	-	-	-	-	0.00	0.4	-	-	-	-	5.2
Sugar beet pectin, 4 hours	-	-	-	-	0.01	1.4	-	-	-	2.7	4.7
Sugar beet pectin, 8 hours	-	-	-	-	0.02	6.3	-	-	-	4.4	4.4
Sugar beet pectin, 24 hours	-	-	-	-	0.00	18.3	-	-	-	1.1	3.6

Supplementary table 5. *A. niger* genes identified as significantly changing across microarray samples by t-test analysis.

Microarray Gene identifier	Locus tag	Description
An00g00148_at	An01g00330	alpha-l-arabinofuranosidase a precursor abfA - Aspergillus niger
An00g03624_at	An01g00450	similarity to 3-hydroxyisobutyrate dehydrogenase mmsB - Pseudomonas aeruginosa
An00g03069_at	An01g00530	proteinase aspergillopepsin II - Aspergillus niger
An00g09289_at	An01g02020	strong similarity to hypothetical protein ssl3291 - Synechocystis sp.
An00g10343_at	An01g02140	strong similarity to plasma membrane H+-biotin symporter VHT1 - Saccharomyces cerevisiae
An00g06723_at	An01g02970	strong similarity to 3-carboxy-cis cis-muconate cycloisomerase pcaB - Bradyrhizobium japonicum
An00g00176_at	An01g03740	D-xylose reductase xyrA - Aspergillus niger
An00g07048_at	An01g04880	strong similarity to alpha-glucosidase II - Bacillus thermoamyloliquefaciens
An00g13480_at	An01g05100	weak similarity to ribonucleoprotein B cp29B - Nicotiana sylvestris
An00g01259_at	An01g05530	hypothetical protein
An00g06985_at	An01g06800	strong similarity to alkaline ceramidase Ypc1p - Saccharomyces cerevisiae
An00g01121_at	An01g07290	hypothetical protein
An00g08417_at	An01g07300	strong similarity to gluconokinase gntV - Escherichia coli
An00g07891_at	An01g09040	strong similarity to DEAD box protein MrDb - Homo sapiens
An00g12703_at	An01g09090	weak similarity to hypothetical protein AAF45855.1 - Drosophila melanogaster
An00g05921_at	An01g09380	similarity to S-layer protein - Clostridium thermocellum
An00g12124_at	An01g09590	strong similarity to voltage-gated potassium channel beta subunit Kv beta 1 - Rattus norvegicus
An00g07956_at	An01g09780	strong similarity to D-lactate dehydrogenase ldhA - Escherischia coli (truncated ORF)
An00g10572_at	An01g09930	strong similarity to propionate catabolic protein PrpD - Salmonella typhimurium
An00g13088_at	An01g09970	weak similarity to Lactobacillus crispatus silent surface layer protein cbsB - Lactobacillus crispatus
An00g08577_at	An01g09980	strong similarity to hemolysin Asp-HS - Aspergillus fumigatus
An00g08723_at	An01g10100	strong similarity to hypothetical aminotransferase SPBC1773.03c - Schizosaccharomyces pombe
An00g10961_at	An01g10350	strong similarity to secreted beta-galactosidase lacA - Aspergillus niger
An00g00384_at	An01g10620	hypothetical protein
An00g03062_s_at	An01g11520	polygalacturonase pgaI - Aspergillus niger (mRNA)
An00g03062_at	An01g11520	polygalacturonase pgaI - Aspergillus niger (mRNA)
An00g06964_at	An01g12170	strong similarity to alcohol dehydrogenase B alcB - Aspergillus nidulans
An00g13953_at	An01g12220	weak similarity to UDPglucose 4-epimerase galE - Escherichia coli
An00g08867_at	An01g12240	strong similarity to hypothetical protein An02g05360 - Aspergillus niger
An00g04221_at	An01g13200	similarity to FLEXHT-10 of patent WO200070047-A2 - Homo sapiens
An00g11300_at	An01g14390	strong similarity to the beta-transducin-like protein HET-E - Podospora anserina
An00g05383_at	An01g15010	similarity to O-methyltransferase omtA - Aspergillus flavus
An00g10553_at	An02g00090	strong similarity to prolidase - Aureobacterium esteraromaticum
An00g12136_at	An02g00140	strong similarity to xylan 1 4-beta-xylosidase xynB - Bacillus subtilis

An00g12307_at	An02g01260	weak similarity to beta transducin-like protein het-e1 - Podospora anserina
An00g08279_at	An02g01480	strong similarity to fluconazole resistance protein FLU1 - Candida albicans
An00g06876_at	An02g02540	strong similarity to acetyl-esterase I from patent R63066 - Aspergillus aculeatus
An00g04920_at	An02g03280	similarity to hypothetical protein UNC-89 - Caenorhabditis elegans
An00g08588_at	An02g03540	strong similarity to hexose transport protein HXT3 - Saccharomyces cerevisiae
An00g11374_at	An02g04830	strong similarity to the extragenic suppressor of the bimD6 mutation (sudD) - Aspergillus nidulans
An00g08372_at	An02g05920	strong similarity to GABA permease UGA4 - Saccharomyces cerevisiae
An00g00615_at	An02g07110	hypothetical protein (mRNA)
An00g03874_at	An02g07590	similarity to beta-N-Acetylglucosaminidase nagA - Streptomyces thermoviolaceus
An00g04986_at	An02g07710	similarity to hypothetical protein Z2629 product - Escherichia coli
An00g04069_at	An02g07720	similarity to dihydrodipicolinate synthase dapA - Escherichia coli
An00g08606_at	An02g08230	strong similarity to high affinity glucose transporter HGT1 - Kluyveromyces lactis
An00g08129_at	An02g09890	strong similarity to EST an_2336 - Aspergillus niger
An00g07698_at	An02g10750	strong similarity to cysteine synthase cysB - Aspergillus nidulans
An00g09311_at	An02g11550	strong similarity to hypothetical protein YDCJ - Escherichia coli
An00g02497_at	An02g12080	hypothetical protein
An00g05481_at	An02g12450	similarity to polygalacturonase XOPG1 - Lycopersicon esculentum
An00g08861_at	An02g13470	strong similarity to hypothetical protein An01g13320 - Aspergillus niger
An00g12330_at	An02g13890	weak similarity to calmodulin- and actin-binding protein h-caldesmon - Gallus gallus
An00g08439_at	An02g14590	strong similarity to glutamate dehydrogenase Gdh2p - Saccharomyces cerevisiae
An00g05989_at	An03g00660	similarity to taurine dioxygenase tauD - Escherichia coli
An00g09458_at	An03g01540	strong similarity to inducible salicylate hydroxylase (NahW) - Pseudomonas stutzeri
An00g08607_at	An03g01620	strong similarity to high affinity glucose transporter HGT1 - Kluyveromyces lactis
An00g11620_at	An03g01750	strong similarity to the monosaccharide transporter Mst1 - Amanita muscaria
An00g06355_at	An03g02160	similarity to the UDP-glucose 4-epimerase Gal10 - Saccharomyces cerevisiae
An00g08975_at	An03g03530	strong similarity to hypothetical protein An16g04320 - Aspergillus niger
An00g09804_at	An03g03640	strong similarity to mitochondrial sulfide dehydrogenase (coenzyme Q2) SPBC2G5.06c - S. pombe
An00g10384_at	An03g05140	strong similarity to polyketide synthase PKS1 - Cochliobolus heterostrophus
An00g10221_at	An03g06660	strong similarity to peptide transporter ptr2 - Arabidopsis thaliana
An00g08201_at	An03g06740	strong similarity to exo-alpha 1 4-polygalacturonase PGX1 - Cochliobolus carbonum
An00g08738_at	An03g06850	strong similarity to hypothetical coiled-coil protein cgrA - Aspergillus nidulans
An00g05276_at	An04g00790	similarity to multifunctional arom protein Aro1p - Saccharomyces cerevisiae
An00g03993_at	An04g02110	similarity to con-8 - Neurospora crassa
An00g09606_at	An04g02220	strong similarity to L-serine dehydratase CHA1 - Saccharomyces cerevisiae
An00g05587_at	An04g03070	similarity to probable transcription activator SPAC139.03 - Schizosaccharomyces pombe
An00g07260_at	An04g03170	strong similarity to beta-glucosidase bglB - Candida wickerhamii

An00g06885_at	An04g03360	strong similarity to acidic Ca(2+)-independent phospholipase A2 aiPLA2 - Rattus norvegicus
An00g03234_at	An04g05790	questionable ORF
An00g09563_at	An04g05880	strong similarity to linoleate diol synthase - Gaeumannomyces graminis
An00g12663_at	An04g06800	weak similarity to hypothetical hypothetical protein C34C6.1 - Caenorhabditis elegans
An00g09525_at	An04g07200	strong similarity to kynureninase - Rattus norvegicus
An00g09457_at	An04g07210	strong similarity to indoleamine 2 3-dioxygenase IDO - Mus musculus
An00g01959_at	An04g07780	hypothetical protein
An00g02164_at	An04g08130	hypothetical protein
An00g10636_at	An04g08250	strong similarity to protein involved in cephalosporin C biosynthesis patent JP09009966-A - Acremonium chrysogenum
An00g04515_at	An04g08880	similarity to hypothetical protein An02g00390 - Aspergillus niger
An00g07143_at	An04g08890	strong similarity to aryl-alcohol oxidase aao - Pleurotus eryngii
An00g09998_s_at	An04g09420	strong similarity to neutral amino acid permease mtr - Neurospora crassa (mRNA)
An00g08943_at	An04g09490	strong similarity to hypothetical protein An12g04090 - Aspergillus niger
An00g07460_at	An04g09890	strong similarity to cell wall alpha-glucan synthase ags1 - Schizosaccharomyces pombe
An00g01232_at	An05g00550	hypothetical protein
An00g08589_at	An05g00730	strong similarity to hexose transport protein HXT3 - Saccharomyces cerevisiae
An00g08558_at	An05g01060	strong similarity to HC-toxin synthetase HTS1 - Cochliobolus carbonum
An00g06808_at	An05g01070	strong similarity to 7-aminocholesterol resistance protein RTA1 - Saccharomyces cerevisiae
An00g11947_at	An05g02370	strong similarity to trichothecene 3-O-acetyltransferase TRI101 - Fusarium sporotrichioides
An00g00144_at	An06g00170	alpha-galactosidase aglA - Aspergillus niger
An00g08597_at	An06g00260	strong similarity to hexose transporter HXT5 - Saccharomyces cerevisiae
An00g07256_at	An06g00290	strong similarity to beta-galactosidase lacA - Aspergillus niger
An00g13945_at	An06g02120	weak similarity to Tyk2 non-receptor tyrosine - Mus musculus
An00g00361_at	An06g02130	hypothetical protein
An00g12711_at	An06g02150	weak similarity to hypothetical protein An01g11330 - Aspergillus niger
An00g08894_at	An07g00070	strong similarity to hypothetical protein An07g00010 - Aspergillus niger
An00g01916_at	An07g00170	hypothetical protein
An00g09602_at	An07g00680	strong similarity to low specificity L-threonine aldolase patent JP03277282-A - Pseudomonas putida
An00g08316_at	An07g00760	strong similarity to formyl-CoA transferase patent WO9816632-A1 - Oxalobacter formigenes
An00g09837_at	An07g00780	strong similarity to monocarboxylate transporter 2 hMCT2 - Homo sapiens
An00g12139_at	An07g01290	strong similarity to xylitol dehydrogenase xdh - Galactocandida mastotermitis
An00g07117_at	An07g01320	strong similarity to antifungal protein precursor paf - Penicillium chrysogenum
An00g08027_at	An07g03140	strong similarity to D-xylulokinase XKS1 - Saccharomyces cerevisiae
An00g06990_at	An07g03470	strong similarity to allantoate permease DAL5 - Saccharomyces cerevisiae
An00g06742_at	An07g04270	strong similarity to 3-methylcrotonyl-CoA carboxylase (MCC) non-biotin-containing beta subunit MCCB - Homo sapiens
An00g06741_at	An07g04300	strong similarity to 3-methylcrotonyl-CoA carboxylase (MCC) biotin-containing alpha subunit MCCA - Homo sapiens

An00g08592_at	An07g04430	strong similarity to hexose transporter Ght2 - Schizosaccharomyces pombe
An00g04504_at	An07g04480	similarity to hypothetical protein An01g02250 - Aspergillus niger
An00g13468_at	An07g05020	weak similarity to regulatory protein UGA3 - Saccharomyces cerevisiae
An00g08315_at	An07g05830	strong similarity to formamidase fmdS - Aspergillus nidulans
An00g08262_at	An07g06240	strong similarity to ferrioxamine B permease sit1 - Saccharomyces cerevisiae
An00g03896_at	An07g06460	similarity to C-7 hydroxycephem methyltransferase coupling protein of patent WO9529253-A1 - Streptomyces lactamdurans
An00g04034_at	An07g06480	similarity to cytochrome 4F8 cyp4F8 - Homo sapiens
An00g03713_at	An07g08940	similarity to acetyl-esterase I of patent WO9502689-A - Aspergillus aculeatus
An00g08318_at	An07g09600	strong similarity to fructosamine-3-kinase FN3K - Homo sapiens
An00g05994_s_at	An08g00810	similarity to tetracyclin resistance protein TetH - Pasteurella multocida
An00g08826_at	An08g00870	strong similarity to hypothetical protein 1A9.230 - Neurospora crassa
An00g04400_at	An08g01490	similarity to hypothetical ankyrin-repeat protein SPCP1E11.10 - Schizosaccharomyces pombe
An00g07055_at	An08g01710	strong similarity to alpha-L-arabinofuranosidase abfA - Bacillus stearothermophilus
An00g10796_at	An08g01720	strong similarity to quinate transport protein QUTD - Aspergillus nidulans
An00g12062_at	An08g01740	strong similarity to uronate dehydrogenase of patent W29217 - Saccharomyces cerevisiae
An00g07093_at	An08g03200	strong similarity to ammonium transport protein Mep2p - Saccharomyces cerevisiae
An00g00095_at	An08g03560	weak similarity to mucin 2 MUC 2- Rattus norvegicus
An00g04765_at	An08g03760	similarity to hypothetical protein Rv3472 - Mycobacterium tuberculosis
An00g10797_at	An08g04040	strong similarity to quinate transport protein QUTD - Aspergillus nidulans
An00g10222_at	An08g04600	strong similarity to peptide transporter Ptr2p - Saccharomyces cerevisiae
An00g05155_at	An08g05380	similarity to mannosylphosphate transferase MNN4 - Saccharomyces cerevisiae
An00g03050_at	An08g05610	nitrate reductase (NADPH) niaD - Aspergillus niger
An00g10027_at	An08g05640	strong similarity to nitrite reductase (NADH) long form niiA - Aspergillus nidulans
An00g10013_at	An08g05670	strong similarity to nitrate permease crnA - Aspergillus nidulans
An00g12047_at	An08g06240	strong similarity to uracil transport protein FUR4 - Schizosaccharomyces pombe
An00g09485_at	An08g06700	strong similarity to iron-phytosiderophore transporter protein yellow stripe 1 ys1 - Zea mays
An00g09705_at	An08g07280	strong similarity to methicillin resistance gene HmrA - Staphylococcus aureus
An00g07149_at	An08g08250	strong similarity to aryl-alcohol oxidase precursor aao - Pleurotus pulmonarius
An00g06715_at	An08g10340	strong similarity to 3 4-dihydroxy-2-butanone 4-phosphate synthase RIB3 - Saccharomyces cerevisiae
An00g06676_s_at	An08g11680	strong similarity to 2 5-dicloro-2 (5-cyclohexadiene-1) 4-diol dehydrogenase linC - Pseudomonas paucimobilis
An00g11495_at	An09g00470	strong similarity to the hypothetical protein encoded by An04g02540 - Aspergillus niger
An00g05849_at	An09g00530	similarity to salicylate hydroxylase nahW - Pseudomonas stutzeri
An00g12328_at	An09g00570	weak similarity to calcium-binding protein P22 - Gallus gallus
An00g05844_at	An09g00590	similarity to salicylate hydroxylase nahG - Pseudomonas putida
An00g05467_at	An09g01050	similarity to platelet-activating factor-acetylhydrolase - Cavia porcellus
An00g03582_at	An09g01170	similarity to 1-aminocyclopropane-1-carboxylate (ACC) oxidase - Oryza sativa

An00g00177_at	An09g01190	endo 1 5-alpha-arabinanase abnA - Aspergillus niger
An00g00168_at	An09g01550	copper amine oxidase AO-I - Aspergillus niger
An00g03550_at	An09g02160	rhamnogalacturonan acetyl esterase rgaeA - Aspergillus niger
An00g10300_at	An09g02180	strong similarity to phospholipase A1 of patent JP10155493-A - Aspergillus oryzae
An00g07303_at	An09g02700	strong similarity to branched-chain alpha-keto acid dehydrogenase E1 alpha subunit BCHEL1 - Homo sapiens
An00g07304_at	An09g02710	strong similarity to branched-chain alpha-keto acid dihydrolipoyl acyltransferase - Gallus gallus
An00g11278_at	An09g03300	strong similarity to the alpha-xylosidase XylS - Sulfolobus solfataricus
An00g09560_at	An09g03900	strong similarity to L-iditol-2 dehydrogenase SORD - Homo sapiens
An00g08473_at	An10g00230	strong similarity to glutathione-independent formaldehyde dehydrogenase fdhA - Pseudomonas putida
An00g12196_x_at	An10g00520	weak similarity to 1-aminocyclopropane-1-carboxylate deaminase - Pseudomonas sp. (truncated ORF)
An00g09047_at	An10g00560	strong similarity to hypothetical protein binA - Aspergillus nidulans
An00g03055-3_at	An10g00870	pectate lyase plyA - Aspergillus niger
An00g10121_at	An11g00070	strong similarity to O-methyltransferase B omtB - Aspergillus parasiticus
An00g06977_at	An11g01120	strong similarity to aldehyde reductase - Sporobolomyces salmonicolor
An00g06207_at	An11g01200	similarity to the hypothetical protein encoded by An11g07670 - Aspergillus niger
An00g12884_at	An11g01400	weak similarity to hypothetical protein L344.14 - Leishmania major
An00g11827_at	An11g02150	strong similarity to transcription factor ARO80 - Saccharomyces cerevisiae
An00g09649_at	An11g02160	strong similarity to maleylacetoacetate isomerase maiA - Aspergillus nidulans
An00g02025_at	An11g02190	hypothetical protein
An00g06775_at	An11g02200	strong similarity to 4-hydroxyphenylpyruvate dioxygenase tcrP - Coccidioides immitis
An00g09729_at	An11g02540	strong similarity to mitochondrial 2-oxoglutarate/malate translocator clone OMT103 - Panicum miliaceum
An00g02720_at	An11g02850	hypothetical protein
An00g06057_at	An11g03120	similarity to the endo-1 4-beta-xylanase XynD - Bacillus polymyxa
An00g10546_at	An11g03270	strong similarity to probable zinc finger protein - Schizosaccharomyces pombe
An00g00138_at	An11g03340	acid alpha-amylase - Aspergillus niger
An00g11234_at	An11g03530	strong similarity to tartrate transporter ttuB - Agrobacterium vitis
An00g11900_at	An11g03640	strong similarity to transmemrane oligopeptide transporter OPT1 - Candida albicans
An00g04497_at	An11g03950	similarity to hypothetical protein AKT2 - Alternaria alternata
An00g04394_at	An11g03970	similarity to hypothetical AAA family ATPase SCD8A.32c - Streptomyces coelicolor
An00g08200_at	An11g04040	strong similarity to exo-alpha 1 4-polygalacturonase PGX1 - Cochliobolus carbonum
An00g07106_at	An11g06060	strong similarity to ankyrin 2 Ank2 - Drosophila melanogaster
An00g07899_at	An11g06140	strong similarity to delta-1-pyrroline-5-carboxylate dehydrogenase p5cdh - Homo sapiens
An00g05063_at	An11g06430	similarity to integral membrane protein PTH11 - Magnaporthe grisea
An00g11483_at	An11g06450	strong similarity to the hypothetical protein encoded by An02g08300 - Aspergillus niger
An00g06987_at	An11g07010	strong similarity to alkane-inducible cytochrome P450 alk2 - Candida tropicalis
An00g08159_at	An11g07040	strong similarity to EST EMBLEST:BE759852 an_2779 - Aspergillus niger

An00g11552_at	An11g07050	strong similarity to the hypothetical protein encoded by An12g10210 - Aspergillus niger
An00g10749_at	An11g07340	strong similarity to putative O-methyl transferase EncK - Streptomyces maritimus
An00g05431_at	An11g07380	similarity to phenazine biosynthesis oxidoreductase phzF - Pseudomonas fluorescens
An00g02011_at	An11g07720	hypothetical protein
An00g04301_at	An11g09240	similarity to Gpi-anchored aspartic protease Yps1 - Saccharomyces cerevisiae
An00g10385_at	An11g09720	strong similarity to polyketide synthase PKS1 - Cochliobolus heterostrophus
An00g04290_at	An11g10610	similarity to glycogenin glucosyltransferase - Oryctolagus cuniculus
An00g09564_at	An12g01320	strong similarity to linoleate diol synthase - Gaeumannomyces graminis
An00g06081_at	An12g02360	similarity to the hippurate hydrolase HipO - Campylobacter jejuni
An00g05879_at	An12g04640	similarity to sequence 28 from Patent WO0032789
An00g00137_at	An12g05010	acetyl xylan esterase aceA - Aspergillus niger
An00g04095_at	An12g05050	similarity to DNA-binding protein amdA - Emericella nidulans
An00g06408_at	An12g05220	similarity to transcription factor nft1 - Schizosaccharomyces pombe
An00g03770_at	An12g05700	similarity to alpha-L-rhamnosidase A precursor RhaA - Aspergillus aculeatus
An00g09555_at	An12g05710	strong similarity to L-fucose permease fucP - Escherichia coli
An00g02965_at	An12g05850	hypothetical protein
An00g03625_at	An12g05870	similarity to 3-hydroxyisobutyrate dehydrogenase mmsB - Pseudomonas aeruginosa
An00g08208_at	An12g07500	strong similarity to exopolygalacturonase pgaX - Aspergillus tubingensis
An00g08208_s_at	An12g07500	strong similarity to exopolygalacturonase pgaX - Aspergillus tubingensis
An00g11950_at	An12g08350	strong similarity to trichothecene efflux pump TRI12 - Fusarium sporotrichioides
An00g07752_at	An12g08630	strong similarity to cytochrome P450 monooxygenase TRI11 - Fusarium sporotrichioides
An00g09538_at	An12g09270	strong similarity to lactose permease LAC12 - Kluyveromyces marxianus var. lactis
An00g09832_at	An12g10150	strong similarity to monoamine oxidase MAO - Oncorhynchus mykiss
An00g05314_at	An12g10560	similarity to negative regulator for expression of galactose-induced genes GAL80 - Saccharomyces cerevisiae
An00g07628_at	An13g00710	strong similarity to copper amine oxidase AO-I - Aspergillus niger
An00g03704_at	An13g00910	similarity to acetate regulatory DNA binding protein FacB - Aspergillus niger
An00g05153_at	An13g00920	similarity to mandelate racemase - Pseudomonas putida
An00g08421_at	An13g00930	strong similarity to glucose 1-dehydrogenase gdhIII - Bacillus megaterium
An00g06963_at	An13g00950	strong similarity to alcohol dehydrogenase B alcB - Emericella nidulans
An00g11455_at	An13g01800	strong similarity to the hypothetical protein An15g07140 - Aspergillus niger
An00g03767_at	An13g02780	similarity to alpha-adducin alpha-ADD - Rattus norvegicus
An00g08258_at	An13g02810	strong similarity to ferric enterobactin transporter protein ENB1 - Saccharomyces cerevisiae
An00g06094_at	An13g02980	similarity to the hypothetical protein An01g08440 - Aspergillus niger
An00g06089_at	An13g02990	similarity to the hypothetical protein An01g00290 - Aspergillus niger
An00g13643_at	An13g03050	weak similarity to the hypothetical protein An04g04070 - Aspergillus niger
An00g08633_at	An13g03110	strong similarity to high-affinity nicotinic acid permease TNA1 - Saccharomyces cerevisiae

An00g07305_at	An13g03150	strong similarity to branched-chain alpha-ketoacid dehydrogenase (BCKDH) E1 beta subunit - Gallus gallus
An00g07928_at	An13g03910	strong similarity to dihydropyrimidinase PYD2 - Saccharomyces kluyveri
An00g04363_at	An14g00520	similarity to homeotic protein HRS1 root-specific - Helianthus annuus
An00g07042_at	An14g01800	strong similarity to alpha-galactosidase - Cyamopsis tetragonoloba
An00g11695_at	An14g02470	strong similarity to the protein PRO304 of patent WO200104311-A1 - Homo sapiens
An00g11896_at	An14g02640	strong similarity to transmembrane protein of patent WO9927105-A2 - Chlamydia pneumoniae
An00g09988_at	An14g02660	strong similarity to necrosis and ethylene inducing protein BH0395 - Bacillus halodurans
An00g08605_at	An14g02740	strong similarity to high affinity glucose transporter HGT1 - Kluyveromyces lactis
An00g11164_at	An14g02870	strong similarity to succinate-semialdehyde dehydrogenase gabD - Escherichia coli
An00g07011_at	An14g02910	strong similarity to allantoate permease DAL5 - Saccharomyces cerevisiae (truncated ORF)
An00g07618_at	An14g02920	strong similarity to conserved hypothetical protein yesR - Bacillus subtilis
An00g04103_at	An14g03220	similarity to DNA-J-like protein SPAC4G9.19 - Schizosaccharomyces pombe
An00g10801_at	An14g04280	strong similarity to quinate transport protein QUTD - Aspergillus nidulans
An00g03056_at	An14g04370	pectin lyase A precursor - Aspergillus niger
An00g02657_at	An14g04550	hypothetical protein (mRNA)
An00g05051_at	An14g05850	similarity to integral membrane protein PTH11 - Magnaporthe grisea
An00g12891_at	An14g06380	weak similarity to hypothetical protein MT0291.4 - Mycobacterium tuberculosis
An00g09993_at	An14g07130	strong similarity to neutral amino acid permease mtr - Neurospora crassa
An00g09083_at	An15g00280	strong similarity to hypothetical protein jhp0295 - Helicobacter pylori
An00g08842_at	An15g00730	strong similarity to hypothetical protein 99H12.280 - Neurospora crassa
An00g10407_at	An15g01810	strong similarity to predicted protein An01g11620 - Aspergillus niger
An00g00828_at	An15g02270	hypothetical protein
An00g00152_at	An15g02300	arabinofuranosidase B abfB from patent EP506190-A - Aspergillus niger
An00g11367_at	An15g03800	strong similarity to the EST an_3228 - Aspergillus niger
An00g09855_at	An15g03940	strong similarity to monosaccharide transporter Mst-1 - Amanita muscaria
An00g08581_at	An15g06140	strong similarity to heterokaryon incompatibility protein HET-C - Neurospora crassa
An00g13576_at	An15g06210	weak similarity to the alpha-2 subunit of propyl-4-hydrolase from patent WO9738121-A1 - Mus sp.
An00g13968_at	An15g06420	weak similarity to urease accessory gene ureD - Bacillus sp.
An00g09270_at	An15g06480	strong similarity to hypothetical protein SPCC18.09c - Schizosaccharomyces pombe
An00g07658_at	An15g07070	strong similarity to cyanate lyase (cynS) - Escherichia coli
An00g08646_at	An15g07190	strong similarity to high-affinity zinc transport protein ZRT1 - Saccharomyces cerevisiae
An00g09444_at	An15g07240	strong similarity to icosapentaenoic acid synthase of patent WO9621735-A1 - Shewanella putrefaciens
An00g10466_at	An15g07360	strong similarity to PRG3 protein of patent WO200012526-A1 - Homo sapiens
An00g10219_at	An15g07510	strong similarity to peptide transport gene CaPTR2 - Candida albicans
An00g05514_at	An15g07790	similarity to predicted protein An11g02730 - Aspergillus niger
An00g09618_at	An16g01880	strong similarity to lysophospholipase - Aspergillus foetidus

An00g00184_at	An16g02170	epoxide hydrolase hyl1 - Aspergillus niger
An00g09044_at	An16g02760	strong similarity to hypothetical protein BH0842 - Bacillus halodurans
An00g06609_at	An16g03700	strong similarity to Phospholipase B from patent US6146869-A - Aspergillus oryzae
An00g08719_at	An16g04760	strong similarity to hypothetical aldehyde reductase 6 alr6 - Colletotrichum gloeosporioides
An00g09402_at	An16g05390	strong similarity to hypothetical RTS beta protein - Homo sapiens
An00g06547_at	An16g06270	similarity to versicolorin reductase verA - Aspergillus nidulans
An00g00181_s_at	An16g06990	endo-polygalacturonase A pgaA - Aspergillus niger (possible sequencing error) (mRNA)
An00g12100_at	An16g07180	strong similarity to vanillin dehydrogenase VDH of patent EP0845532
An00g13746_at	An16g08760	weak similarity to the latent nuclear antigen from ORF 73 - Kaposi s sarcoma-associated herpesvirus
An00g07381_at	An16g09010	strong similarity to carboxypeptidase I protein of patent WO9814599-A1 - Aspergillus oryza
An00g08419_at	An16g09070	strong similarity to glucosamine-6-phosphate deaminase protein of patent WO9835047-A1 - Escherichia coli
An00g07069_at	An17g00010	strong similarity to amine oxidase AMO - Hansenula polymorpha
An00g01536_at	An17g00700	hypothetical protein
An00g13910_at	An17g00800	weak similarity to transcriptional activator prtT of patent WO200020596-A1 - Aspergillus niger
An00g05044_at	An18g00400	similarity to inorganic phosphate transporter PT1 - Nicotiana tabacum
An00g08561_at	An18g00600	strong similarity to heat shock protein 30 hsp30 - Neurospora crassa (truncated ORF)
An00g07375_at	An18g01580	strong similarity to carbonic anhydrase pca1 - Porphyridium purpureum
An00g00136_at	An18g01670	6-phosphofructokinase pfkA - Aspergillus niger
An00g10794_at	An18g01700	strong similarity to quinate transport protein QUTD - Aspergillus nidulans
An00g06305_at	An18g03360	similarity to the proteophosphoglycan ppg1 - Leishmania major
An00g11618_at	An18g03380	strong similarity to the mitochondrial thioredoxin Trx3 - Saccharomyces cerevisiae
An00g07932_at	An18g04160	strong similarity to dihydroxy-acid dehydratase Ilv3 - Saccharomyces cerevisiae
An00g10198_at	An18g04810	strong similarity to PATENTPROT R59792 exo-polygalacturonase PGX - Aspergillus tubingensis
An00g06829_at	An18g05120	strong similarity to a subunit of succinyl-CoA:benzylsuccinate CoA-transferase bbsF- Thauera aromatica
An00g06971_at	An18g05480	strong similarity to alcohol oxidase AOX1 - Pichia pastoris
An00g09556_at	An18g06310	strong similarity to L-fucose permease fucP - Escherichia coli
An00g08560_at	An18g06650	strong similarity to heat shock protein 30 hsp30 - Aspergillus nidulans

Supplementary table 6. Raw, and normalized expression values of the genes , identified in a transcriptional cluster on all tested carbon sources. Control values derived by GeneSpring for the normalization of the raw data are indicated. Affymetrix flags assigning the significance of the detected signal as present, absent or marginal are shown as P, A, and M, respectively. The t-test p-values corresponding to the change in signal strength relative to the median of the gene across conditions (differential expression) are indicated as well.

	rhamnose 2 hours					rhamnose 4 hours				
Gene Name	normalized signal	control value	raw signal	t-test p-value	flags	normalized signal	control value	raw signal	t-test p-value	flags
An14g04370	0.336	136.1	45.7	0.441	A	0.782	127.8	99.9	0.682	A
An02g07720	7.856	593.1	4,660	0.0147	P	2.242	556.7	1,248	0.126	P
An02g07710	5.203	266.2	1,385	0.0277	P	0.93	249.9	232.4	0.88	P
An04g00790	0.908	122.3	111	0.849	P	0.931	114.8	106.9	0.883	P
An02g12450	0.458	140.5	64.3	0.474	A	0.479	131.9	63.2	0.477	A
An02g02540	1.09	105.9	115.4	0.842	A	0.155	99.39	15.4	0.407	A
An11g01120	0.663	1,041	690.3	0.577	P	0.0145	977	14.2	0.344	A
An08g01710	1.066	478.5	510	0.882	P	0.296	449.1	133.1	0.427	P
An11g04040	0.366	27.62	10.1	0.493	A	0.691	25.92	17.9	0.646	A
An03g06740	0.471	11.05	5.2	0.634	A	0.444	10.37	4.6	0.612	A
An12g07500	0.444	11.05	4.9	0.621	A	0.386	10.37	4	0.586	A
An03g01620	0.571	190	108.5	0.522	A	0.757	178.3	135	0.655	A
An16g05390	0.757	498.4	377.1	0.657	P	0.14	467.8	65.6	0.402	A
An07g00780	0.751	43.78	32.9	0.677	A	9.427	41.09	387.4	0.0121	A
An14g04280	0.988	308.4	304.8	0.979	P	1.012	289.5	292.9	0.979	P
	rhamnose 8 hours					rhamnose 24 hours				
Gene Name	normalized signal	control value	raw signal	t-test p-value	flags	normalized signal	control value	raw signal	t-test p-value	flags
An14g04370	1.001	126.1	126.2	0.998	A	1.182	108.1	127.8	0.777	A
An02g07720	2.135	549.2	1,173	0.119	P	0.334	471	157.4	0.52	P
An02g07710	1.07	246.6	263.8	0.851	P	0.536	211.4	113.4	0.604	P
An04g00790	0.77	113.3	87.2	0.627	P	1.261	97.13	122.5	0.686	P
An02g12450	0.818	130.1	106.5	0.685	A	0.658	111.6	73.4	0.675	A
An02g02540	0.172	98.07	16.9	0.386	A	1.085	84.09	91.2	0.897	A
An11g01120	0.358	963.9	345.4	0.4	A	0.174	826.6	143.8	0.465	A
An08g01710	0.377	443.1	167.1	0.404	P	1.674	380	636	0.363	P
An11g04040	0.626	25.58	16	0.589	A	0.556	21.93	12.2	0.631	A
An03g06740	0.264	10.23	2.7	0.545	A	1.003	8.772	8.8	0.997	A
An12g07500	0.557	10.23	5.7	0.681	A	0.353	8.772	3.1	0.587	A
An03g01620	0.957	175.9	168.4	0.913	A	0.726	150.8	109.5	0.724	A
An16g05390	0.666	461.5	307.4	0.526	P	0.206	395.8	81.7	0.477	A

An07g00780	0.935	40.55	37.9	0.886	A	0.253	34.77	8.8	0.497	A
An14g04280	0.917	285.6	261.8	0.835	P	0.344	244.9	84.2	0.523	P
	xylose 2 hours					xylose 4 hours				
	normalized signal	control value	raw signal	t-test p-value	flags	normalized signal	control value	raw signal	t-test p-value	flags
An14g04370	0.845	123.6	104.4	0.796	A	0.269	99.11	26.7	0.451	A
An02g07720	0.193	538.4	103.9	0.443	A	0.0757	431.8	32.7	0.399	A
An02g07710	0.44	241.7	106.4	0.513	P	0.369	193.8	71.6	0.476	P
An04g00790	0.831	111	92.3	0.781	P	0.727	89.06	64.7	0.664	P
An02g12450	0.785	127.6	100.2	0.733	A	0.814	102.3	83.3	0.75	A
An02g02540	0.185	96.14	17.8	0.442	A	0.227	77.1	17.5	0.442	A
An11g01120	5.279	945	4,989	0.0329	P	1.297	757.9	983.1	0.566	P
An08g01710	6.868	434.4	2,984	0.0219	P	0.643	348.4	224.1	0.599	P
An11g04040	0.794	25.07	19.9	0.759	A	2.586	20.11	52	0.122	A
An03g06740	0.279	10.03	2.8	0.532	A	0.373	8.043	3	0.57	A
An12g07500	0.798	10.03	8	0.815	A	0.995	8.043	8	0.995	A
An03g01620	32.22	172.5	5,556	0.0026	P	4.826	138.3	667.4	0.0358	P
An16g05390	0.122	452.5	55	0.422	A	0.183	362.9	66.4	0.431	A
An07g00780	0.629	39.75	25	0.615	A	0.656	31.88	20.9	0.62	A
An14g04280	0.349	280	97.7	0.483	P	0.353	224.6	79.3	0.471	A
	xylose 8 hours					xylose 24 hours				
	normalized signal	control value	raw signal	t-test p-value	flags	normalized signal	control value	raw signal	t-test p-value	flags
An14g04370	0.569	104.8	59.6	0.505	A	0.734	155.1	113.8	0.742	A
An02g07720	0.184	456.5	83.9	0.402	A	0.119	675.7	80.2	0.449	A
An02g07710	0.344	204.9	70.4	0.426	P	0.393	303.3	119.1	0.552	P
An04g00790	0.688	94.13	64.8	0.583	P	1.44	139.4	200.7	0.532	P
An02g12450	0.748	108.1	80.9	0.635	A	1.122	160.1	179.7	0.857	A
An02g02540	0.16	81.5	13	0.402	A	0.276	120.7	33.3	0.51	A
An11g01120	1.599	801.1	1,281	0.275	P	0.131	1,186	155.6	0.455	A
An08g01710	0.8	368.3	294.5	0.688	P	0.836	545.2	456	0.829	P
An11g04040	0.974	21.26	20.7	0.962	A	0.728	31.47	22.9	0.743	A
An03g06740	0.353	8.501	3	0.585	A	0.453	12.59	5.7	0.609	A
An12g07500	0.729	8.501	6.2	0.787	A	0.373	12.59	4.7	0.575	A
An03g01620	9.52	146.2	1,392	0.0104	P	0.407	216.4	88.1	0.558	A
An16g05390	0.741	383.6	284.2	0.625	P	0.0535	567.8	30.4	0.411	A
An07g00780	0.255	33.7	8.6	0.434	A	0.473	49.89	23.6	0.589	A
An14g04280	0.461	237.4	109.5	0.457	P	0.292	351.4	102.5	0.515	M

	sorbitol 2 hours					sorbitol 4 hours				
	normalized signal	control value	raw signal	t-test p-value	flags	normalized signal	control value	raw signal	t-test p-value	flags
An14g04370	0.696	121.4	84.5	0.686	A	0.961	175.1	168.3	0.936	A
An02g07720	0.102	529	54.2	0.429	A	0.683	763.1	521	0.607	P
An02g07710	0.313	237.5	74.3	0.499	P	0.702	342.6	240.5	0.622	P
An04g00790	0.665	109.1	72.6	0.664	A	0.68	157.4	107	0.607	A
An02g12450	0.667	125.3	83.6	0.665	A	0.766	180.8	138.4	0.681	A
An02g02540	0.257	94.45	24.3	0.482	A	0.266	136.3	36.2	0.437	A
An11g01120	0.104	928.4	96.8	0.43	A	1.148	1,339	1,537	0.751	P
An08g01710	0.367	426.8	156.5	0.517	P	0.331	615.7	203.6	0.449	P
An11g04040	0.857	24.63	21.1	0.837	A	2.395	35.54	85.1	0.137	A
An03g06740	0.548	9.852	5.4	0.646	A	0.612	14.21	8.7	0.69	A
An12g07500	1.086	9.852	10.7	0.91	A	0.359	14.21	5.1	0.567	A
An03g01620	0.645	169.4	109.3	0.65	A	0.851	244.4	207.9	0.777	A
An16g05390	0.0486	444.5	21.6	0.398	A	1.495	641.3	958.9	0.365	P
An07g00780	1.065	39.05	41.6	0.916	A	1.395	56.34	78.6	0.463	A
An14g04280	0.369	275.1	101.5	0.518	P	1.648	396.9	653.9	0.282	P
	sorbitol 8 hours					sorbitol 24 hours				
	normalized signal	control value	raw signal	t-test p-value	flags	normalized signal	control value	raw signal	t-test p-value	flags
An14g04370	0.519	141.5	73.5	0.431	A	0.795	103	81.9	0.782	A
An02g07720	0.818	616.7	504.6	0.661	P	0.261	448.7	117.1	0.494	A
An02g07710	0.765	276.8	211.7	0.596	P	0.539	201.4	108.6	0.604	P
An04g00790	0.624	127.2	79.3	0.484	P	1.069	92.53	98.9	0.916	P
An02g12450	0.87	146.1	127.1	0.744	A	0.868	106.3	92.3	0.852	A
An02g02540	0.159	110.1	17.5	0.375	A	1.716	80.12	137.5	0.344	A
An11g01120	0.852	1,082	922.6	0.711	P	0.234	787.5	184.1	0.485	A
An08g01710	0.623	497.6	310.2	0.476	P	1.175	362	425.4	0.784	P
An11g04040	0.752	28.72	21.6	0.688	A	1.685	20.89	35.2	0.388	A
An03g06740	0.331	11.49	3.8	0.58	A	1.161	8.357	9.7	0.865	A
An12g07500	0.548	11.49	6.3	0.684	A	0.443	8.357	3.7	0.641	A
An03g01620	1.086	197.5	214.6	0.805	A	0.381	143.7	54.8	0.536	A
An16g05390	1.227	518.2	635.7	0.539	P	0.274	377.1	103.2	0.499	A
An07g00780	0.868	45.53	39.5	0.777	A	0.356	33.12	11.8	0.535	A
An14g04280	0.981	320.7	314.5	0.957	P	0.363	233.3	84.7	0.529	A
	fructose 2 hours					fructose 4 hours				
	normalized signal	control value	raw signal	t-test p-value	flags	normalized signal	control value	raw signal	t-test p-value	flags

An14g04370	0.177	133.6	23.7	0.419	A	1.138	176.7	201.1	0.729	A
An02g07720	0.188	582.3	109.3	0.419	A	1.166	769.9	897.6	0.675	P
An02g07710	0.345	261.4	90.2	0.451	P	1.29	345.6	445.7	0.494	P
An04g00790	0.629	120.1	75.5	0.569	M	0.494	158.8	78.5	0.455	P
An02g12450	0.552	138	76.2	0.525	A	0.991	182.4	180.7	0.982	A
An02g02540	0.175	104	18.2	0.419	A	0.25	137.5	34.4	0.402	A
An11g01120	0.592	1,022	604.7	0.544	A	1.429	1,351	1,931	0.358	P
An08g01710	0.31	469.8	145.6	0.443	P	0.388	621.1	240.8	0.419	P
An11g04040	0.771	27.12	20.9	0.722	A	0.681	35.85	24.4	0.632	A
An03g06740	0.369	10.85	4	0.577	A	0.753	14.34	10.8	0.8	A
An12g07500	0.793	10.85	8.6	0.819	A	2.971	14.34	42.6	0.153	A
An03g01620	0.113	186.5	21.1	0.404	A	1.043	246.6	257.1	0.914	P
An16g05390	0.341	489.3	166.8	0.449	A	2.103	646.9	1,361	0.13	P
An07g00780	0.689	42.99	29.6	0.629	A	3.371	56.83	191.6	0.058	A
An14g04280	0.388	302.8	117.5	0.462	P	2.218	400.4	888.1	0.116	P
			fructose 8 hours					fructose 24 hours		
	normalized signal	control value	raw signal	t-test p-value	flags	normalized signal	control value	raw signal	t-test p-value	flags
An14g04370	0.574	122.9	70.5	0.447	A	0.471	138.6	65.3	0.56	A
An02g07720	0.268	535.4	143.5	0.366	A	0.142	603.9	85.5	0.446	A
An02g07710	0.402	240.3	96.6	0.384	P	0.511	271.1	138.4	0.577	P
An04g00790	0.619	110.4	68.3	0.473	P	0.904	124.5	112.6	0.884	P
An02g12450	1.113	126.8	141.2	0.743	A	1.009	143.1	144.4	0.988	A
An02g02540	0.163	95.6	15.6	0.371	A	1.331	107.8	143.5	0.596	A
An11g01120	0.724	939.7	680.6	0.543	P	0.258	1,060	273	0.484	M
An08g01710	0.412	432	178	0.385	P	0.937	487.2	456.4	0.922	P
An11g04040	0.75	24.93	18.7	0.683	A	0.562	28.12	15.8	0.629	A
An03g06740	0.231	9.972	2.3	0.535	A	0.453	11.25	5.1	0.642	A
An12g07500	0.542	9.972	5.4	0.68	A	0.293	11.25	3.3	0.568	A
An03g01620	0.453	171.5	77.7	0.398	A	0.484	193.4	93.7	0.566	A
An16g05390	0.621	449.9	279.5	0.465	P	0.23	507.5	116.7	0.475	A
An07g00780	0.754	39.52	29.8	0.633	A	0.191	44.58	8.5	0.47	A
An14g04280	0.437	278.4	121.6	0.391	P	0.356	314.1	111.7	0.515	M
			galacturonic acid 2 hours					galacturonic acid 4 hours		
	normalized signal	control value	raw signal	t-test p-value	flags	normalized signal	control value	raw signal	t-test p-value	flags
An14g04370	19	199.8	3,798	0.0039	P	20.62	196.5	4,052	0.00408	P
An02g07720	13.82	870.7	12,031	0.00593	P	15.08	856.2	12,915	0.00617	P

An02g07710	34.85	390.9	13,623	0.00175	P	25.93	384.4	9,966	0.003	P
An04g00790	18.43	179.6	3,310	0.00408	P	10.34	176.6	1,826	0.0106	P
An02g12450	54.64	206.3	11,272	0.000998	P	27.5	202.9	5,579	0.00279	P
An02g02540	29.28	155.5	4,552	0.00223	P	10.22	152.9	1,562	0.0108	P
An11g01120	5.914	1,528	9,038	0.0205	P	5.739	1,503	8,624	0.0252	P
An08g01710	11.74	702.5	8,248	0.00745	P	5.562	690.8	3,842	0.0264	P
An11g04040	11.35	40.55	460.2	0.0102	A	12.86	39.87	512.8	0.00921	A
An03g06740	613.2	16.22	9,944	0.000127	P	306.2	15.95	4,883	0.000278	P
An12g07500	609.4	16.22	9,883	0.000128	P	255.7	15.95	4,078	0.000344	P
An03g01620	15.06	278.9	4,200	0.00531	P	6.5	274.2	1,782	0.0208	P
An16g05390	22.36	731.7	16,359	0.0031	P	21.36	719.5	15,370	0.00387	P
An07g00780	25.47	64.28	1,637	0.00295	P	23.1	63.21	1,460	0.00377	P
An14g04280	24.41	452.8	11,051	0.00277	P	26.53	445.3	11,814	0.00291	P
	galacturonic acid 8 hours					galacturonic acid 24 hours				
	normalized signal	control value	raw signal	t-test p-value	flags	normalized signal	control value	raw signal	t-test p-value	flags
An14g04370	22.81	149.1	3,402	0.00306	P	1.539	161.1	247.9	0.446	A
An02g07720	17.79	649.8	11,561	0.00419	P	0.834	701.7	585.3	0.82	P
An02g07710	28.42	291.7	8,290	0.00227	P	1.524	315	480	0.456	P
An04g00790	12.58	134	1,685	0.0069	P	1.713	144.7	247.9	0.349	P
An02g12450	19.6	153.9	3,018	0.00374	P	1.234	166.2	205.2	0.719	P
An02g02540	23.91	116	2,774	0.00292	P	0.915	125.3	114.7	0.904	A
An11g01120	6.837	1,140	7,796	0.0163	P	0.388	1,232	478.4	0.542	P
An08g01710	7.321	524.2	3,838	0.0147	P	1.084	566.2	613.6	0.898	P
An11g04040	9.353	30.26	283	0.0142	A	1.692	32.68	55.3	0.378	A
An03g06740	315.1	12.1	3,813	0.000308	P	20.51	13.07	268	0.00828	P
An12g07500	352.2	12.1	4,262	0.000271	P	13.9	13.07	181.6	0.014	A
An03g01620	5.678	208.1	1,182	0.0219	P	0.503	224.7	113.1	0.591	A
An16g05390	24.67	546	13,470	0.00272	P	1.508	589.7	889	0.467	P
An07g00780	20.97	47.97	1,006	0.00393	P	2.293	51.8	118.8	0.19	A
An14g04280	31.9	337.9	10,778	0.00195	P	2.16	364.9	788.4	0.209	P
	polygalacturonic acid 2 hours					polygalacturonic acid 4 hours				
	normalized signal	control value	raw signal	t-test p-value	flags	normalized signal	control value	raw signal	t-test p-value	flags
An14g04370	6.759	121.7	822.7	0.0198	P	10.56	120.3	1,271	0.00962	P
An02g07720	17.58	530.3	9,324	0.00504	P	11.76	524.3	6,168	0.00818	P
An02g07710	31.89	238.1	7,592	0.00231	P	17.84	235.3	4,199	0.00463	P
An04g00790	14.33	109.4	1,568	0.00673	P	7.101	108.1	767.7	0.0172	P

An02g12450	58.74	125.6	7,381	0.00107	P	13.99	124.2	1,738	0.00649	P
An02g02540	11.97	94.69	1,134	0.00867	P	6.57	93.61	615	0.0195	P
An11g01120	10.17	930.8	9,469	0.0108	P	3.041	920.1	2,798	0.0696	P
An08g01710	24.88	427.9	10,646	0.00318	P	0.853	423	360.9	0.769	P
An11g04040	15.01	24.7	370.8	0.00749	A	10.85	24.41	265	0.0112	A
An03g06740	375.7	9.877	3,711	0.000221	P	207.8	9.764	2,029	0.000432	P
An12g07500	613.4	9.877	6,059	0.000125	P	230.6	9.764	2,252	0.000382	P
An03g01620	55.14	169.9	9,367	0.00115	P	1.345	167.9	225.8	0.475	P
An16g05390	25.48	445.7	11,357	0.00308	P	21.66	440.6	9,541	0.00357	P
An07g00780	2.112	39.15	82.7	0.168	A	9.984	38.7	386.4	0.0113	A
An14g04280	25.77	275.8	7,107	0.00304	P	22.74	272.6	6,200	0.00335	P
	polygalacturonic acid 8 hours					polygalacturonic acid 24 hours				
	normalized signal	control value	raw signal	t-test p-value	flags	normalized signal	control value	raw signal	t-test p-value	flags
An14g04370	7.863	136.1	1,070	0.0117	P	1.516	112.7	170.8	0.366	A
An02g07720	14.21	593.1	8,428	0.00492	P	1.914	490.9	939.6	0.202	P
An02g07710	20.16	266.2	5,368	0.00308	P	2.537	220.4	559.1	0.109	P
An04g00790	10.12	122.3	1,238	0.00813	P	2.494	101.2	252.5	0.115	P
An02g12450	11.88	140.5	1,669	0.00644	P	1.181	116.3	137.3	0.712	A
An02g02540	9.932	105.9	1,052	0.00843	P	1.804	87.64	158.1	0.236	A
An11g01120	4.877	1,041	5,076	0.024	P	0.422	861.5	363.3	0.483	P
An08g01710	3.685	478.5	1,763	0.0386	P	1.063	396	421.1	0.896	P
An11g04040	9.045	27.62	249.8	0.0134	A	0.936	22.86	21.4	0.919	A
An03g06740	250.9	11.05	2,772	0.000372	P	8.74	9.142	79.9	0.0299	A
An12g07500	214.1	11.05	2,366	0.000449	P	10.31	9.142	94.3	0.0236	P
An03g01620	3.421	190	649.9	0.0444	P	0.771	157.2	121.2	0.696	P
An16g05390	22.07	498.4	11,000	0.00272	P	1.579	412.5	651.3	0.326	P
An07g00780	13.86	43.78	606.8	0.00609	P	5.594	36.24	202.7	0.0302	A
An14g04280	24.5	308.4	7,558	0.00238	P	2.583	255.3	659.3	0.106	P
	sugar beet pectin 2 hours					sugar beet pectin4 hours				
	normalized signal	control value	raw signal	t-test p-value	flags	normalized signal	control value	raw signal	t-test p-value	flags
An14g04370	2.788	112.7	314.3	0.0943	A	56.42	101.3	5,715	0.00116	P
An02g07720	15.91	491.2	7,813	0.00617	P	16.31	441.3	7,199	0.00576	P
An02g07710	26.08	220.5	5,750	0.00319	P	22.67	198.1	4,491	0.00371	P
An04g00790	7.724	101.3	782.4	0.0173	P	13.99	91	1,273	0.00717	P
An02g12450	23.74	116.4	2,763	0.00363	P	59.97	104.5	6,269	0.00108	P
An02g02540	5.847	87.7	512.8	0.0265	P	19.1	78.79	1,505	0.00471	P

An11g01120	7.006	862.1	6,040	0.0199	P	9.712	774.5	7,521	0.0119	P
An08g01710	16.65	396.3	6,598	0.0058	P	22.51	356	8,015	0.00374	P
An11g04040	2.387	22.87	54.6	0.145	A	12.27	20.55	252.2	0.00995	A
An03g06740	167.6	9.149	1,534	0.000541	P	498.6	8.219	4,098	0.000153	P
An12g07500	565.6	9.149	5,175	0.00013	P	507.2	8.219	4,169	0.00015	P
An03g01620	47.22	157.3	7,429	0.0015	P	37.54	141.3	5,305	0.00193	P
An16g05390	22.06	412.8	9,105	0.00398	P	27.32	370.8	10,131	0.0029	P
An07g00780	0.565	36.26	20.5	0.566	A	5.271	32.58	171.7	0.032	A
An14g04280	21.57	255.5	5,510	0.0041	P	27.95	229.5	6,414	0.00282	P
	sugar beet pectin 8 hours					sugar beet pectin 24 hours				
	normalized signal	control value	raw signal	t-test p-value	flags	normalized signal	control value	raw signal	t-test p-value	flags
An14g04370	12	102.7	1,232	0.00838	P	2.699	102.4	276.3	0.107	M
An02g07720	17.11	447.3	7,654	0.00509	P	6.18	446	2,756	0.0257	P
An02g07710	24.59	200.8	4,938	0.00315	P	8.544	200.2	1,710	0.0158	P
An04g00790	18.66	92.26	1,722	0.00459	P	5.477	91.98	503.8	0.0314	P
An02g12450	38.3	106	4,059	0.00179	P	1.863	105.7	196.9	0.237	P
An02g02540	34.87	79.87	2,785	0.00203	P	7.028	79.63	559.7	0.0215	P
An11g01120	6.288	785.1	4,937	0.0214	P	1.257	782.8	983.6	0.633	P
An08g01710	16.92	360.9	6,108	0.00517	P	2.847	359.8	1,024	0.096	P
An11g04040	11.53	20.83	240.1	0.011	A	4.81	20.77	99.9	0.0475	A
An03g06740	846.3	8.332	7,051	9.20E-05	P	64.14	8.307	532.8	0.00229	P
An12g07500	642.3	8.332	5,351	0.000126	P	37.45	8.307	311.1	0.0045	P
An03g01620	26	143.3	3,726	0.00293	P	2.639	142.8	377	0.112	P
An16g05390	22.86	375.9	8,595	0.00346	P	3.571	374.8	1,338	0.0633	P
An07g00780	3.352	33.02	110.7	0.0669	A	14.73	32.92	485.1	0.00806	P
An14g04280	20.48	232.6	4,764	0.00401	P	5.414	231.9	1,256	0.0317	P

fructose pre-culture					
Gene Name	normalized signal	control value	raw signal	t-test p-value	flags
An14g04370	0.999	110.7	110.6	0.998	A
An02g07720	0.267	482.4	128.7	0.409	A
An02g07710	0.584	216.6	126.5	0.505	P
An04g00790	0.672	99.49	66.9	0.566	M
An02g12450	0.931	114.3	106.4	0.875	A

An02g02540	0.169	86.14	14.6	0.4	A
An11g01120	0.471	846.7	399	0.455	P
An08g01710	0.359	389.2	139.9	0.425	P
An11g04040	0.712	22.47	16	0.659	A
An03g06740	0.245	8.985	2.2	0.531	A
An12g07500	0.779	8.985	7	0.815	A
An03g01620	0.319	154.5	49.3	0.418	A
An16g05390	0.758	405.4	307.4	0.637	P
An07g00780	0.601	35.61	21.4	0.545	A
An14g04280	0.438	250.9	109.9	0.445	P

Supplementary table 7. *A. niger* genes identified as differentially expressed. Normalized signal shows the data values for each gene after RMA preprocessing and normalization of the raw data averaged over the values obtained from each replicate experiment for the given condition.

Loccus tag	normalized signal fructose	normalized signal galacturonic acid	ANOVA p-value	Description	*A. nidulans* ortholog
An01g00330	0.443 (0.393 to 0.512)	2.381 (1.488 to 3.559)	0.01	alpha-l-arabinofuranosidase a precursor abfA - A. niger	
An01g00560	1.412 (1.379 to 1.454)	0.518 (0.403 to 0.621)	0.01	strong similarity to microsomal signal peptidase SPC21 - Canis familaris	AN10354.3
An01g01550	0.569 (0.507 to 0.659)	1.51 (1.341 to 1.69)	0	strong similarity to catalase cat1 - A. fumigatus	AN9339.3
An01g01620	1.658 (1.537 to 1.894)	0.343 (0.251 to 0.463)	0	strong similarity to high-affinity zinc transporter ZRT1 - .S cerevisiae	AN8337.3
An01g01630	0.226 (0.129 to 0.357)	2.09 (1.643 to 2.485)	0.01	strong similarity to hypothetical protein An09g00510 - A. niger	
An01g01720	1.831 (1.326 to 3.23)	0.604 (0.543 to 0.674)	0.03	strong similarity to bleomycin hydrolase BLH1 - .S cerevisiae	AN6399.3
An01g01740	1.434 (1.112 to 1.886)	0.709 (0.537 to 0.888)	0.04	weak similarity to troponin C - C. elegans	
An01g01750	2.193 (1.855 to 2.827)	0.138 (0.131 to 0.145)	0	similarity to lysosomal protease CLN2 - R. norvegicus	
An01g01820	2.02 (1.578 to 2.313)	0.319 (0.25 to 0.422)	0	catalase R catR - A. niger	
An01g01830	1.433 (1.376 to 1.539)	0.528 (0.45 to 0.624)	0	strong similarity to catalase/peroxidase cpeB - Streptomyces reticuli	AN7388.3
An01g03900	2.177 (1.127 to 3.501)	0.615 (0.432 to 0.873)	0.05	strong similarity to ATP-binding cassette multidrug transport protein atrA – E. nidulans	
An01g04260	1.771 (1.17 to 2.251)	0.678 (0.453 to 0.83)	0.04	strong similarity to cytosine deaminase FCA1 - C. albicans	
An01g04320	1.631 (1.403 to 2.145)	0.516 (0.394 to 0.597)	0.01	strong similarity to COPII vesicle coat component protein Erv46p - .S cerevisiae	AN2738.3
An01g04600	1.414 (1.272 to 1.688)	0.535 (0.305 to 0.728)	0.04	PDI related protein A prpA - A. niger	AN0248.3
An01g05820	1.531 (1.307 to 2.054)	0.647 (0.592 to 0.693)	0.01	strong similarity to H.protocatechuate operon enzyme hpcE - E. coli	

An01g05960	0.365 (0.342 to 0.391)	1.627 (1.609 to 1.651)	0	similarity to cyanovirin-N CV-N - Nostoc ellipsosporum	AN3481.3
An01g06150	0.484 (0.416 to 0.628)	3.017 (1.372 to 5.155)	0.02	strong similarity to phe-inhibited DAHP synthase aroG - A. nidulans	AN0354.3
An01g06330	0.665 (0.557 to 0.777)	1.479 (1.223 to 1.704)	0.01	similarity to transcriptional activator UPC2 - .S cerevisiae	AN0364.3
An01g06670	1.369 (1.251 to 1.52)	0.567 (0.337 to 0.749)	0.04	strong similarity to peptidylprolyl isomerase FKBP-21 – N. crassa	AN8343.3
An01g07150	1.917 (1.257 to 2.568)	0.631 (0.471 to 0.743)	0.02	strong similarity to cycloheximide resistance protein CYHR - C. maltosa	
An01g07300	0.713 (0.613 to 0.817)	1.649 (1.183 to 2.064)	0.02	strong similarity to gluconokinase gntV - E. coli	AN3641.3
An01g07730	0.151 (0.129 to 0.188)	2.934 (1.812 to 4.107)	0	weak similarity to TcSL-2 protein precursor - Toxocara cani	
An01g08430	1.657 (1.519 to 1.949)	0.455 (0.432 to 0.481)	0	strong similarity to methylmalonate-semialdehyde dehydrogenase MMSDH - R. norvegicus	AN3591.3
An01g08670	1.422 (1.392 to 1.472)	0.483 (0.392 to 0.608)	0	similarity to nucleolin C23 - Cricetulus griseus	
An01g09270	0.525 (0.322 to 0.704)	1.338 (1.296 to 1.369)	0.03	strong similarity to isocitrate lyase acuD - Emericella nidulans	AN5634.3
An01g09410	0.486 (0.468 to 0.51)	1.632 (1.49 to 1.86)	0	similarity to Kruppel-like transcription factor biklf - Danio rerio	AN5659.3
An01g09520	1.538 (1.301 to 1.674)	0.677 (0.662 to 0.699)	0	strong similarity to mitogen-activated protein kinase MPKA - A. nidulans	AN5666.3
An01g09610	1.479 (1.326 to 1.777)	0.46 (0.364 to 0.674)	0.01	strong similarity to gamma subunit of translation elongation factor eEF-1 - H. sapiens	AN9304.3
An01g09780	1.379 (1.139 to 1.675)	0.514 (0.394 to 0.861)	0.04	strong similarity to D-lactate dehydrogenase ldhA - Escherischia coli (truncated ORF)	AN0628.3
An01g09940	2.562 (1.178 to 4.562)	0.666 (0.579 to 0.822)	0.05	strong similarity to glyoxysomal citrate synthase - Cucurbita sp.	
An01g10190	0.632 (0.535 to 0.792)	1.404 (1.208 to 1.55)	0.01	similarity to mitochondrial tricarboxylate carrier - R. sp.	AN10445.3
An01g10350	0.172 (0.147 to 0.206)	2.194 (1.794 to 2.677)	0	strong similarity to secreted beta-galactosidase lacA - A. niger	AN0980.3
An01g10580	2.3 (1.639 to 3.306)	0.291 (0.243 to 0.361)	0	strong similarity to ribonuclease T2 precursor rntB - A. oryzae	
An01g11320	1.562 (1.291 to 2.134)	0.586 (0.464 to 0.709)	0.02	similarity to dopa decarboxylase 3 DDC3 patent WO9960136-A1 - A. oryzae	
An01g11330	1.848 (1.354 to 3.189)	0.524 (0.432 to 0.646)	0.02	strong similarity to hypothetical protein An06g02150 - A. niger	AN0021.3
An01g11340	2.24 (1.651 to 3.941)	0.313 (0.279 to 0.349)	0.01	strong similarity to methionyl aminopeptidase MAP1 - .S cerevisiae	
An01g11520	0.624 (0.611 to 0.65)	1.405 (1.35 to 1.508)	0	polygalacturonase pgaI - A. niger (mRNA)	
An01g11580	1.574 (1.321 to 1.951)	0.635 (0.556 to 0.679)	0.01	similarity to hypothetical protein An01g11570 - A. niger	
An01g11640	1.584 (1.491 to 1.653)	0.424 (0.301 to 0.509)	0.01	strong similarity to high affinity low capacity ammonia permease MEP2 - .S cerevisiae	AN10097.3
An01g11680	1.633 (1.214 to 2.589)	0.705 (0.648 to 0.786)	0.04	strong similarity to carboxy-cis cis-muconate cyclase CMLE - Neurospora crassa	AN1151.3
An01g12150	0.709 (0.564 to 0.807)	1.733 (1.193 to 2.264)	0.03	beta-galactosidase lacA+(EC 3.2.1.23) - A. niger	AN0756.3
An01g12220	0.474 (0.446 to 0.524)	1.531 (1.476 to 1.643)	0	weak similarity to UDPglucose 4-epimerase galE - E. coli	AN0746.3
An01g12310	1.475 (1.193 to 1.74)	0.698 (0.648 to 0.807)	0.01	similarity to hydroxyquinol 1 2-dioxygenase BAA82713.1 - Arthrobacter sp.	AN0764.3
An01g12470	0.723 (0.614 to 0.788)	1.527 (1.212 to 2.219)	0.03	similarity to Atu related protein - Neurospora crassa	AN0781.3
An01g13290	1.419 (1.333 to 1.481)	0.47 (0.311 to 0.667)	0.02	strong similarity to choline permease HNM1 - .S cerevisiae	AN0856.3
An01g13630	1.469 (1.33 to 1.764)	0.596 (0.486 to 0.67)	0.01	weak similarity to the flavoredoxin flr - Desulfovibrio gigas	AN0876.3
An01g13810	1.588 (1.244 to 2.425)	0.74 (0.712 to 0.756)	0.04	strong similarity to urea amidolyase DUR1 2 - .S cerevisiae	AN0887.3
An01g14100	1.386 (1.298 to 1.517)	0.6 (0.533 to 0.702)	0	weak similarity to stress protein Herp - Mus musculus	AN0909.3
An01g14350	0.73 (0.647 to 0.798)	2.288 (1.202 to 4.012)	0.05	similarity to the probable transcription factor Upc2 - .S cerevisiae (truncated ORF)	
An01g14360	0.695 (0.663 to 0.741)	1.763 (1.259 to 2.098)	0.01	strong similarity to hypothetical protein 68B2.10 - Neurospora crassa (truncated ORF)	AN0928.3
An01g14390	1.504 (1.23 to 1.805)	0.686 (0.565 to 0.77)	0.01	strong similarity to the beta-transducin-like protein HET-E - Podospora anserina	AN10921.3

An01g14440	0.836 (0.788 to 0.877)	1.698 (1.123 to 2.171)	0.04	similarity to extracellular matrix protein lustrin A - Haliotis rufescens (truncated ORF)	
An02g00140	0.171 (0.147 to 0.195)	1.879 (1.805 to 1.939)	0	strong similarity to xylan 1 4-beta-xylosidase xynB - B. subtilis	AN6751.3
An02g00180	0.531 (0.482 to 0.586)	1.471 (1.414 to 1.564)	0	strong similarity to phosphate-repressible phosphate permease pho-4 – N. crassa	AN3781.3
An02g00890	0.695 (0.639 to 0.727)	1.405 (1.273 to 1.659)	0.01	strong similarity to phosphoserine transaminase SER1 - .S cerevisiae	AN10298.3
An02g01480	1.883 (1.666 to 2.199)	0.242 (0.13 to 0.334)	0.01	strong similarity to fluconazole resistance protein FLU1 - C. albicans	AN6277.3
An02g02060	1.394 (1.242 to 1.739)	0.668 (0.598 to 0.758)	0.01	strong similarity to alcohol dehydrogenase of patent EP 0845532-A 19	AN10793.3
An02g02540	0.187 (0.153 to 0.239)	1.856 (1.761 to 1.908)	0	strong similarity to acetyl-esterase I from patent R63066 - A. aculeatus	AN5930.3
An02g02660	0.554 (0.457 to 0.65)	1.528 (1.35 to 1.867)	0.01	strong similarity to the protein required for filamentous growth cell polarity (and cellular elongation Dfg5 - .S cerevisiae) mRNA	AN0393.3
An02g03200	1.59 (1.201 to 1.938)	0.793 (0.788 to 0.799)	0.02	strong similarity to the hypothetical protein encoded by An04g09720 - A. niger	
An02g03240	1.569 (1.504 to 1.665)	0.446 (0.362 to 0.496)	0	strong similarity to UDP-N-acetylglucosamine--dolichyl-phosphate N-acetylglucosaminephosphotransferase Alg7 - .S cerevisiae	AN5888.3
An02g03320	1.355 (1.214 to 1.472)	0.674 (0.542 to 0.786)	0.01	strong similarity to peroxisomal acetyl-CoA C-acyltransferase - Yarrowia lipolytica	AN5878.3
An02g03540	1.602 (1.49 to 1.714)	0.178 (0.0987 to 0.51)	0.02	strong similarity to hexose transport protein HXT3 - .S cerevisiae	AN5860.3
An02g03590	1.379 (1.093 to 1.645)	0.647 (0.501 to 0.907)	0.04	strong similarity to UDP-glucose-hexose-1-phosphate uridylyltransferase GAL7 - .S cerevisiae	AN6182.3
An02g04000	0.675 (0.655 to 0.696)	1.383 (1.304 to 1.434)	0	strong similarity to pyruvate dehydrogenase kinase isoform 2 - Zea mays	AN6207.3
An02g04690	1.737 (1.657 to 1.845)	0.292 (0.229 to 0.343)	0	strong similarity to serine-type carboxypeptidase I cdpS - A. saitoi	
An02g05640	3.375 (1.744 to 9.635)	0.252 (0.246 to 0.256)	0.02	strong similarity to hypothetical protein SPAC11D3.03c - Schizo.S pombe	AN6335.3
An02g06150	0.589 (0.512 to 0.659)	1.648 (1.341 to 2.355)	0.01	strong similarity to branched-chain amino acids aminotransferase BAT2 - .S cerevisiae	AN5957.3
An02g06320	1.634 (1.385 to 1.808)	0.427 (0.224 to 0.615)	0.03	strong similarity to hydroxymethylglutaryl-CoA synthase HMGS - .S cerevisiae	AN4923.3
An02g06910	1.577 (1.393 to 1.681)	0.537 (0.467 to 0.607)	0	strong similarity to probable membrane protein YIL108w - .S cerevisiae	AN4880.3
An02g07250	0.61 (0.513 to 0.827)	1.425 (1.173 to 1.926)	0.03	arginase argA - A. niger	AN2901.3
An02g07320	1.533 (1.392 to 1.615)	0.599 (0.588 to 0.608)	0	strong similarity to AU-specific RNA-binding protein / enoyl-CoA hydratase AUH - H. sapiens	AN2896.3
An02g07650	1.524 (1.306 to 1.842)	0.603 (0.522 to 0.694)	0.01	strong similarity to phosphoglucomutase pgmB - A. nidulans	AN2867.3
An02g07710	0.057 (0.046 to 0.073)	2.001 (1.927 to 2.097)	0	similarity to hypothetical protein Z2629 product - E. coli	AN2858.3
An02g07720	0.072 (0.056 to 0.11)	1.906 (1.893 to 1.927)	0	similarity to dihydrodipicolinate synthase dapA - E. coli	AN2859.3
An02g07890	0.539 (0.472 to 0.651)	1.883 (1.349 to 2.817)	0.01	transcription factor pacC - A. niger	AN2855.3
An02g08450	1.433 (1.279 to 1.707)	0.696 (0.682 to 0.721)	0.01	secretory gene NsfA - A. niger	AN3098.3
An02g08520	1.356 (1.135 to 1.886)	0.536 (0.421 to 0.865)	0.05	strong similarity to allantoicase alc - Neurospora crassa	AN3104.3
An02g08660	1.456 (1.426 to 1.475)	0.391 (0.185 to 0.574)	0.04	strong similarity to hypothetical protein H04M03.4 - C. elegans	AN3112.3
An02g09060	1.631 (1.37 to 2.211)	0.507 (0.369 to 0.63)	0.01	similarity disease associated protein kinase DAPK-3 from patent WO9858052-A2 - H. sapiens	AN3181.3
An02g09240	0.623 (0.43 to 0.792)	1.314 (1.208 to 1.409)	0.03	strong similarity to ribose-phosphate pyrophosphokinase Prs5 - .S cerevisiae	AN3169.3
An02g09530	2.691 (1.506 to 4.146)	0.468 (0.433 to 0.494)	0.01	similarity to integral membrane protein Pth11 - M. grisea strain G-11	
An02g09540	2.352 (1.444 to 3.068)	0.404 (0.289 to 0.556)	0.01	strong similarity to the choline transport protein Hnm1 - .S cerevisiae	
An02g10310	1.417 (1.291 to 1.623)	0.461 (0.344 to 0.709)	0.02	strong similarity to glycogen synthase - Neurospora crassa	AN8010.3

An02g10460	1.398 (1.289 to 1.623)	0.662 (0.581 to 0.711)	0.01	similarity to protein fragment SEQ ID NO: 63238 patent EP1033405-A2 – A. thaliana	AN8024.3
An02g10660	1.552 (1.227 to 2.356)	0.716 (0.677 to 0.773)	0.03	strong similarity to methionine adenosyltransferase regulatory beta subunit MAT II - H. sapiens	AN8032.3
An02g10980	0.474 (0.408 to 0.57)	1.549 (1.43 to 1.776)	0	weak similarity to hypothetical protein An07g02310 - A. niger	AN8942.3
An02g11190	1.757 (1.123 to 2.229)	0.73 (0.602 to 0.877)	0.04	strong similarity to protein fragment SEQ ID NO: 75556 of patent EP1033405-A2 - Zea mays	AN8050.3
An02g11320	0.303 (0.266 to 0.333)	1.812 (1.667 to 2.104)	0	strong similarity to the protein fragment SEQ ID NO: 15392 from patent EP1033405-A2 - Arabidopsis thaliana	AN2951.3
An02g11330	0.476 (0.387 to 0.559)	1.675 (1.441 to 1.846)	0	similarity to the developmentally regulated spherulin 4 - Physarum polycephalum	AN2952.3
An02g11360	0.328 (0.294 to 0.351)	1.801 (1.649 to 2.042)	0	strong similarity to the putative endo alpha-1 4 polygalactosaminidase precusor gene - Pseudomonas sp.	AN2953.3
An02g11390	0.279 (0.167 to 0.389)	1.773 (1.611 to 1.907)	0.01	strong similarity to hypothetical protein - Deinococcus radiodurans	AN2954.3
An02g11410	0.38 (0.335 to 0.423)	1.862 (1.577 to 2.028)	0	strong similarity to hypothetical protein An12g10440 - A. niger	
An02g12450	0.051 (0.044 to 0.064)	2.013 (1.936 to 2.156)	0	similarity to polygalacturonase XOPG1 - Lycopersicon esculentum	
An02g12620	0.579 (0.458 to 0.697)	1.457 (1.303 to 1.795)	0.01	strong similarity to copper metallochaperone COX17 - .S cerevisiae	AN4863.3
An02g12940	1.478 (1.289 to 1.762)	0.623 (0.555 to 0.711)	0.01	strong similarity to EST an_3344 EMBLEST:BE759052 - A. niger	
An02g13220	0.33 (0.293 to 0.379)	1.817 (1.621 to 2.104)	0	strong similarity to lysophospholipase phospholipase B - P. notatum	
An02g13250	1.383 (1.258 to 1.486)	0.683 (0.603 to 0.742)	0	strong similarity to water channel protein aquaporin 3 AQP3 - R. norvegicus	AN0830.3
An02g13410	1.391 (1.287 to 1.601)	0.552 (0.331 to 0.713)	0.04	similarity to acetyl-coenzyme A transporter AT-1 - H. sapiens	AN4836.3
An02g13450	1.389 (1.297 to 1.443)	0.641 (0.538 to 0.703)	0	weak similarity to CAP59 protein - Cryptococcus neoformans	
An02g13910	1.452 (1.291 to 1.577)	0.62 (0.476 to 0.709)	0.01	strong similarity to hypothetical protein SPBP4H10.20 - Schizo.S pombe	AN7492.3
An02g13920	1.383 (1.25 to 1.667)	0.667 (0.604 to 0.75)	0.01	strong similarity to ubiquitin thiolesterase YUH1 - .S cerevisiae	AN7491.3
An02g14380	1.653 (1.441 to 1.812)	0.509 (0.479 to 0.559)	0	hexokinase hxk - A. niger (mRNA)	AN7459.3
An02g14500	0.602 (0.485 to 0.716)	2.301 (1.284 to 3.467)	0.02	similarity to hypothetical protein An11g03520 - A. niger	
An02g14520	0.567 (0.347 to 0.763)	1.379 (1.237 to 1.487)	0.04	strong similarity to GMP synthase (glutamine-hydrolyzing) Gua1p - .S cerevisiae	AN5566.3
An02g14560	1.387 (1.25 to 1.698)	0.596 (0.398 to 0.75)	0.03	identity to oligosaccharyltransferase alpha subunit ostA - A. niger	AN7472.3
An03g00230	1.409 (1.247 to 1.621)	0.618 (0.447 to 0.753)	0.02	strong similarity to GABA permease gabA - A. nidulans	
An03g00460	0.37 (0.198 to 0.573)	2.201 (1.427 to 2.81)	0.02	strong similarity to the 6-hydroxy-D-nicotine oxidase 6-HDNO - Arthrobacter oxidans	AN10930.3
An03g00470	0.721 (0.693 to 0.756)	1.637 (1.244 to 1.941)	0.01	similarity to hypothetical protein An12g09260 - A. niger	
An03g00500	0.559 (0.556 to 0.565)	2.357 (1.435 to 3.21)	0.01	strong similarity to the diglycosidase related protein SEQ ID NO:10 of patent WO200018931-A1 - A. fumigatus	
An03g00640	1.945 (1.775 to 2.297)	0.109 (0.0477 to 0.225)	0.01	similarity to neutral amino acid permease mtr - Neurospora crassa (truncated ORF)	
An03g00660	1.951 (1.858 to 2.13)	0.117 (0.0941 to 0.142)	0	similarity to taurine dioxygenase tauD - E. coli	
An03g00680	1.813 (1.627 to 1.998)	0.329 (0.289 to 0.373)	0	strong similarity to multidrug resistance protein FNX1 - S pombe	
An03g00690	2.052 (1.515 to 3.326)	0.373 (0.303 to 0.485)	0.01	hypothetical protein (mRNA)	
An03g00720	1.366 (1.358 to 1.372)	0.402 (0.203 to 0.642)	0.04	strong similarity to expressed sequence tag seq id no:4295 of patent WO200056762-A2 - A. niger	AN11083.3
An03g00770	2.102 (1.346 to 2.717)	0.544 (0.467 to 0.654)	0.01	strong similarity to allergic bronchopulmonary aspergillosis allergen rAsp f 4 of patent	AN8461.3

				WO9828624-A1 - A. fumigatus	
An03g00840	0.681 (0.647 to 0.698)	1.665 (1.302 to 2.476)	0.02	strong similarity to cDNA clone an_3113 - A. niger	
An03g01010	2.322 (1.761 to 3.698)	0.188 (0.165 to 0.239)	0	strong similarity to lysosomal pepstatin insensitive protease (CLN2) - H. sapiens	
An03g01050	1.558 (1.223 to 1.791)	0.716 (0.647 to 0.777)	0.01	similarity to endo-beta-1 4-glucanase - B. polymyxa	AN9166.3
An03g01060	0.0952 (0.083 to 0.11)	4.045 (1.892 to 6.565)	0	hypothetical protein	
An03g01290	1.481 (1.204 to 2.215)	0.734 (0.649 to 0.796)	0.04	strong similarity to probable large secreted protein - Streptomyces coelicolor	AN9156.3
An03g01310	0.572 (0.374 to 0.747)	1.356 (1.253 to 1.484)	0.03	strong similarity to CTP synthetase (URA7) - .S cerevisiae	AN0490.3
An03g01620	0.424 (0.336 to 0.568)	1.878 (1.432 to 3.043)	0.01	strong similarity to high affinity glucose transporter HGT1 - Kluyveromyces lactis	AN9173.3
An03g01680	1.679 (1.53 to 1.994)	0.41 (0.375 to 0.47)	0	hypothetical protein (mRNA)	
An03g02270	1.536 (1.154 to 2.297)	0.733 (0.657 to 0.846)	0.04	similarity to the dihydropyridine receptor Fgalpha1S - Rana catesbeiana	
An03g03050	1.229 (1.151 to 1.299)	0.597 (0.394 to 0.849)	0.05	strong similarity to cDNA gene trap ankyrin repeat containing protein Gtar - Mus musculus	
An03g03300	2.071 (1.572 to 3.562)	0.34 (0.286 to 0.428)	0.01	similarity to almond N-glycosidase from patent R80982 - Prunus dulcis	AN10715.3
An03g03400	0.682 (0.652 to 0.708)	1.529 (1.292 to 2.085)	0.01	strong similarity to hypothetical protein An09g01460 - A. niger (mRNA)	AN8462.3
An03g03520	0.644 (0.567 to 0.703)	1.833 (1.297 to 2.56)	0.02	similarity to AM-toxin synthetase AMT - A. alternata	AN6236.3
An03g03530	0.323 (0.232 to 0.4)	2.092 (1.6 to 2.621)	0	strong similarity to hypothetical protein An16g04320 - A. niger	AN6239.3
An03g03540	0.361 (0.319 to 0.383)	2.062 (1.617 to 2.609)	0	similarity to aerobactin biosynthesis protein iucB - E. coli	AN6234.3
An03g03550	0.616 (0.532 to 0.679)	1.567 (1.321 to 1.955)	0.01	strong similarity to carnitine racemase caiD - E. coli	
An03g03560	0.314 (0.293 to 0.353)	1.777 (1.647 to 1.845)	0	strong similarity to ferrichrome-type siderophore transporter ARN1 - .S cerevisiae	AN8365.3
An03g03620	0.15 (0.144 to 0.156)	2.148 (1.844 to 2.505)	0	strong similarity to multidrug resistance protein atrD - A. nidulans	AN6237.3
An03g03680	0.528 (0.443 to 0.608)	2.206 (1.392 to 3.171)	0.01	strong similarity to hypothetical protein An13g03400 - A. niger	
An03g04230	1.602 (1.255 to 2.389)	0.646 (0.558 to 0.745)	0.02	strong similarity to cytoplasmic form of aminopeptidase P - H. sapiens	AN11005.3
An03g04410	1.496 (1.38 to 1.65)	0.551 (0.485 to 0.62)	0	strong similarity to UDP-glucose:dolichyl-phosphate glucosyltransferase ALG5 - .S cerevisiae	AN7715.3
An03g04940	1.509 (1.403 to 1.743)	0.452 (0.278 to 0.597)	0.02	strong similarity to Erv41 - .S cerevisiae	AN7679.3
An03g05130	0.599 (0.378 to 0.787)	1.894 (1.213 to 2.756)	0.04	strong similarity to hypothetical protein An15g02140 - A. niger	
An03g05140	0.643 (0.531 to 0.854)	1.792 (1.146 to 2.81)	0.04	strong similarity to polyketide synthase PKS1 - Cochliobolus heterostrophus	
An03g05200	1.686 (1.448 to 2.08)	0.433 (0.362 to 0.552)	0.01	strong similarity to carboxypeptidase I protein of patent WO9814599-A1 - A. oryzae	
An03g05210	1.539 (1.445 to 1.722)	0.419 (0.319 to 0.555)	0.01	strong similarity to reticuline oxidase bbe1 - Eschscholzia californica	AN10388.3
An03g05260	0.212 (0.192 to 0.242)	2.982 (1.758 to 4.938)	0	similarity to chitosanase csnA - A. oryzae	AN11051.3
An03g05530	0.703 (0.642 to 0.765)	3.106 (1.235 to 5.278)	0.05	strong similarity to endo-beta-1 4-glucanase EGIII-like patent WO9931255-A2 - Emericella desertoru	
An03g06480	1.858 (1.332 to 3.303)	0.612 (0.553 to 0.668)	0.03	strong similarity to Steroid monooxygenase smo - Rhodococcus rhodochrous	
An03g06550	1.746 (1.287 to 2.135)	0.558 (0.399 to 0.713)	0.02	glucan 1 4-alpha-glucosidase glaA - A. niger	AN7402.3
An03g06660	0.363 (0.281 to 0.553)	1.99 (1.447 to 3.18)	0.01	strong similarity to peptide transporter ptr2 - Arabidopsis thaliana	AN8903.3
An03g06740	0.0938 (0.0869 to 0.107)	2.58 (1.893 to 3.173)	0	strong similarity to exo-alpha 1 4-polygalacturonase PGX1 - Cochliobolus carbonum	AN8891.3
An03g06820	1.81 (1.3 to 3.489)	0.584 (0.529 to 0.7)	0.04	similarity to negative regulatory protein Hex2 - .S cerevisiae	

An03g06830	1.344 (1.302 to 1.385)	0.665 (0.63 to 0.698)	0	strong similarity to hypothetical protein B24P7.10 - Neurospora crassa	AN8883.3
An03g06850	0.493 (0.414 to 0.561)	1.888 (1.439 to 2.657)	0.01	strong similarity to hypothetical coiled-coil protein cgrA - A. nidulans	AN11145.3
An03g06860	0.558 (0.495 to 0.6)	1.903 (1.4 to 2.266)	0.01	strong similarity to mitochondrial transport protein ARG11 - .S cerevisiae	AN8881.3
An04g00410	1.374 (1.268 to 1.526)	0.684 (0.621 to 0.732)	0	strong similarity to dipeptidyl peptidase III - R. norvegicus	AN10540.3
An04g00480	0.609 (0.573 to 0.681)	1.751 (1.319 to 2.791)	0.02	weak similarity to the vitellogenin II precursor - Gallus gallus	AN4324.3
An04g00610	0.667 (0.631 to 0.718)	1.417 (1.282 to 1.56)	0	strong similarity to the hydroxymethylglutaryl-coenzyme A reductase Hmg1 – S. pombe (truncated ORF)	
An04g00790	0.121 (0.114 to 0.129)	2.169 (1.871 to 2.596)	0	similarity to multifunctional arom protein Aro1p - .S cerevisiae	AN10544.3
An04g01170	0.748 (0.728 to 0.767)	1.502 (1.233 to 1.97)	0.02	strong similarity to the protein probably involved in the synthesis of Fe-S clusters Isu2 - .S cerevisiae	AN4655.3
An04g02610	1.536 (1.44 to 1.682)	0.335 (0.242 to 0.56)	0.01	strong similarity to succinate-semialdehyde dehydrogenase NAD(P)+ gabD - E. coli	AN3829.3
An04g02670	1.459 (1.286 to 1.569)	0.633 (0.574 to 0.714)	0	strong similarity to NADPH-dependent aldehyde reductase – S. salmonicolor	
An04g02760	0.625 (0.571 to 0.669)	2.207 (1.331 to 3.176)	0.02	strong similarity to starvation-sensing protein RspA - E. coli	
An04g02850	1.51 (1.403 to 1.619)	0.543 (0.479 to 0.597)	0	similarity to the X-Pro dipeptidyl-peptidase IV - XantH.nas maltophilia	
An04g03180	1.324 (1.289 to 1.385)	0.652 (0.557 to 0.711)	0	hypothetical protein	AN1719.3
An04g03190	1.534 (1.376 to 1.898)	0.558 (0.49 to 0.624)	0.01	weak similarity to hypothetical protein F28C11.17 - Arabidopsis thaliana	AN1718.3
An04g03400	1.703 (1.386 to 2.443)	0.589 (0.562 to 0.614)	0.01	strong similarity to aldehyde dehydrogenase aldA - A. niger	AN1689.3
An04g03930	1.476 (1.344 to 1.706)	0.596 (0.508 to 0.656)	0	lysine aminopeptidase apsA - A. niger	AN1638.3
An04g04510	0.585 (0.468 to 0.822)	1.434 (1.178 to 1.862)	0.03	similarity to the putative transcription factor hric - Cladosporium fulvum	AN1812.3
An04g04630	0.598 (0.498 to 0.713)	2.018 (1.287 to 2.66)	0.02	strong similarity to capsule protein CAP59 - Cryptococcus neoformans	
An04g05440	1.478 (1.204 to 1.891)	0.623 (0.497 to 0.796)	0.02	strong similarity to xanthine dehydrogenase XDH - H. sapiens	AN5613.3
An04g05880	0.589 (0.498 to 0.751)	1.52 (1.249 to 1.707)	0.01	strong similarity to linoleate diol synthase - Gaeumannomyces graminis	AN1967.3
An04g06290	0.431 (0.386 to 0.491)	1.605 (1.509 to 1.662)	0	weak similarity to C2H2 zinc finger protein zas1 - Schizo.S pombe	
An04g06750	1.838 (1.221 to 2.321)	0.45 (0.286 to 0.779)	0.03	similarity to hypothetical transmembrane protein - C. albicans	
An04g06910	1.548 (1.296 to 1.927)	0.521 (0.418 to 0.704)	0.01	transcriptional regulator amyR - A. niger	AN2016.3
An04g06920	2.526 (1.532 to 3.65)	0.442 (0.426 to 0.468)	0.01	extracellular alpha-glucosidase aglU - A. niger	AN2017.3
An04g06990	1.58 (1.444 to 1.67)	0.465 (0.34 to 0.556)	0.01	strong similarity to alpha 1 2-mannosidase IC - H. sapiens	AN2045.3
An04g07200	0.516 (0.436 to 0.622)	1.765 (1.378 to 2.363)	0.01	strong similarity to kynureninase - R. norvegicus	AN1857.3
An04g08880	0.185 (0.154 to 0.231)	2.113 (1.769 to 2.498)	0	similarity to hypothetical protein An02g00390 - A. niger	
An04g09230	0.553 (0.5 to 0.598)	1.712 (1.402 to 1.941)	0	strong similarity to Na+/H+-exchanging protein Nha1p - .S cerevisiae	AN7250.3
An04g09360	0.474 (0.444 to 0.506)	2.843 (1.494 to 6.725)	0.03	strong similarity to hypothetical protein CC0812 - Caulobacter crescentus	AN2834.3
An04g09550	1.548 (1.434 to 1.791)	0.4 (0.218 to 0.566)	0.02	strong similarity to 4-Hydroxyacetophenone monooxygenase hapE - Pseudomonas fluorescens	AN7228.3
An04g09690	0.53 (0.498 to 0.546)	1.486 (1.454 to 1.51)	0	strong similarity to pectin methylesterase PME1 - A. aculeatus	AN3390.3
An04g09890	1.314 (1.27 to 1.381)	0.632 (0.547 to 0.73)	0	strong similarity to cell wall alpha-glucan synthase ags1 - Schizo.S pombe	
An05g00220	0.57 (0.481 to 0.681)	1.751 (1.319 to 2.187)	0.01	strong similarity to L-ornithine N5-hydroxylase psbA - Pseudomonas sp. B10	AN5823.3
An05g00340	0.679 (0.562 to 0.759)	2.223 (1.241 to 3.4)	0.03	strong similarity to cis-epoxysuccinate hydrolase (LMGP-18079) of patent EP911392-A1 - Rhodococcus rhodochrous	AN5830.3

An05g00410	1.987 (1.252 to 2.551)	0.522 (0.423 to 0.748)	0.02	strong similarity to the serine hydroxymethyl-transferase I Shm1 - C. albicans	AN10745.3
An05g00870	0.668 (0.608 to 0.76)	1.471 (1.24 to 2.022)	0.02	strong similarity to purine utilization positive regulator UaY - Emericella nidulans	AN6173.3
An05g00880	1.412 (1.323 to 1.541)	0.561 (0.418 to 0.677)	0.01	strong similarity to dnaJ protein H.log SCJ1 - .S cerevisiae	AN6170.3
An05g00930	0.615 (0.563 to 0.679)	2.29 (1.321 to 3.201)	0.02	strong similarity to NADP-dependent malate dehydrogenase mdh - H. sapiens	AN6168.3
An05g01060	0.607 (0.56 to 0.677)	1.78 (1.323 to 2.431)	0.01	strong similarity to HC-toxin synthetase HTS1 - Cochliobolus carbonum	AN7884.3
An05g01070	0.475 (0.385 to 0.646)	1.766 (1.354 to 2.018)	0.01	strong similarity to 7-aminocholesterol resistance protein RTA1 - .S cerevisiae	AN2669.3
An05g01100	0.632 (0.607 to 0.654)	1.816 (1.346 to 2.518)	0.01	strong similarity to HC-toxin biosynthesis protein TOXF - Cochliobolus carbonum	
An05g01210	0.709 (0.678 to 0.745)	1.911 (1.255 to 2.67)	0.02	strong similarity to 1-acyldihydroxyacetone-phosphate reductase Ayr1p - .S cerevisiae	AN3276.3
An05g01290	2.157 (1.617 to 2.91)	0.372 (0.366 to 0.383)	0	strong similarity to hexose transport protein HXT3 - .S cerevisiae	AN3357.3
An05g01750	1.602 (1.39 to 1.958)	0.505 (0.364 to 0.61)	0.01	strong similarity to mannosyltransferase Hoc1p - .S cerevisiae	
An05g01760	1.385 (1.309 to 1.467)	0.554 (0.366 to 0.691)	0.02	similarity to hard surface induced protein 3 chip3 - Glomerella cingulata	
An05g01770	1.584 (1.506 to 1.746)	0.452 (0.388 to 0.494)	0	hypothetical protein	
An05g01850	0.48 (0.399 to 0.624)	1.593 (1.376 to 1.909)	0.01	similarity to monocarboxylate transporter MCT2 - H. sapiens	
An05g02370	1.433 (1.209 to 1.859)	0.66 (0.588 to 0.791)	0.02	strong similarity to trichothecene 3-O-acetyltransferase TRI101 - Fusarium sporotrichioides	AN9265.3
An06g00190	1.723 (1.51 to 1.936)	0.377 (0.293 to 0.49)	0	strong similarity to lysosomal pepstatin insensitive protease CLN2 - H. sapiens	
An06g00290	0.652 (0.607 to 0.749)	1.371 (1.251 to 1.543)	0.01	strong similarity to beta-galactosidase lacA - A. niger	
An06g00300	1.854 (1.314 to 2.319)	0.544 (0.383 to 0.686)	0.02	similarity to phosphate/phosphoenolpyruvate translocator TABPPT8 - Nicotiana tabacum	
An06g00310	1.353 (1.204 to 1.642)	0.584 (0.471 to 0.796)	0.02	similarity to carboxypeptidase D - P. janthinellum	
An06g00600	0.602 (0.544 to 0.702)	1.519 (1.298 to 2.029)	0.01	similarity to short-chain alcohol dehydrogenase adhA - A. parasiticus	AN5331.3
An06g00660	3.188 (1.535 to 9.705)	0.362 (0.296 to 0.465)	0.03	strong similarity to 5-oxoprolinase OPLA - R. norvegicus (truncated ORF)	AN3972.3
An06g02270	1.517 (1.347 to 1.767)	0.515 (0.326 to 0.653)	0.02	similarity to arabinose transport protein araE - E. coli	
An07g00130	1.573 (1.263 to 1.915)	0.492 (0.38 to 0.737)	0.02	similarity to conserved hypothetical protein ynaD - B. subtilis	AN8539.3
An07g00440	0.509 (0.417 to 0.564)	1.873 (1.436 to 3.139)	0.02	strong similarity to secretory lipase LIP2 - C. albicans	AN1799.3
An07g00760	0.607 (0.523 to 0.673)	1.564 (1.327 to 1.936)	0.01	strong similarity to formyl-CoA transferase patent WO9816632-A1 – O. formigenes	AN0058.3
An07g00780	0.528 (0.418 to 0.637)	1.491 (1.363 to 1.653)	0.01	strong similarity to monocarboxylate transporter 2 hMCT2 - H. sapiens	
An07g01200	1.483 (1.23 to 2.041)	0.727 (0.671 to 0.77)	0.02	strong similarity to enoyl reductase lovC - A. terreus	
An07g01290	2.122 (1.593 to 2.759)	0.371 (0.353 to 0.407)	0	strong similarity to xylitol dehydrogenase xdh - GalactoC. mastotermitis	AN2666.3
An07g01950	1.512 (1.467 to 1.587)	0.314 (0.227 to 0.533)	0.01	strong similarity to purine permease broad specificity uapC - Emericella nidulans	AN6730.3
An07g02380	1.511 (1.312 to 1.831)	0.662 (0.642 to 0.688)	0.01	weak similarity to T-2 toxin biosynthesis protein TRI7 - Fusarium sporotrichioides	
An07g03050	1.421 (1.328 to 1.573)	0.544 (0.478 to 0.672)	0.01	similarity to putative integral membrane protein SCC53.26c - Streptomyces coelicolor	AN8786.3
An07g03100	1.469 (1.387 to 1.618)	0.537 (0.44 to 0.613)	0	strong similarity to esterase D ESD - H. sapiens	AN8782.3
An07g03570	1.598 (1.516 to 1.761)	0.23 (0.115 to 0.484)	0.02	strong similarity to sorbitol utilization protein sou2 - C. albicans	AN8819.3
An07g04270	1.247 (1.118 to 1.509)	0.597 (0.459 to 0.882)	0.04	strong similarity to 3-methylcrotonyl-CoA carboxylase (MCC) non-biotin-containing beta subunit MCCB - H. sapiens	AN4687.3
An07g04280	1.911 (1.417 to 2.352)	0.509 (0.429 to 0.583)	0.01	strong similarity to isovaleryl-coenzyme A dehydrogenase AtIVD – A. thaliana	AN4688.3
An07g04300	1.782 (1.34 to 2.27)	0.623 (0.582 to 0.66)	0.01	strong similarity to 3-methylcrotonyl-CoA carboxylase (MCC) biotin-containing alpha	AN4690.3

				subunit MCCA - H. sapiens	
An07g04360	1.398 (1.188 to 1.707)	0.694 (0.604 to 0.812)	0.01	hypothetical protein (mRNA)	
An07g04430	2.158 (1.497 to 4.085)	0.375 (0.245 to 0.503)	0.02	strong similarity to hexose transporter Ght2 - Schizo.S pombe	
An07g04480	0.504 (0.477 to 0.53)	1.729 (1.47 to 2.064)	0	similarity to hypothetical protein An01g02250 - A. niger	AN1591.3
An07g04490	0.668 (0.626 to 0.752)	1.522 (1.248 to 2.12)	0.02	weak similarity to trans-Golgi p230 - H. sapiens	AN1590.3
An07g04850	1.601 (1.572 to 1.64)	0.384 (0.325 to 0.428)	0	hypothetical protein	
An07g05260	0.507 (0.397 to 0.757)	1.338 (1.243 to 1.421)	0.02	hypothetical protein	AN4654.3
An07g05370	1.366 (1.173 to 1.739)	0.656 (0.521 to 0.827)	0.03	strong similarity to hypothetical protein Y105C5B.9 - C. elegans	AN4647.3
An07g05520	1.546 (1.339 to 1.952)	0.524 (0.406 to 0.661)	0.01	strong similarity to O-methyltransferase B omtB - A. parasiticus	
An07g05830	2.165 (1.353 to 4.584)	0.576 (0.467 to 0.647)	0.04	strong similarity to formamidase fmdS - A. nidulans	AN4577.3
An07g06230	0.659 (0.618 to 0.711)	1.442 (1.289 to 1.65)	0	hypothetical protein	
An07g06240	0.159 (0.153 to 0.17)	2.091 (1.83 to 2.538)	0	strong similarity to ferrioxamine B permease sit1 - .S cerevisiae	AN6238.3
An07g06700	0.677 (0.642 to 0.721)	1.378 (1.279 to 1.55)	0	weak similarity to hypothetical protein SPBC24C6.08c - Schizo.S pombe	AN4598.3
An07g06820	1.656 (1.324 to 2.434)	0.626 (0.557 to 0.676)	0.02	strong similarity to probable dehydrogenase - Xanthobacter sp	
An07g07280	1.325 (1.203 to 1.489)	0.598 (0.402 to 0.797)	0.03	strong similarity to hydroxyquinol 1 2-dioxygenase dxnF - Sphingomonas sp.	AN4532.3
An07g07340	1.346 (1.26 to 1.508)	0.643 (0.557 to 0.74)	0.01	strong similarity to luminal ER-protein retention receptor ERD2 – K. marxianus	AN4528.3
An07g08030	1.54 (1.418 to 1.782)	0.463 (0.375 to 0.582)	0.01	serine carboxypeptidase pepF - A. niger	AN2555.3
An07g08940	0.277 (0.252 to 0.327)	1.76 (1.673 to 1.878)	0	similarity to acetyl-esterase I of patent WO9502689-A - A. aculeatus	AN9442.3
An07g09190	1.617 (1.202 to 2.028)	0.737 (0.637 to 0.798)	0.02	strong similarity to very long-chain fatty acyl-CoA synthetase FAT1 - .S cerevisiae	AN5192.3
An07g09390	1.732 (1.135 to 2.171)	0.727 (0.537 to 0.865)	0.04	strong similarity to nuclear movement protein nudC - A. nidulans	AN5181.3
An08g00210	0.374 (0.308 to 0.547)	1.546 (1.453 to 1.741)	0.01	strong similarity to glycerol-3-phosphate dehydrogenase gdm1 - Mus musculus	AN1396.3
An08g01250	2.18 (1.585 to 3.578)	0.315 (0.264 to 0.415)	0.01	weak similarity to COP1-interacting protein 7 CIP7 - Arabidopsis thaliana	
An08g01710	0.0943 (0.0834 to 0.113)	2.14 (1.887 to 2.507)	0	strong similarity to alpha-L-arabinofuranosidase abfA - B. stearothermophilus	AN1277.3
An08g01720	0.438 (0.358 to 0.629)	2.22 (1.371 to 3.85)	0.02	strong similarity to quinate transport protein QUTD - A. nidulans	AN1276.3
An08g01740	1.365 (1.257 to 1.459)	0.651 (0.608 to 0.743)	0	strong similarity to uronate dehydrogenase of patent W29217 - .S cerevisiae	AN1274.3
An08g03200	2.073 (1.442 to 3.763)	0.395 (0.305 to 0.558)	0.02	strong similarity to ammonium transport protein Mep2p - .S cerevisiae	AN1181.3
An08g03750	1.408 (1.257 to 1.587)	0.645 (0.57 to 0.743)	0.01	strong similarity to fluconazole resistance protein FLU1 - C. albicans	
An08g03760	1.883 (1.693 to 2.197)	0.227 (0.167 to 0.307)	0	similarity to hypothetical protein Rv3472 - Mycobacterium tuberculosis	
An08g03770	1.358 (1.214 to 1.597)	0.676 (0.523 to 0.786)	0.02	weak similarity to mucin MUC5AC - H. sapiens	
An08g03960	1.378 (1.272 to 1.597)	0.639 (0.527 to 0.728)	0.01	strong similarity to probable edoplasmic reticulum associated protein - Schizo.S pombe	AN1117.3
An08g04040	0.101 (0.091 to 0.125)	2.183 (1.875 to 2.605)	0	strong similarity to quinate transport protein QUTD - A. nidulans	AN1109.3
An08g04390	2.788 (1.358 to 4.104)	0.572 (0.537 to 0.642)	0.02	strong similarity to glycine decarboxylase subunit H protein FUN40 -.S cerevisiae	AN1074.3
An08g04490	2.072 (1.597 to 3.039)	0.26 (0.203 to 0.403)	0.01	similarity to putative serine peptidase - Oryza sativa	
An08g04640	2.418 (1.679 to 4.522)	0.251 (0.221 to 0.321)	0.01	strong similarity to putative lysosomal pepstatin insensitive protease CLN2 - Canis familiaris	
An08g05610	2.844 (1.947 to 5.18)	0.052 (0.05 to 0.0533)	0	nitrate reductase (NADPH) niaD - A. niger	AN1006.3

An08g05640	2.85 (1.923 to 5.059)	0.05 (0.037 to 0.077)	0	strong similarity to nitrite reductase (NADH) long form niiA - A. nidulans	AN1007.3
An08g05670	3.234 (1.726 to 9.454)	0.232 (0.191 to 0.274)	0.02	strong similarity to nitrate permease crnA - A. nidulans	AN1008.3
An08g06240	1.609 (1.314 to 2.056)	0.598 (0.517 to 0.686)	0.01	strong similarity to uracil transport protein FUR4 - Schizo.S pombe	AN0660.3
An08g06890	1.706 (1.288 to 2.524)	0.559 (0.478 to 0.712)	0.02	weak similarity to 3-demethylubiquinone-9 3-O-methyltransferase ubiG- E. coli	
An08g07290	1.439 (1.261 to 1.802)	0.662 (0.546 to 0.739)	0.01	aldehyde dehydrogenase aldA - A. niger	AN0554.3
An08g07890	1.478 (1.415 to 1.516)	0.57 (0.554 to 0.585)	0	strong similarity to 3-hydroxyisobutyrate dehydrogenase MmsB – P. aeruginosa	AN0593.3
An08g08780	1.393 (1.295 to 1.532)	0.642 (0.595 to 0.705)	0	hypothetical protein	AN5445.3
An08g08840	1.63 (1.424 to 1.998)	0.384 (0.285 to 0.576)	0.01	strong similarity to glutamate decarboxylase 1 GAD 1 - Arabidopsis thaliana	AN5447.3
An08g09610	1.538 (1.459 to 1.657)	0.472 (0.408 to 0.541)	0	similarity to mutanase mutA - P. purpurogenum	
An08g10600	0.676 (0.621 to 0.714)	1.493 (1.286 to 1.688)	0	strong similarity to ABC transporter ATM1 - .S cerevisiae	AN5528.3
An08g11030	1.454 (1.334 to 1.664)	0.645 (0.623 to 0.666)	0	acid phosphatase aph 3-phytase phyB - A. niger	
An08g11060	0.627 (0.529 to 0.783)	1.262 (1.217 to 1.286)	0.01	weak similarity to hypothetical protein T29A15.10 - Arabidopsis thaliana	
An09g00360	0.652 (0.625 to 0.679)	1.933 (1.321 to 2.868)	0.02	similarity to the hypothetical protein encoded by An07g04880 - A. niger	AN7985.3
An09g00630	1.436 (1.308 to 1.631)	0.678 (0.667 to 0.692)	0	weak similarity to the hypothetical protein encoded by An07g03660 - A. niger	AN7937.3
An09g00650	1.668 (1.299 to 2.357)	0.517 (0.332 to 0.701)	0.03	similarity to hypothetical protein F9K20.18 - Arabidopsis thaliana	AN3877.3
An09g00660	2.63 (1.916 to 4.48)	0.075 (0.071 to 0.084)	0	strong similarity to protein isp4 - Schizo.S pombe	
An09g00670	2.008 (1.927 to 2.089)	0.062 (0.057 to 0.073)	0	strong similarity to beta (1-3) glucanosyltransferase Gel3p - A. fumigatus	
An09g01190	0.424 (0.393 to 0.464)	2.048 (1.536 to 2.412)	0	endo 1 5-alpha-arabinanase abnA - A. niger	AN2534.3
An09g01210	0.407 (0.318 to 0.665)	2.03 (1.335 to 4.515)	0.04	strong similarity to 2-keto-3-deoxygluconate oxidoreductase kduD – E. chrysanthemi	AN2402.3
An09g01220	0.205 (0.19 to 0.236)	1.986 (1.764 to 2.127)	0	similarity to the hypothetical protein encoded by An04g00640 - A. niger	AN2326.3
An09g01240	0.29 (0.273 to 0.3)	1.902 (1.7 to 2.198)	0	strong similarity to phospholipase B - P. notatum	AN7792.3
An09g03300	0.317 (0.233 to 0.379)	1.743 (1.621 to 1.929)	0	strong similarity to the alpha-xylosidase XylS - Sulfolobus solfataricus	
An09g03650	0.51 (0.394 to 0.594)	2.94 (1.406 to 4.366)	0.02	weak similarity to hypothetical protein Ta0309 - Thermoplasma acidophilum	
An09g03660	0.583 (0.567 to 0.594)	1.73 (1.406 to 2.074)	0	strong similarity to neutral amino acid permease (mtr) - Neurospora crassa	
An09g04280	0.708 (0.605 to 0.788)	1.591 (1.212 to 2.358)	0.03	strong similarity to tRNA splicing protein SPL1 - C. maltosa	AN2508.3
An09g05240	1.263 (1.158 to 1.351)	0.442 (0.293 to 0.842)	0.05	weak similarity to utr1 protein H.log SPCC24B10.02c - Schizo.S pombe	AN8181.3
An09g05420	1.437 (1.329 to 1.516)	0.589 (0.462 to 0.671)	0.01	similarity to signal peptidase subunit SPC3 - .S cerevisiae	AN9460.3
An09g05520	0.461 (0.426 to 0.504)	2.642 (1.496 to 4.824)	0.01	hypothetical protein	
An09g05550	0.05 (0.047 to 0.052)	3.075 (1.948 to 4.253)	0	hypothetical protein	
An09g05880	1.471 (1.297 to 1.758)	0.582 (0.424 to 0.703)	0.02	strong similarity to alpha-glucosidase ModA - Dictyostelium discoideum	AN11054.3
An09g05940	0.211 (0.083 to 0.337)	2.345 (1.663 to 2.938)	0.02	weak similarity to thioredoxin reductase trxB - Eubacterium acidaminophilum	AN8218.3
An09g06130	1.378 (1.347 to 1.417)	0.5 (0.331 to 0.653)	0.02	hypothetical protein	
An09g06300	0.632 (0.401 to 0.826)	1.463 (1.174 to 1.743)	0.04	similarity to adrenodoxin precursor protein - Sus scrofa	
An09g06800	1.331 (1.253 to 1.41)	0.637 (0.529 to 0.747)	0.01	strong similarity to the leucyl aminopeptidase Ape2 - .S cerevisiae	AN4282.3
An10g00210	0.729 (0.684 to 0.824)	1.666 (1.176 to 2.071)	0.02	hypothetical protein	
An10g00620	0.513 (0.479 to 0.565)	2.11 (1.435 to 3.395)	0.01	strong similarity to putative branched-chain amino acid aminotransferase ToxF - Cochliobolus carbonum	AN7876.3

An10g00630	0.527 (0.411 to 0.709)	1.475 (1.291 to 1.835)	0.01	strong similarity to the alpha subunit of the fatty acid synthase fasA - A. nidulans	AN7880.3
An10g00830	0.509 (0.449 to 0.544)	1.531 (1.456 to 1.575)	0	strong similarity to the hypothetical protein encoded by An02g13140 - A. niger	
An10g00870	0.585 (0.546 to 0.609)	1.481 (1.391 to 1.544)	0	pectate lyase plyA - A. niger	AN7646.3
An11g00070	0.698 (0.638 to 0.762)	1.603 (1.238 to 1.911)	0.01	strong similarity to O-methyltransferase B omtB - A. parasiticus	
An11g00220	0.69 (0.629 to 0.816)	1.648 (1.184 to 2.502)	0.03	similarity to ferric reductase cfl1 - C. albicans	AN6400.3
An11g01100	1.948 (1.483 to 2.273)	0.446 (0.362 to 0.517)	0	strong similarity to high-affinity glucose transporter HGT1 - Klyveromyces lactis (truncated ORF)	AN2466.3
An11g01120	0.573 (0.522 to 0.634)	1.51 (1.366 to 1.626)	0	strong similarity to aldehyde reductase - Sporobolomyces salmonicolor	AN7193.3
An11g01190	0.559 (0.412 to 0.705)	1.369 (1.295 to 1.495)	0.01	similarity to protein participating in extracellular/cell surface phenomena ECM33 - .S cerevisiae	AN7191.3
An11g01240	1.7 (1.556 to 1.916)	0.404 (0.36 to 0.444)	0	similarity to protein required for filamentous growth DFG5 - .S cerevisiae	
An11g01810	0.666 (0.591 to 0.807)	1.587 (1.193 to 2.5)	0.04	weak similarity to probable membrane protein YBR005w - .S cerevisiae	AN3935.3
An11g02200	0.272 (0.261 to 0.286)	3.179 (1.714 to 10.48)	0.02	strong similarity to 4-hydroxyphenylpyruvate dioxygenase tcrP - Coccidioides immitis	AN1899.3
An11g02380	1.354 (1.208 to 1.438)	0.666 (0.488 to 0.792)	0.02	strong similarity to mannose-1-phosphate guanylyltransferase MPG1 - Hypocrea jecorina	AN1911.3
An11g03110	1.441 (1.233 to 1.961)	0.665 (0.605 to 0.767)	0.02	strong similarity to the methanol dehydrogenase Mdh - B. methanolicus C1	AN1868.3
An11g03500	0.479 (0.434 to 0.504)	1.803 (1.496 to 2.179)	0	strong similarity to L-lactate dehydrogenase precursor CYB2 - .S cerevisiae	
An11g03860	0.506 (0.465 to 0.584)	1.65 (1.416 to 2.224)	0.01	similarity to mitochondrial ATP-dependent protease ftsH2 - Cyanidioschyzon merolae	
An11g04040	0.592 (0.536 to 0.635)	1.518 (1.365 to 1.765)	0	strong similarity to exo-alpha 1 4-polygalacturonase PGX1 - Cochliobolus carbonum	
An11g04720	0.588 (0.495 to 0.744)	1.529 (1.256 to 2.135)	0.02	strong similarity to the aromatic amino acid decarboxylase Ddc - Sorangium cellulosum	AN2091.3
An11g04890	0.638 (0.556 to 0.762)	1.756 (1.238 to 2.948)	0.03	similarity to multicatalytic endopeptidase regulator Rpn5 - Drosophila melanogaster	AN2107.3
An11g04900	0.194 (0.174 to 0.229)	1.846 (1.771 to 1.931)	0	hypothetical protein	AN3492.3
An11g04990	0.806 (0.739 to 0.907)	1.73 (1.093 to 2.325)	0.05	similarity to RAN-binding protein KAP123 - .S cerevisiae (truncated ORF)	AN2120.3
An11g05680	0.569 (0.543 to 0.588)	1.538 (1.412 to 1.743)	0	similarity to cytochrome P450 3A13 - Mus musculus	
An11g06150	1.751 (1.455 to 2.505)	0.46 (0.413 to 0.545)	0.01	strong similarity to proline permease prnB - A. nidulans	AN1732.3
An11g07010	0.6 (0.463 to 0.811)	1.425 (1.189 to 1.854)	0.03	strong similarity to alkane-inducible cytochrome P450 alk2 - C. tropicalis	
An11g07040	0.034 (0.027 to 0.04)	3.227 (1.96 to 4.485)	0	strong similarity to EST EMBLEST:BE759852 an_2779 - A. niger	
An11g07270	0.709 (0.621 to 0.787)	1.652 (1.213 to 2.045)	0.02	strong similarity to D-mandelate dehydrogenase D-MDH - Rhodotorula graminis	AN5030.3
An11g07310	0.123 (0.105 to 0.162)	4.424 (1.838 to 8.62)	0.01	strong similarity to polyketide synthase PKS1 - Colletotrichum lagenarium	AN6000.3
An11g07320	0.27 (0.247 to 0.294)	6.791 (1.706 to 23.6)	0.02	similarity to glyoxalase II GLO2 - .S cerevisiae	AN6001.3
An11g07330	0.247 (0.231 to 0.259)	5.461 (1.741 to 16.56)	0.02	similarity to 6-hydroxynicotinic acid mono-oxygenase 6-HNAMO of patent W10018 - Pseudomonas fluorescens	AN6002.3
An11g07340	0.071 (0.068 to 0.073)	5.68 (1.927 to 15.83)	0.01	strong similarity to putative O-methyl transferase EncK - Streptomyces maritimus	
An11g07350	0.524 (0.522 to 0.526)	1.736 (1.474 to 1.911)	0	similarity to transcriptional regulator amdR - A. oryzae	
An11g07730	0.111 (0.105 to 0.123)	2.213 (1.877 to 2.874)	0	similarity to mucin 4 (MUC4) - H. sapiens	
An11g07830	2.912 (1.574 to 8.985)	0.402 (0.366 to 0.426)	0.04	strong similarity to putative peptide transporter MTD1 (mtd1) - Schizophyllum commune	AN6452.3
An11g08080	1.413 (1.356 to 1.466)	0.587 (0.498 to 0.644)	0	strong similarity to cDNA O-methyltransferase mt-I - A. parasiticus	

An11g08290	0.462 (0.424 to 0.496)	1.866 (1.504 to 2.227)	0	similarity to pJOY3 ferulic acid hydroxylase FAH patent CA2270417-A1 – B. napus	
An11g09170	0.539 (0.432 to 0.713)	1.663 (1.287 to 1.988)	0.01	similarity to the secreted aspartic proteinase SAP8 - C. albicans	AN3321.3
An11g09700	1.254 (1.163 to 1.394)	0.551 (0.428 to 0.837)	0.03	similarity to siroheme synthase cysG - E. coli	AN4774.3
An11g10300	0.493 (0.367 to 0.805)	1.447 (1.195 to 1.909)	0.03	weak similarity to hypothetical alpha-methylacyl-CoA racemase HSA - H. sapiens	
An11g10470	1.36 (1.209 to 1.452)	0.666 (0.5 to 0.791)	0.02	strong similarity to glutaryl-CoA dehydrogenase GCHD - H. sapiens	AN2762.3
An11g10540	0.441 (0.313 to 0.565)	1.929 (1.435 to 2.907)	0.01	strong similarity to ras-associated protein mra1 - Schizo.S pombe	AN2759.3
An11g10970	0.618 (0.561 to 0.676)	1.756 (1.324 to 2.551)	0.01	similarity to monocarboxylate transporter MCT2 - Mesocricetus auratus	AN10410.3
An12g00100	1.385 (1.14 to 1.915)	0.648 (0.547 to 0.86)	0.04	weak similarity to putative polysaccharide synthase H.log cap3B – S. pneumoniae	AN9069.3
An12g00240	1.535 (1.394 to 1.86)	0.548 (0.515 to 0.606)	0	strong similarity to translational inhibitor uk14 - H. sapiens (truncated ORF)	AN9080.3
An12g00600	1.767 (1.425 to 1.987)	0.479 (0.41 to 0.575)	0	similarity to monodehydroascorbate reductase MDA - Cucumis sativus	AN9103.3
An12g00820	1.316 (1.225 to 1.48)	0.649 (0.456 to 0.775)	0.03	strong similarity to UTP--glucose-1-phosphate uridylyltransferase UGP1 - .S cerevisiae	AN9148.3
An12g01020	1.813 (1.452 to 2.473)	0.541 (0.532 to 0.548)	0.01	strong similarity to acetamidase amdS - Emericella nidulans	AN9138.3
An12g01260	2.007 (1.45 to 3.26)	0.472 (0.391 to 0.55)	0.01	strong similarity to nitrilase NIT1 - Arabidopsis thaliana	
An12g01270	1.625 (1.489 to 1.828)	0.452 (0.386 to 0.511)	0	similarity to hypothetical alanine--tRNA ligase alaS - Streptomyces coelicolor	AN11110.3
An12g01280	1.627 (1.303 to 1.938)	0.685 (0.661 to 0.697)	0.01	strong similarity to argininosuccinate synthase argG - Streptomyces clavuligerus	
An12g02070	0.0866 (0.08 to 0.095)	3.089 (1.905 to 4.415)	0	similarity to the ribonuclease T1 precursor RntA - A. oryzae	
An12g02450	1.447 (1.397 to 1.514)	0.484 (0.362 to 0.603)	0.01	strong similarity to alpha-glucan synthase mok1 - Schizo.S pombe	
An12g02460	1.683 (1.454 to 1.842)	0.483 (0.381 to 0.546)	0	strong similarity to alpha-amylase - A. niger	
An12g02520	0.665 (0.648 to 0.683)	1.411 (1.317 to 1.615)	0	strong similarity to clavulanic acid dehydrogenase patent WO9503416-A - Streptomyces clavuligerus	
An12g03300	1.984 (1.599 to 3.048)	0.245 (0.192 to 0.401)	0.01	strong similarity to aspartic protease pr1 - Phaffia rhodozyma	AN8102.3
An12g03370	0.522 (0.451 to 0.592)	1.852 (1.408 to 2.507)	0.01	weak similarity to tol-dependent translocation system component tolB - E. coli	
An12g04020	0.443 (0.334 to 0.526)	1.809 (1.474 to 2.221)	0.01	strong similarity to acetyl-CoA carboxylase SPAC56E4.04c - Schizo.S pombe	AN6126.3
An12g04640	0.583 (0.513 to 0.677)	1.369 (1.323 to 1.452)	0	similarity to sequence 28 from Patent WO0032789	AN6064.3
An12g04990	0.54 (0.462 to 0.584)	1.788 (1.416 to 2.056)	0	strong similarity to glucitol 6-phosphate dehydrogenase gutD - Clostridium beijerinckii	
An12g05510	0.512 (0.466 to 0.547)	2.118 (1.453 to 2.696)	0.01	strong similarity to triacetylfusarinine C transporter TAF1 - .S cerevisiae	
An12g05960	1.669 (1.475 to 2.129)	0.484 (0.429 to 0.525)	0	strong similarity to dipeptidyl peptidase II DPPII - R. norvegicus	AN7231.3
An12g05990	1.355 (1.275 to 1.488)	0.654 (0.598 to 0.725)	0	weak similarity to hypothetical protein - Pichia angusta	
An12g07260	0.702 (0.654 to 0.807)	2.478 (1.193 to 3.662)	0.04	strong similarity to putative protein SC4B10.22 - Streptomyces coelicolor	AN7130.3
An12g07500	0.04 (0.035 to 0.043)	2.13 (1.957 to 2.521)	0	strong similarity to exopolygalacturonase pgaX - A. tubingensis	AN8761.3
An12g07750	1.799 (1.175 to 2.295)	0.773 (0.73 to 0.825)	0.03	similarity to serine repeat antigen BAA78500.1 - Plasmodium falciparum	
An12g08270	0.466 (0.396 to 0.545)	1.658 (1.455 to 1.841)	0	strong similarity to L-lactate 2-monooxygenase LA2M - Mycobacterium smegmati	AN8744.3
An12g08320	0.644 (0.507 to 0.731)	1.539 (1.269 to 1.761)	0.01	strong similarity to high-affinity nicotinic acid permease TNA1 - .S cerevisiae	AN11116.3
An12g08610	1.461 (1.317 to 1.605)	0.448 (0.354 to 0.683)	0.01	glucokinase GlkA - A. niger	AN8689.3
An12g09520	0.538 (0.447 to 0.71)	1.421 (1.29 to 1.708)	0.01	weak similarity to the hypothetical protein An02g13400 - A. niger	AN8958.3
An12g09860	1.529 (1.41 to 1.717)	0.538 (0.457 to 0.59)	0	weak similarity to cerebral cell adhesion molecule CerCAM - H. sapiens	
An12g09920	1.316 (1.282 to 1.378)	0.626 (0.541 to 0.718)	0	weak similarity to PG123 patent WO9929870-A1 - Porphorymonas gingivalis	

An12g10000	1.58 (1.447 to 1.852)	0.435 (0.33 to 0.553)	0.01	strong similarity to GABA permease gabA - Aspergillsu nidulans	AN3347.3
An12g10350	1.548 (1.405 to 1.639)	0.495 (0.347 to 0.595)	0.01	strong similarity to the hypothetical protein An15g07090 - A. niger (mRNA)	
An13g00620	1.431 (1.293 to 1.728)	0.613 (0.466 to 0.707)	0.02	strong similarity to 80K protein H precursor G19P1 - H. sapiens	AN10702.3
An13g00710	1.516 (1.363 to 1.692)	0.555 (0.441 to 0.637)	0.01	strong similarity to copper amine oxidase AO-I - A. niger	AN5690.3
An13g00840	1.548 (1.264 to 2.232)	0.447 (0.339 to 0.736)	0.03	strong similarity to amino acid transport protein GAP1 - .S cerevisiae	AN5678.3
An13g01250	0.444 (0.372 to 0.531)	1.728 (1.469 to 1.908)	0	strong similarity to the yeast siderophore-iron transporter Enb1 - .S cerevisiae.	AN3763.3
An13g01520	0.45 (0.231 to 0.643)	1.787 (1.357 to 2.196)	0.03	hypothetical protein	
An13g01530	2.154 (1.57 to 2.911)	0.379 (0.32 to 0.43)	0	weak similarity to hypothetical protein ycnE - B. subtilis	AN3616.3
An13g01750	1.819 (1.507 to 2.035)	0.4 (0.289 to 0.493)	0.01	acid phosphatase aphA - A. niger	AN9186.3
An13g01920	1.698 (1.153 to 2.327)	0.773 (0.679 to 0.847)	0.03	strong similarity to acetyl-CoA C-acetyltransferase precursor - R. norvegicus	AN10512.3
An13g01960	1.35 (1.281 to 1.446)	0.516 (0.326 to 0.719)	0.03	strong similarity to alcohol dehydrogenase alkJ - Pseudomonas putida	
An13g02130	0.571 (0.455 to 0.671)	1.517 (1.329 to 1.723)	0.01	strong similarity to aspartic proteinase Yps3p - .S cerevisiae	AN6487.3
An13g02390	0.515 (0.459 to 0.555)	2.041 (1.445 to 2.449)	0.01	similarity to fluconazole resistance protein FLU1 - C. albicans	AN8534.3
An13g02480	1.494 (1.187 to 2.114)	0.649 (0.527 to 0.813)	0.03	strong similarity to polyamine oxidase PAO - Zea mays	
An13g02940	0.035 (0.025 to 0.042)	2.409 (1.958 to 2.824)	0	strong similarity to enoyl reductase lovC - A. terreus	
An13g02970	0.061 (0.059 to 0.064)	3.594 (1.936 to 5.906)	0	weak similarity to the hypothetical protein An08g03800 - A. niger	
An13g02980	0.033 (0.017 to 0.072)	2.32 (1.928 to 2.835)	0	similarity to the hypothetical protein An01g08440 - A. niger	
An13g02990	0.184 (0.125 to 0.306)	2.594 (1.694 to 4.497)	0.01	similarity to the hypothetical protein An01g00290 - A. niger	
An13g03000	0.219 (0.172 to 0.252)	2.78 (1.748 to 4.729)	0.01	strong similarity to n-alkane-inducible cytochrome P450 protein ALK1 - Yarrowia lipolytica	AN6057.3
An13g03040	0.040 (0.03 to 0.055)	2.433 (1.945 to 3.769)	0	strong similarity to enniatin synthetase esyn1 - Fusarium scirpi	
An13g03050	0.133 (0.098 to 0.222)	2.32 (1.778 to 2.89)	0	weak similarity to the hypothetical protein An04g04070 - A. niger	AN9209.3
An13g03150	1.639 (1.201 to 2.068)	0.61 (0.516 to 0.799)	0.02	strong similarity to branched-chain alpha-ketoacid dehydrogenase (BCKDH) E1 beta subunit - Gallus gallus	AN8559.3
An13g03220	1.764 (1.351 to 2.493)	0.495 (0.431 to 0.649)	0.01	strong similarity to vacuolar polyamine transporter TPO1 - .S cerevisiae	
An13g03950	1.468 (1.206 to 1.782)	0.675 (0.531 to 0.794)	0.02	similarity to the hypothetical protein An09g00320 - A. niger	
An13g03970	0.685 (0.505 to 0.799)	1.393 (1.201 to 1.552)	0.02	similarity to glutathione-dependent formaldehyde dehydrogenase FDH – M. marinus	AN8406.3
An14g00260	1.333 (1.252 to 1.389)	0.646 (0.597 to 0.748)	0	similarity to stimulatory GDP/GTP exchange protein GDS - Bos taurus	AN10882.3
An14g00860	0.678 (0.558 to 0.751)	1.543 (1.249 to 1.958)	0.02	strong similarity to triacylglycerol lipase 5 precursor LIP5 - C. rugosa	
An14g01130	0.193 (0.187 to 0.197)	1.916 (1.803 to 2.094)	0	strong similarity to rhamnogalacturonase B precursor rhgB - A. aculeatus	AN7135.3
An14g01370	1.968 (1.516 to 3.111)	0.443 (0.413 to 0.484)	0.01	hypothetical protein	
An14g01420	1.184 (1.129 to 1.256)	0.561 (0.43 to 0.871)	0.04	strong similarity to probable membrane protein YMR221c - .S cerevisiae	AN7138.3
An14g01440	1.543 (1.368 to 1.935)	0.529 (0.451 to 0.632)	0.01	strong similarity to succinate-semialdehyde dehydrogenase SSADH - R. norvegicus	AN7141.3
An14g02590	1.306 (1.085 to 1.491)	0.632 (0.488 to 0.915)	0.04	strong similarity to GABA permease gabA - A. nidulans	AN7150.3
An14g02670	0.617 (0.535 to 0.688)	1.45 (1.312 to 1.532)	0	strong similarity to endoglucanase IV egl4 - Trichoderma reesei	
An14g02720	0.317 (0.196 to 0.467)	1.591 (1.533 to 1.688)	0.01	strong similarity to neutral amino acid permease mtr - Neurospora crassa	AN3207.3
An14g02740	0.043 (0.040 to 0.045)	2.156 (1.955 to 2.422)	0	strong similarity to high affinity glucose transporter HGT1 - Kluyveromyces lactis	

An14g03030	1.444 (1.149 to 1.642)	0.663 (0.466 to 0.851)	0.03	similarity to spermidine/spermine N(1)-acetyltransferase SAT - H. sapiens	AN2479.3
An14g03310	1.517 (1.453 to 1.638)	0.503 (0.457 to 0.547)	0	strong similarity to D-amino acid oxidase DAO1 patent EP0969088-A/2 – T. variabilis	AN7187.3
An14g03870	1.568 (1.43 to 1.653)	0.345 (0.221 to 0.57)	0.01	strong similarity to cytochrome P450 monooxygenase avnA - A. parasiticus	
An14g04040	0.629 (0.488 to 0.881)	1.326 (1.119 to 1.74)	0.04	strong similarity to hypothetical protein SPAC1093.01 with conserved domain PF01535 DUF17 - S pombe (mRNA)	AN10285.3
An14g04190	1.583 (1.434 to 1.928)	0.556 (0.543 to 0.566)	0	strong similarity to 1 4-alpha-glucan branching enzyme glc3 - .S cerevisiae	AN2314.3
An14g04280	0.136 (0.098 to 0.182)	2.067 (1.818 to 2.339)	0	strong similarity to quinate transport protein QUTD - A. nidulans	AN7667.3
An14g04370	0.27 (0.254 to 0.281)	1.993 (1.719 to 2.354)	0	pectin lyase A precursor - A. niger	AN2331.3
An14g04530	0.5 (0.481 to 0.518)	1.768 (1.482 to 2.414)	0.01	similarity to hypothetical protein actVA-ORF4 - Streptomyces coelicolor	
An14g04640	1.52 (1.437 to 1.657)	0.486 (0.39 to 0.563)	0	weak similarity to hypothetical protein F10B6.27 - Arabidopsis thaliana	AN6885.3
An14g04710	2.292 (1.806 to 3.621)	0.13 (0.0976 to 0.194)	0	aspartic proteinase aspergillopepsin I pepA - A. niger	AN6888.3
An14g04910	0.64 (0.545 to 0.75)	1.513 (1.25 to 2.201)	0.02	weak similarity to GABA-A receptor epsilon-like subunit Epsilon - R. norvegicus	AN6899.3
An14g05670	0.444 (0.408 to 0.474)	2.593 (1.526 to 3.567)	0.01	similarity to AK-toxin regulating protein AktR-1 - A. alternata	AN2673.3
An14g05910	1.597 (1.249 to 2.377)	0.633 (0.551 to 0.751)	0.02	strong similarity to mannosyltransferase ALG2 - .S cerevisiae	AN6874.3
An14g06340	1.421 (1.185 to 1.631)	0.66 (0.538 to 0.815)	0.02	strong similarity to hydroxyacylglutathione hydrolase RSP29 - R. norvegicus	AN6840.3
An14g06500	0.119 (0.063 to 0.313)	2.056 (1.687 to 2.747)	0.01	strong similarity to glycerone kinase isoform I DAK1 - Schizo.S pombe	AN0034.3
An14g06950	0.364 (0.344 to 0.401)	2.44 (1.599 to 3.264)	0	strong similarity to fluconazole resistance protein FLU1 - C. albicans	AN8089.3
An14g07200	2.991 (1.307 to 4.851)	0.667 (0.645 to 0.693)	0.03	strong similarity to catalase catC - A. nidulans	
An15g00410	1.583 (1.386 to 1.709)	0.533 (0.443 to 0.614)	0	strong similarity to acetate-inducible gene aciA - Emericella nidulans	AN6525.3
An15g00610	1.507 (1.307 to 1.946)	0.636 (0.537 to 0.693)	0.01	strong similarity to imidazoleglycerolphosphate dehydratase igh – T. harzianum	AN6536.3
An15g00640	1.462 (1.301 to 1.831)	0.666 (0.618 to 0.699)	0.01	strong similarity to hypothetical NADH oxidoreductase complex I subunit - C. elegans	
An15g01420	1.558 (1.256 to 1.995)	0.576 (0.377 to 0.744)	0.03	strong similarity to the glucosidase I Cwh41 - .S cerevisiae	AN6606.3
An15g01500	2.242 (1.465 to 3.478)	0.359 (0.211 to 0.535)	0.02	strong similarity to the fructose symporter FSY1 - .S pastorianus	AN2794.3
An15g01620	0.542 (0.49 to 0.604)	3.733 (1.396 to 7.011)	0.03	weak similarity to mucin-like protein MUC1 - .S cerevisiae	AN6625.3
An15g01780	1.444 (1.286 to 1.547)	0.66 (0.591 to 0.714)	0	strong similarity to 2-methylcitrate dehydratase PrpD - Salmonella typhimurium	AN6639.3
An15g02250	0.5 (0.426 to 0.544)	2.05 (1.456 to 2.898)	0.01	hypothetical protein	
An15g02300	0.537 (0.504 to 0.602)	1.706 (1.398 to 2.511)	0.01	arabinofuranosidase B abfB from patent EP506190-A - A. niger	AN1571.3
An15g03800	3.121 (1.663 to 6.425)	0.295 (0.273 to 0.337)	0.01	strong similarity to the EST an_3228 - A. niger	AN7539.3
An15g03940	2.133 (1.814 to 2.721)	0.165 (0.142 to 0.186)	0	strong similarity to monosaccharide transporter Mst-1 - Amanita muscaria	AN1797.3
An15g06280	0.415 (0.358 to 0.452)	1.934 (1.548 to 2.619)	0	strong similarity to the Aspergillopepsin I precursor PepA - A. niger (truncated ORF)	AN2157.3
An15g06350	1.419 (1.294 to 1.655)	0.618 (0.493 to 0.706)	0.01	weak similarity to the splicing coactivator subunit SRm300 - H. sapiens	AN2151.3
An15g06420	0.516 (0.37 to 0.615)	1.641 (1.385 to 2.297)	0.02	weak similarity to urease accessory gene ureD - B. sp.	AN2144.3
An15g06470	1.307 (1.229 to 1.426)	0.542 (0.317 to 0.771)	0.05	similarity to signal sequence receptor alpha chain - Canis lupus familiaris	AN2140.3
An15g07070	1.835 (1.63 to 2.294)	0.351 (0.321 to 0.37)	0	strong similarity to cyanate lyase (cynS) - E. coli	AN7331.3
An15g07460	0.608 (0.53 to 0.696)	1.501 (1.304 to 1.925)	0.01	strong similarity to oligopeptide transporter (OPT1) - C. albicans	AN7702.3
An15g07510	2.285 (1.454 to 5.52)	0.509 (0.447 to 0.546)	0.04	strong similarity to peptide transport gene CaPTR2 - C. albicans	AN8915.3
An15g07560	1.748 (1.312 to 2.035)	0.658 (0.627 to 0.688)	0.01	similarity to hypothetical protein (dag11) - Agaricus bisporus	AN7378.3

An15g07730	1.579 (1.259 to 2.413)	0.679 (0.594 to 0.741)	0.03	strong similarity to 3-isopropylmalate dehydratase (leu1) - Rhizopus niveus	
An16g00670	0.0704 (0.054 to 0.11)	2.285 (1.89 to 3.067)	0	weak similarity to transferrin - Oncorhynchus mykiss	
An16g00960	1.461 (1.336 to 1.601)	0.62 (0.572 to 0.664)	0	strong similarity to actin-modulating protein severin - Dictyostelium discoideum	AN1306.3
An16g01000	4.059 (1.83 to 14)	0.153 (0.145 to 0.17)	0.01	strong similarity to phthalate transporter (ophD) - Burkholderia cepacia	
An16g01030	2.228 (1.519 to 4.205)	0.467 (0.451 to 0.481)	0.02	strong similarity to phenylacetate hydroxylase pahA - P. chrysogenum	AN8078.3
An16g01490	0.62 (0.537 to 0.816)	1.4 (1.184 to 1.718)	0.02	similarity to ubiquitin carboxyl-terminal hydrolase uch2 - Schizo.S pombe	AN8074.3
An16g01600	1.412 (1.228 to 1.589)	0.682 (0.611 to 0.772)	0.01	similarity to monosaccharide transporter 1 pmt1 - Petunia hybrida	AN4621.3
An16g01610	1.343 (1.265 to 1.441)	0.65 (0.606 to 0.735)	0	weak similarity to gamma-butyrobetaine hydroxylase BBH - R. norvegicus	AN4620.3
An16g01630	0.649 (0.54 to 0.846)	1.514 (1.154 to 2.234)	0.04	strong similarity to enoyl reductase lovC - A. terreus	
An16g01780	0.68 (0.622 to 0.735)	1.604 (1.265 to 2.029)	0.01	similarity to CAP22 gene product - Colletotrichum gloeosporioides	
An16g01820	1.297 (1.225 to 1.385)	0.614 (0.387 to 0.775)	0.05	strong similarity to inorganic phosphate transporter and regulator of Pho81p PHO88 - .S cerevisiae	AN8040.3
An16g02000	1.577 (1.423 to 1.778)	0.257 (0.168 to 0.577)	0.02	strong similarity to gamma-amino-n-butyrate (GABA) permease gabA - A. nidulans	AN2962.3
An16g02240	0.562 (0.451 to 0.647)	1.838 (1.353 to 2.384)	0.01	similarity to phenylcoumaran benzylic ether reductase PT1 - Pinus taeda	
An16g02560	1.67 (1.304 to 2.382)	0.658 (0.608 to 0.696)	0.02	strong similarity to probable beta-lactamase XF1621 - Xylella fastidiosa	
An16g02760	0.268 (0.213 to 0.324)	1.914 (1.676 to 2.338)	0	strong similarity to hypothetical protein BH0842 - B. halodurans	AN8149.3
An16g03430	1.372 (1.225 to 1.64)	0.657 (0.502 to 0.775)	0.02	strong similarity to the novobiocin biosynthetic gene novR - Streptomyces spheroides	AN5007.3
An16g03690	1.709 (1.397 to 2.429)	0.444 (0.342 to 0.603)	0.01	strong similarity to CCC1 protein - .S cerevisiae	AN4990.3
An16g03700	1.424 (1.326 to 1.601)	0.221 (0.125 to 0.674)	0.04	strong similarity to Phospholipase B from patent US6146869-A - A. oryzae	
An16g03710	0.438 (0.412 to 0.474)	2.015 (1.526 to 2.503)	0	weak similarity to cDNA clone i7e03a1.f1 - A. nidulans	AN4989.3
An16g04060	1.387 (1.361 to 1.424)	0.604 (0.585 to 0.639)	0	similarity to hypothetical protein BAB55393.1 - H. sapiens	
An16g04160	1.407 (1.259 to 1.586)	0.625 (0.496 to 0.741)	0.01	strong similarity to the galactokinase Gal1 - .S cerevisiae	AN4957.3
An16g05290	1.657 (1.29 to 2.602)	0.534 (0.369 to 0.71)	0.03	strong similarity to hypothetical purine-cytosine permease FCY2 - .S cerevisiae	AN10767.3
An16g05390	0.0943 (0.076 to 0.14)	1.877 (1.86 to 1.911)	0	strong similarity to hypothetical RTS beta protein - H. sapiens	AN6035.3
An16g05880	1.866 (1.178 to 2.453)	0.732 (0.65 to 0.822)	0.03	strong similarity to neutral amino acid transporter Mtr1 - Neurospora crassa	
An16g05990	0.551 (0.527 to 0.586)	1.689 (1.414 to 2.092)	0	strong similarity to 5 EST of cDNA clone an_3135 - A. niger	
An16g06010	2.11 (1.265 to 3.015)	0.635 (0.538 to 0.735)	0.02	similarity to phosphoglycerate mutase pgm - Zymomonas mobilis	
An16g06140	0.245 (0.216 to 0.313)	2.036 (1.687 to 2.548)	0	strong similarity to hypothetical protein B24P11.210 - Neurospora crassa	
An16g06720	0.499 (0.309 to 0.701)	1.78 (1.299 to 2.103)	0.02	strong similarity to HC-toxin peptide synthase HTS - Cochliobolus carbonum	
An16g06740	0.609 (0.508 to 0.713)	1.339 (1.287 to 1.441)	0.01	strong similarity to cadmium resistance protein YCF1 - .S cerevisiae (truncated ORF)	
An16g06990	0.163 (0.147 to 0.182)	2.061 (1.818 to 2.514)	0	endo-polygalacturonase A pgaA - A. niger (possible sequencing error) (mRNA)	
An16g07390	1.52 (1.388 to 1.704)	0.548 (0.451 to 0.612)	0	strong similarity to endoplasmatic reticulum signal peptidase subunit SPC2 - .S cerevisiae	AN1525.3
An16g07950	0.592 (0.531 to 0.718)	1.763 (1.282 to 2.326)	0.01	hypothetical protein	
An16g08080	0.464 (0.41 to 0.494)	1.784 (1.506 to 2.335)	0	strong similarity to transporter of Patent WO0100804 - Corynebacterium glutamicum	AN1481.3
An16g08150	1.54 (1.282 to 2.085)	0.557 (0.49 to 0.718)	0.01	strong similarity to dipeptidyl-peptidase V DPP V - A. fumigatus	AN10198.3
An16g08570	1.432 (1.269 to 1.761)	0.559 (0.328 to 0.731)	0.04	strong similarity to oligosaccharyl transferase (OTase) stt3 subunit - Schizo.S pombe	AN1455.3

An17g00210	1.488 (1.305 to 1.728)	0.368 (0.235 to 0.695)	0.02	strong similarity to hypothetical oxidoreductase SPBC113.03 - Schizo.S pombe	AN2208.3
An17g00550	1.68 (1.285 to 2.701)	0.684 (0.644 to 0.715)	0.03	weak similarity to the dTDP-glucose 4 6-dehydratase protein_id CAB05932.1 - Streptococcus pneumoniae	AN2225.3
An17g00910	1.866 (1.71 to 2.085)	0.197 (0.148 to 0.29)	0	strong similarity to 4-aminobutyrate transaminase (gatA) - A. nidulans	AN2248.3
An17g01000	0.599 (0.46 to 0.701)	1.799 (1.299 to 2.174)	0.01	strong similarity to hypothetical protein sll1024 - Synechocystis sp.	AN2251.3
An17g01770	0.516 (0.37 to 0.663)	1.618 (1.337 to 1.984)	0.01	strong similarity to multidrug resistance protein MDR1 - A. fumigatus (truncated ORF)	AN2300.3
An17g01925	0.653 (0.542 to 0.768)	1.408 (1.232 to 1.782)	0.02	strong similarity to the ion homeostasis modulating kinase Hal4 - .S cerevisiae (mRNA)	AN8830.3
An18g00260	1.258 (1.19 to 1.334)	0.618 (0.448 to 0.81)	0.03	strong similarity to cytochrome P450 monooxygenase avnA - A. parasiticus	
An18g00340	2.034 (1.351 to 2.594)	0.6 (0.551 to 0.649)	0.01	hypothetical protein	
An18g00400	2.324 (1.793 to 3.315)	0.179 (0.166 to 0.207)	0	similarity to inorganic phosphate transporter PT1 - Nicotiana tabacum	AN2864.3
An18g00740	1.363 (1.232 to 1.527)	0.672 (0.613 to 0.768)	0.01	hypothetical protein	AN3244.3
An18g01320	0.577 (0.497 to 0.773)	1.28 (1.227 to 1.316)	0.01	strong similarity to extracellular protease precursor BAR1 - .S cerevisiae	
An18g02020	1.463 (1.282 to 1.882)	0.624 (0.529 to 0.718)	0.01	putative disulfide isomerase tigA - A. niger	AN0075.3
An18g02600	0.775 (0.763 to 0.791)	1.741 (1.209 to 2.851)	0.05	hypothetical protein	AN5399.3
An18g02690	0.455 (0.391 to 0.527)	1.798 (1.473 to 2.165)	0	strong similarity to dihydrogeodin oxidase DHGO - A. terreus	
An18g02700	0.61 (0.574 to 0.632)	1.95 (1.368 to 2.479)	0.01	similarity to cytochrome P-450 cyp509A1 - Cunninghamella elegans	AN1598.3
An18g02710	0.362 (0.305 to 0.488)	1.539 (1.512 to 1.566)	0	strong similarity to ent-Kaurene synthase - Phaeosphaeria sp.	AN1594.3
An18g02730	0.384 (0.325 to 0.431)	1.963 (1.569 to 2.498)	0	similarity to transmembrane protein PTH11 - M. grisea	AN3257.3
An18g03570	0.395 (0.279 to 0.71)	1.658 (1.29 to 2.625)	0.03	beta-glucosidase bgl1 - A. niger	AN4102.3
An18g03920	1.672 (1.416 to 2.164)	0.564 (0.54 to 0.584)	0.01	strong similarity to the defender against apoptotic cell death DAD1 - H. sapiens	AN4031.3
An18g03930	0.619 (0.531 to 0.686)	1.347 (1.314 to 1.376)	0	similarity to cutinase transcription factor 1 alpha CTFalpha - Fusarium solani	AN10504.3
An18g04040	2.225 (1.409 to 3.43)	0.455 (0.394 to 0.591)	0.01	similarity to the alkaline phosphatase (9A1A) patent WO9748416-A1 - Methanococcus igneus	AN4025.3
An18g04160	1.478 (1.384 to 1.529)	0.392 (0.202 to 0.616)	0.03	strong similarity to dihydroxy-acid dehydratase Ilv3 - .S cerevisiae	AN4058.3
An18g04420	1.44 (1.175 to 1.99)	0.566 (0.42 to 0.825)	0.03	similarity to glycogenin 1 GYG - H. sapiens	AN4082.3
An18g04810	0.367 (0.342 to 0.413)	1.888 (1.587 to 2.219)	0	strong similarity to PATENTPROT R59792 exo-polygalacturonase PGX - A. tubingensis	
An18g05910	1.435 (1.259 to 1.64)	0.644 (0.548 to 0.741)	0.01	strong similarity to probable glycosyl transferase SPCC330.08 - Schizo.S pombe	AN5725.3
An18g06220	1.459 (1.281 to 1.779)	0.666 (0.606 to 0.719)	0.01	strong similarity to alpha-mannosidase MNS1 - .S cerevisiae	AN5748.3

Supplementary table 8. *A. nidulans* genes identified as differentially expressed. Normalized signal shows the data values for each gene after RMA preprocessing and normalization of the raw signal averaged over the values obtained from each replicate experiment for the given condition.

Loccus tag	normalized signal fructose	normalized signal galacturonic acid	ANOVA p-value	Gene description	*A. niger* ortholog
AN0036	0.502 (0.261 to 0.894)	1.975 (1.33 to 3.796)	0.03	(AN0036.2) hypothetical protein	
AN0058	0.287 (0.095 to 0.487)	1.646 (1.448 to 1.838)	0.04	(AN0058.2) hypothetical protein	An07g00760
AN0197	0.414 (0.233 to 0.966)	1.994 (1.161 to 2.753)	0.03	(AN0197.2) hypothetical protein	

AN0259	1.411 (1.143 to 1.645)	0.655 (0.482 to 0.809)	0.00	(AN0259.2) hypothetical protein	An01g04710
AN0401	0.463 (0.307 to 0.83)	1.583 (1.174 to 2.552)	0.04	(AN0401.2) hypothetical protein	
AN0402	0.513 (0.423 to 0.815)	1.475 (1.287 to 1.658)	0.01	(AN0402.2) hypothetical protein	
AN0498	0.497 (0.396 to 0.983)	1.693 (1.233 to 2.993)	0.03	(AN0498.2) hypothetical protein	An01g14690
AN0656	0.462 (0.166 to 0.619)	2.927 (1.195 to 5.513)	0.05	(AN0656.2) hypothetical protein	
AN0746	0.644 (0.54 to 0.874)	1.39 (1.148 to 1.529)	0.01	(AN0746.2) hypothetical protein	An01g12220
AN0761	1.82 (0.856 to 4.042)	0.322 (0.217 to 0.512)	0.01	(AN0761.2) hypothetical protein	An16g01250
AN0824	0.592 (0.482 to 0.722)	1.417 (1.268 to 1.821)	0.00	(AN0824.2) conserved hypothetical protein	An01g12960
AN0875	0.405 (0.266 to 0.803)	1.595 (1.196 to 2.017)	0.04	(AN0875.2) hypothetical protein	An01g13580
AN0941	1.376 (1.185 to 1.524)	0.583 (0.447 to 0.808)	0.02	(AN0941.2) hypothetical protein	An01g10930
AN0987	0.498 (0.282 to 0.688)	1.999 (1.251 to 2.876)	0.03	(AN0987.2) hypothetical protein	
AN1109	0.057 (0.027 to 0.075)	2.215 (1.87 to 2.674)	0.00	(AN1109.2) hypothetical protein	An08g04040
AN1276	0.405 (0.205 to 0.601)	1.519 (1.345 to 1.639)	0.03	(AN1276.2) hypothetical protein	An08g01720
AN1277	0.474 (0.355 to 0.739)	1.444 (1.13 to 1.802)	0.03	(AN1277.2) hypothetical protein	An08g01710
AN1320	0.313 (0.2 to 0.634)	2.128 (1.436 to 2.883)	0.00	(AN1320.2) hypothetical protein	
AN1396	0.636 (0.508 to 0.787)	1.441 (1.212 to 1.634)	0.00	(AN1396.2) hypothetical protein	An08g00210
AN1426	1.499 (1.189 to 2.099)	0.695 (0.618 to 0.892)	0.03	(AN1426.2) hypothetical protein	An16g09010
AN1477	0.633 (0.475 to 0.975)	1.398 (0.993 to 1.734)	0.03	(AN1477.2) hypothetical protein	
AN1566	0.394 (0.194 to 0.602)	2.182 (1.393 to 4.046)	0.04	(AN1566.2) hypothetical protein	
AN1579	0.654 (0.517 to 0.811)	1.502 (1.175 to 2.151)	0.01	(AN1579.2) predicted protein	
AN1619	0.448 (0.325 to 0.865)	1.57 (1.208 to 2.356)	0.02	(AN1619.2) hypothetical protein	An02g14730
AN1641	0.32 (0.208 to 0.808)	2.39 (0.861 to 3.619)	0.04	(AN1641.2) hypothetical protein	An18g01040
AN1738	0.585 (0.432 to 1.022)	1.216 (1.073 to 1.451)	0.05	(AN1738.2) hypothetical protein	
AN1797	1.601 (1.259 to 1.917)	0.453 (0.364 to 0.704)	0.02	(AN1797.2) hypothetical protein	An15g03940
AN1895	0.23 (0.142 to 0.466)	1.614 (1.424 to 1.761)	0.02	(AN1895.2) MAAI_EMENI Maleylacetoacetate isomerase (MAAI)	An11g02160
AN1896	0.17 (0.0888 to 0.525)	1.729 (1.494 to 2.282)	0.04	(AN1896.2) hypothetical protein similar to fumarylacetoacetate hydrolase	An11g02170
AN1897	0.215 (0.129 to 0.38)	1.864 (1.433 to 2.395)	0.01	(AN1897.2) HGD_EMENI Homogentisate 1,2-dioxygenase	An11g02180
AN1898	0.066 (0.029 to 0.271)	1.866 (1.711 to 1.972)	0.02	(AN1898.2) hypothetical protein	An11g02190
AN1899	0.085 (0.013 to 0.241)	2.851 (1.629 to 3.799)	0.03	(AN1899.2) hypothetical protein	An11g02200
AN1917	0.538 (0.465 to 0.604)	1.659 (1.323 to 1.944)	0.00	(AN1917.2) hypothetical protein	An11g02540
AN1918	0.595 (0.535 to 0.663)	1.389 (1.3 to 1.509)	0.00	(AN1918.2) hypothetical protein similar to phosphoenolpyruvate carboxykinase	An11g02550
AN2217	0.383 (0.145 to 0.561)	1.588 (1.375 to 1.753)	0.03	(AN2217.2) hypothetical protein	An17g00300
AN2331	0.249 (0.156 to 0.522)	2.215 (1.464 to 4.166)	0.02	(AN2331.2) hypothetical protein	An14g04370
AN2351	2.099 (0.922 to 3.082)	0.719 (0.615 to 0.927)	0.05	(AN2351.2) hypothetical protein	
AN2395	0.659 (0.556 to 0.755)	1.487 (1.27 to 1.792)	0.01	(AN2395.2) hypothetical protein	An02g00610
AN2402	0.38 (0.21 to 0.525)	1.779 (1.383 to 2.034)	0.00	(AN2402.2) hypothetical protein	An09g01210
AN2438	1.341 (1.188 to 1.576)	0.641 (0.583 to 0.73)	0.00	(AN2438.2) hypothetical protein	An11g00490

AN2533	0.171 (0.117 to 0.34)	2.085 (1.584 to 3.105)	0.01	(AN2533.2) hypothetical protein	
AN2557	1.531 (1.15 to 2.055)	0.594 (0.43 to 0.97)	0.04	(AN2557.2) hypothetical protein	An03g01920
AN2601	0.123 (0.047 to 0.171)	2.084 (1.749 to 2.43)	0.01	(AN2601.2) hypothetical protein	An06g00620
AN2602	0.5 (0.354 to 0.987)	1.653 (1.229 to 3.197)	0.01	(AN2602.2) hypothetical protein	
AN2604	0.396 (0.191 to 0.574)	1.717 (1.185 to 2.095)	0.03	(AN2604.2) predicted protein	
AN2609	0.479 (0.37 to 0.753)	1.417 (1.221 to 1.506)	0.01	(AN2609.2) hypothetical protein	
AN2621	0.541 (0.377 to 0.646)	1.421 (1.263 to 1.492)	0.01	(AN2621.2) ACVS_EMENI N-(5-amino-5-carboxypentanoyl)-L-cysteinyl-D-valine synthase (Delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase) (ACV synthetase) (ACVS)	
AN2622	0.549 (0.449 to 0.713)	1.638 (1.205 to 2.767)	0.04	(AN2622.2) IPNS_EMENI Isopenicillin N synthetase (IPNS) (Isopenicillin N synthase)	
AN2637	0.694 (0.494 to 0.84)	1.398 (1.012 to 1.622)	0.02	(AN2637.2) predicted protein	An01g13470
AN2652	0.519 (0.227 to 0.865)	1.43 (1.115 to 1.658)	0.01	(AN2652.2) hypothetical protein	An08g10210
AN2704	0.653 (0.511 to 0.773)	1.327 (1.159 to 1.498)	0.02	(AN2704.2) hypothetical protein	
AN2794	1.487 (1.269 to 1.632)	0.621 (0.575 to 0.667)	0.00	(AN2794.2) hypothetical protein	An15g01500
AN2828	0.642 (0.531 to 0.885)	1.53 (1.179 to 2.24)	0.01	(AN2828.2) hypothetical protein	
AN2858	0.11 (0.0714 to 0.138)	2.062 (1.847 to 2.334)	0.00	(AN2858.2) hypothetical protein	An02g07710
AN2859	0.157 (0.099 to 0.326)	1.899 (1.585 to 2.395)	0.01	(AN2859.2) hypothetical protein	An02g07720
AN2860	0.047 (0.023 to 0.070)	2.068 (1.792 to 2.525)	0.00	(AN2860.2) hypothetical protein	
AN2884	1.312 (1.07 to 1.632)	0.65 (0.505 to 1.004)	0.04	(AN2884.2) predicted protein	An02g07410
AN3085	0.559 (0.39 to 0.682)	1.511 (1.167 to 1.752)	0.00	(AN3085.2) hypothetical protein	An02g08050
AN3195	0.095 (0.048 to 0.326)	2.226 (1.613 to 3.291)	0.03	(AN3195.2) hypothetical protein	An14g02910
AN3196	0.297 (0.204 to 0.358)	2.206 (1.598 to 2.712)	0.01	(AN3196.2) hypothetical protein	An14g02920
AN3197	0.161 (0.097 to 0.667)	2.082 (1.564 to 2.986)	0.02	(AN3197.2) hypothetical protein	
AN3229	1.346 (0.684 to 1.768)	0.672 (0.528 to 1.018)	0.02	(AN3229.2) hypothetical protein	
AN3265	1.805 (1.299 to 3.253)	0.467 (0.171 to 0.676)	0.04	(AN3265.2) hypothetical protein	
AN3303	2.064 (1.118 to 4.207)	0.406 (0.226 to 0.792)	0.05	(AN3303.2) predicted protein	
AN3357	1.539 (1.182 to 2.346)	0.408 (0.261 to 0.99)	0.05	(AN3357.2) hypothetical protein	An05g01290
AN3429	0.575 (0.487 to 0.787)	1.625 (1.252 to 1.961)	0.01	(AN3429.2) hypothetical protein	An11g10800
AN3505	0.61 (0.497 to 0.835)	1.611 (1.142 to 3.254)	0.04	(AN3505.2) hypothetical protein	An16g00170
AN3566	0.485 (0.2 to 0.623)	1.544 (1.245 to 2.133)	0.03	(AN3566.2) hypothetical protein similar to AF233287_1 alpha-mannosidase IC	
AN3567	0.299 (0.251 to 0.386)	1.851 (1.566 to 2.275)	0.00	(AN3567.2) hypothetical protein	
AN3641	0.468 (0.38 to 0.603)	1.456 (1.324 to 1.545)	0.01	(AN3641.2) hypothetical protein	An01g07300
AN3776	0.192 (0.094 to 0.734)	1.841 (1.363 to 2.954)	0.05	(AN3776.2) hypothetical protein	An13g03680
AN3916	0.464 (0.402 to 0.62)	1.54 (1.352 to 1.747)	0.00	(AN3916.2) hypothetical protein	
AN4003	0.125 (0.090 to 0.259)	2.027 (1.695 to 2.701)	0.00	(AN4003.2) hypothetical protein	An01g03030
AN4025	1.496 (1.403 to 1.636)	0.522 (0.392 to 0.603)	0.00	(AN4025.2) hypothetical protein	An18g04040
AN4119	1.324 (1.157 to 1.482)	0.63 (0.529 to 0.875)	0.02	(AN4119.2) hypothetical protein	An05g01840

AN4131	0.726 (0.543 to 0.948)	1.477 (1.103 to 1.78)	0.05	(AN4131.2) hypothetical protein	
AN4180	0.377 (0.31 to 0.562)	1.562 (1.441 to 1.813)	0.01	(AN4180.2) hypothetical protein	An05g00850
AN4348	0.718 (0.536 to 0.889)	1.493 (0.969 to 2.062)	0.03	(AN4348.2) hypothetical protein	
AN4355	0.558 (0.389 to 0.907)	1.396 (1.136 to 1.69)	0.04	(AN4355.2) hypothetical protein	An01g01520
AN4420	0.702 (0.555 to 1.042)	1.436 (1.101 to 1.654)	0.02	(AN4420.2) hypothetical protein	
AN4424	0.532 (0.311 to 0.644)	1.793 (1.303 to 2.26)	0.01	(AN4424.2) hypothetical protein	An04g08560
AN4641	2.17 (1.769 to 3.244)	0.151 (0.092 to 0.209)	0.01	(AN4641.2) hypothetical protein	
AN4792	0.513 (0.396 to 0.586)	1.936 (1.366 to 2.391)	0.01	(AN4792.2) hypothetical protein	An11g09520
AN4860	0.066 (0.046 to 0.089)	1.935 (1.853 to 2.038)	0.00	(AN4860.2) hypothetical protein	An02g12505
AN5040	0.607 (0.447 to 0.976)	1.555 (0.905 to 2.181)	0.04	(AN5040.2) predicted protein	An15g02450
AN5050	0.603 (0.446 to 0.708)	1.526 (1.278 to 1.807)	0.01	(AN5050.2) hypothetical protein	An02g08230
AN5109	0.539 (0.228 to 0.708)	1.616 (1.164 to 1.924)	0.04	(AN5109.2) hypothetical protein	An07g10290
AN5146	0.59 (0.484 to 0.78)	1.375 (1.281 to 1.528)	0.01	(AN5146.2) hypothetical protein	
AN5187	1.589 (1.132 to 1.753)	0.544 (0.266 to 0.777)	0.00	(AN5187.2) hypothetical protein	An07g09740
AN5330	0.39 (0.313 to 0.48)	2.108 (1.443 to 2.977)	0.01	(AN5330.2) hypothetical protein	
AN5331	0.608 (0.491 to 0.836)	1.285 (1.128 to 1.476)	0.03	(AN5331.2) hypothetical protein	An06g00600
AN5379	0.592 (0.452 to 0.731)	1.604 (1.272 to 1.933)	0.01	(AN5379.2) hypothetical protein	An03g00380
AN5408	0.483 (0.376 to 0.625)	1.588 (1.344 to 1.898)	0.01	(AN5408.2) predicted protein	An03g00790
AN5461	0.559 (0.441 to 0.671)	1.425 (1.202 to 1.631)	0.00	(AN5461.2) predicted protein	An01g00570
AN5589	0.628 (0.42 to 0.867)	1.344 (1.131 to 1.522)	0.03	(AN5589.2) hypothetical protein	An04g04890
AN5604	0.547 (0.403 to 0.861)	1.325 (1.038 to 1.729)	0.04	(AN5604.2) hypothetical protein similar to AF525021_1 fructose-1,6-bisphosphatase	An04g05300
AN5634	0.417 (0.326 to 0.668)	1.577 (1.214 to 1.831)	0.02	(AN5634.2) ACEA_EMENI Isocitrate lyase (Isocitrase) (ICL)	An01g09270
AN5669	0.661 (0.507 to 0.794)	1.355 (1.209 to 1.622)	0.01	(AN5669.2) hypothetical protein	An06g01370
AN5698	0.593 (0.476 to 0.868)	1.438 (1.243 to 1.794)	0.02	(AN5698.2) hypothetical protein	An13g00590
AN5733	0.031 (0.015 to 0.054)	2.17 (1.956 to 2.491)	0.00	(AN5733.2) hypothetical protein	
AN5734	0.084 (0.060 to 0.135)	2.133 (1.796 to 2.572)	0.00	(AN5734.2) hypothetical protein	
AN5853	0.688 (0.265 to 0.879)	1.573 (1.158 to 2.093)	0.01	(AN5853.2) hypothetical protein	
AN5860	1.452 (1.254 to 1.57)	0.508 (0.365 to 0.69)	0.02	(AN5860.2) hypothetical protein similar to monosaccharide transporter	An02g03540
AN5885	1.35 (1.179 to 1.46)	0.573 (0.445 to 0.861)	0.02	(AN5885.2) hypothetical protein	An02g03260
AN5986	0.325 (0.193 to 0.715)	1.547 (1.238 to 2.075)	0.05	(AN5986.2) hypothetical protein	An16g04770
AN6035	0.044 (0.028 to 0.06)	2.115 (1.841 to 2.454)	0.00	(AN6035.2) hypothetical protein	An16g05390
AN6064	0.594 (0.561 to 0.652)	1.474 (1.348 to 1.592)	0.00	(AN6064.2) hypothetical protein	An12g04640
AN6095	0.399 (0.326 to 0.56)	1.655 (1.346 to 1.936)	0.01	(AN6095.2) hypothetical protein	An16g06950
AN6148	0.279 (0.148 to 0.947)	1.635 (1.063 to 2.118)	0.04	(AN6148.2) hypothetical protein	An12g03860
AN6274	1.654 (1.286 to 1.911)	0.197 (0.101 to 0.658)	0.03	(AN6274.2) hypothetical protein	An13g01130
AN6277	1.812 (1.213 to 2.265)	0.648 (0.479 to 0.75)	0.03	(AN6277.2) hypothetical protein	An02g01480

AN6411	0.482 (0.397 to 0.576)	2.402 (1.419 to 4.626)	0.02	(AN6411.2) hypothetical protein	
AN6412	0.669 (0.54 to 1.041)	1.409 (1.096 to 1.748)	0.04	(AN6412.2) hypothetical protein	An07g01940
AN6413	1.404 (1.201 to 1.681)	0.518 (0.38 to 0.804)	0.03	(AN6413.2) hypothetical protein	An02g00390
AN6442	1.571 (1.145 to 2.119)	0.698 (0.596 to 0.842)	0.03	(AN6442.2) hypothetical protein	An15g07550
AN6472	1.335 (1.067 to 1.68)	0.611 (0.321 to 0.825)	0.02	(AN6472.2) hypothetical protein	An04g09650
AN6624	1.308 (1.003 to 1.627)	0.549 (0.337 to 0.862)	0.03	(AN6624.2) hypothetical protein	An15g01640
AN6642	0.491 (0.368 to 0.728)	1.544 (1.27 to 2.221)	0.03	(AN6642.2) hypothetical protein	An15g01830
AN6653	0.263 (0.192 to 0.464)	1.618 (1.467 to 1.807)	0.01	(AN6653.2) MASY_EMENI Malate synthase, glyoxysomal	An15g01860
AN6693	0.715 (0.6 to 0.785)	1.531 (1.116 to 1.973)	0.02	(AN6693.2) hypothetical protein	
AN6751	0.09 (0.0513 to 0.248)	2.853 (1.723 to 3.798)	0.01	(AN6751.2) hypothetical protein	An02g00140
AN6774	0.401 (0.163 to 0.723)	1.773 (1.476 to 2.579)	0.00	(AN6774.2) hypothetical protein	An02g07580
AN6805	1.917 (1.007 to 2.6)	0.6 (0.491 to 0.966)	0.04	(AN6805.2) hypothetical protein	An02g07880
AN6856	1.543 (1.224 to 1.815)	0.623 (0.43 to 0.744)	0.01	(AN6856.2) hypothetical protein	An14g06050
AN7042	0.447 (0.343 to 0.685)	1.523 (1.283 to 2.143)	0.02	(AN7042.2) hypothetical protein	An14g00810
AN7061	0.452 (0.37 to 0.695)	1.476 (1.206 to 1.903)	0.01	(AN7061.2) hypothetical protein	
AN7062	0.434 (0.316 to 0.806)	1.678 (1.3 to 3.129)	0.01	(AN7062.2) hypothetical protein	
AN7119	0.488 (0.291 to 0.955)	1.848 (0.777 to 2.498)	0.01	(AN7119.2) hypothetical protein	
AN7156	0.12 (0.0572 to 0.389)	1.947 (1.64 to 2.748)	0.02	(AN7156.2) hypothetical protein	An03g05240
AN7287	0.198 (0.137 to 0.387)	1.729 (1.58 to 2.043)	0.01	(AN7287.2) hypothetical protein	An04g09030
AN7315	0.468 (0.391 to 0.492)	1.81 (1.497 to 2.331)	0.00	(AN7315.2) hypothetical protein	
AN7324	0.531 (0.357 to 0.655)	1.619 (1.287 to 2.413)	0.01	(AN7324.2) hypothetical protein	An08g04590
AN7358	0.686 (0.52 to 0.797)	1.478 (1.166 to 1.818)	0.02	(AN7358.2) hypothetical protein	An15g06700
AN7522	0.337 (0.204 to 0.752)	1.514 (1.283 to 1.819)	0.02	(AN7522.2) hypothetical protein	An08g09940
AN7667	0.035 (0.024to 0.073)	2.064 (1.714 to 2.467)	0.00	(AN7667.2) hypothetical protein	An14g04280
AN7735	0.7 (0.543 to 0.79)	1.405 (1.152 to 1.731)	0.01	(AN7735.2) hypothetical protein	An03g04190
AN7792	0.643 (0.471 to 0.792)	1.641 (1.249 to 2.551)	0.03	(AN7792.2) hypothetical protein	An09g01240
AN7828	0.0141 (0.01 to 0.019)	2.332 (1.828 to 2.723)	0.00	(AN7828.2) hypothetical protein	
AN7859	0.607 (0.334 to 1.302)	1.675 (0.87 to 2.385)	0.04	(AN7859.2) predicted protein	
AN7932	0.361 (0.206 to 0.62)	1.681 (1.088 to 2.376)	0.03	(AN7932.2) hypothetical protein	
AN7990	1.769 (1.509 to 2.08)	0.347 (0.229 to 0.477)	0.01	(AN7990.2) hypothetical protein	An11g06780
AN8043	1.805 (1.15 to 3.015)	0.665 (0.543 to 0.818)	0.03	(AN8043.2) hypothetical protein	An16g01850
AN8089	0.591 (0.378 to 0.665)	1.533 (1.309 to 1.929)	0.01	(AN8089.2) hypothetical protein	An14g06950
AN8149	0.465 (0.364 to 0.601)	1.611 (1.313 to 2.056)	0.00	(AN8149.2) hypothetical protein	An16g02760
AN8154	0.772 (0.653 to 0.909)	1.803 (0.937 to 2.554)	0.05	(AN8154.2) hypothetical protein	
AN8241	0.569 (0.482 to 0.664)	1.59 (1.2 to 2.128)	0.01	(AN8241.2) hypothetical protein similar to chitinase	An09g06400
AN8344	0.517 (0.325 to 0.615)	1.576 (1.204 to 1.8)	0.00	(AN8344.2) hypothetical protein	
AN8345	0.489 (0.398 to 0.723)	1.469 (1.127 to 1.743)	0.00	(AN8345.2) predicted protein	

AN8347	0.303 (0.254 to 0.347)	1.838 (1.54 to 2.065)	0.00	(AN8347.2) hypothetical protein	
AN8400	0.363 (0.264 to 0.672)	1.578 (1.367 to 2.179)	0.03	(AN8400.2) hypothetical protein	An11g09600
AN8403	0.294 (0.152 to 0.803)	1.44 (1.284 to 1.829)	0.05	(AN8403.2) hypothetical protein	
AN8404	0.019 (0.01 to 0.0841)	2.172 (1.871 to 2.734)	0.01	(AN8404.2) hypothetical protein	An19g00400
AN8416	1.485 (1.156 to 1.821)	0.668 (0.447 to 0.963)	0.01	(AN8416.2) hypothetical protein	An13g03930
AN8430	1.419 (1.135 to 1.844)	0.657 (0.499 to 0.757)	0.01	(AN8430.2) hypothetical protein	An18g00330
AN8433	1.279 (1.052 to 1.553)	0.631 (0.491 to 0.95)	0.05	(AN8433.2) hypothetical protein	
AN8515	1.477 (1.2 to 2.153)	0.616 (0.321 to 0.77)	0.05	(AN8515.2) hypothetical protein	
AN8518	1.446 (1.182 to 2.268)	0.669 (0.476 to 0.809)	0.04	(AN8518.2) hypothetical protein	
AN8612	0.637 (0.424 to 0.941)	1.323 (1.148 to 1.654)	0.05	(AN8612.2) hypothetical protein	
AN8646	1.377 (0.959 to 1.698)	0.66 (0.445 to 0.793)	0.02	(AN8646.2) hypothetical protein	
AN8755	0.64 (0.465 to 0.74)	1.308 (1.262 to 1.36)	0.01	(AN8755.2) hypothetical protein	An12g07630
AN8761	0.0123 (0.01 to 0.015)	2.226 (1.726 to 2.791)	0.00	(AN8761.2) hypothetical protein	An12g07500
AN8771	0.417 (0.186 to 0.638)	1.487 (0.939 to 1.742)	0.05	(AN8771.2) hypothetical protein	
AN8812	0.56 (0.453 to 0.659)	1.447 (1.306 to 1.678)	0.00	(AN8812.2) hypothetical protein	
AN8910	0.537 (0.461 to 0.622)	1.776 (1.305 to 2.215)	0.01	(AN8910.2) hypothetical protein	
AN8915	1.542 (1.15 to 2.002)	0.473 (0.38 to 0.681)	0.01	(AN8915.2) hypothetical protein	An15g07510
AN8977	0.503 (0.371 to 1.059)	1.554 (1.245 to 2.126)	0.03	(AN8977.2) hypothetical protein	An10g00900
AN8984	1.468 (0.906 to 1.817)	0.695 (0.218 to 0.875)	0.02	(AN8984.2) hypothetical protein	An14g05850
AN8994	0.473 (0.355 to 0.544)	1.754 (1.309 to 2.006)	0.00	(AN8994.2) hypothetical protein	
AN9007	0.52 (0.222 to 0.694)	1.603 (1.213 to 2.022)	0.04	(AN9007.2) hypothetical protein	An12g08630
AN9165	0.221 (0.185 to 0.268)	1.98 (1.665 to 2.469)	0.00	(AN9165.2) hypothetical protein	
AN9206	1.554 (0.771 to 3.793)	0.575 (0.36 to 0.663)	0.02	(AN9206.2) hypothetical protein	An12g02060
AN9211	0.657 (0.526 to 0.93)	1.587 (1.07 to 2.116)	0.04	(AN9211.2) predicted protein	
AN9276	0.646 (0.431 to 1.118)	2.164 (1.002 to 3.402)	0.03	(AN9276.2) hypothetical protein	
AN9305	1.447 (1.201 to 2.13)	0.718 (0.548 to 0.805)	0.02	(AN9305.2) hypothetical protein	An08g05350
AN9320	0.298 (0.196 to 0.893)	2.398 (0.812 to 3.897)	0.03	(AN9320.2) hypothetical protein	
AN9330	0.449 (0.33 to 0.658)	1.777 (1.454 to 2.455)	0.00	(AN9330.2) hypothetical protein	
AN9340	0.528 (0.361 to 0.839)	1.519 (1.083 to 2.173)	0.05	(AN9340.2) TREA_EMENI Acid trehalase precursor (Alpha,alpha-trehalase) (Alpha,alpha-trehalose glucohydrolase)	An01g01540
AN9361	0.275 (0.212 to 0.412)	2.014 (1.532 to 2.862)	0.01	(AN9361.2) hypothetical protein	An05g01800
AN9369	1.46 (1.196 to 2.354)	0.665 (0.274 to 0.768)	0.02	(AN9369.2) hypothetical protein	
AN9383	0.063 (0.034 to 0.229)	2.22 (1.688 to 3.455)	0.02	(AN9383.2) hypothetical protein	
AN9525	0.513 (0.445 to 0.682)	1.667 (1.388 to 2.157)	0.00	(AN9525.2) hypothetical protein	

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Finally, I would like to thank my family and my parents Maria and Stefan who taught me so much, my uncle Boris, who opened my mind for the world of science, and Kerstin and Kees for all the help, attention, and acceptance.

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Elena

About the author

Elena Stefanova Martens-Uzunova was born on the 15 of April 1973, in the old town of Plovdiv, Bulgaria. In 1992, she completed her secondary education – gymnasium. In the same year, she started her MSc studies in Biology at the University of Plovdiv "Paisii Hilendarski", Bulgaria. After she concluded the program of general courses, Elena joined the 2 years specialization of "Plant Biotechnology" at the department of Plant Physiology and Molecular Biology. In 1998, she received a TEMPUS grant in education and training from the trans-European programme of cooperation in higher education, which enabled her to perform a six months long internship at the Department of Molecular Biology at Plant Research International (PRI) in Wageningen, The Netherlands. During this period, she completed the practical work on her master thesis entitled "Developing of Hybrid B.t. Genes, Expression of Their Products In *Escherichia Coli*, and Bioassay" and in 1999, she received her MSc degree in Biology and Plant Biotechnology.

In 2001, Elena started working on a fellowship project "Self Retting Flax - Expression of Hydrolytic Enzymes from Thermostable Microorganisms in Plants" between the Laboratory of Microbiology at Wageningen University and the Business Unit "Cell cybernetics" at PRI-DLO, Wageningen. During this time, she came in touch with the world of microbial carbohydrate modifying enzymes.

In 2002, Elena started her PhD study in the group of Fungal Genomics, Laboratory of Microbiology at Wageningen University. This research was part of the IOP Genomics project "CarbNet - Disclosing the Carbohydrate Modifying Network of *Aspergillus niger* by Functional Genomics". The results from this project are summariesed in this thesis. In 2007, Elena was appointed as a post-doctoral researcher at the Laboratory of Microbiology at Wageningen University.

Currently, Elena is working as a post-doctoral researcher in cancer biology at the Erasmus University Medical Center in Rotterdam, the Netherlands.

Overview of completed training activities

Graduate school VLAG	Year	ETCS
Discipline specific activities		
Courses		
Bioinformatics I, Wageningen University	2002	2.9
Bioinformatics II, Wageningen University	2003	4.2
Summer course Glycosciences, VLAG	2002	1.4
Affymetrix workshop, Wageningen	2003	0.6
GeneSpring Workshop, Berlin, Germany	2003	1.1
Analysis of microarray expression data, Leiden University	2004	0.6
Systems Biology: "Principles of ~omics data analysis	2005	1.1
Meetings		
10th Netherlands Biotechnology Congress, Ede, The Netherlands (*poster*)	2004	1.1
2nd Symposium Kluyver centre for genomics and industrial fermentation, Nordwijkerhout (*presentation*)	2005	1.1
4th International *Aspergillus* meeting, Assilomar, USA (*poster*)	2005	1.1
23rd Fungal Genetics Conference, Assilomar, USA (*presentation*)	2005	1.9
12th European Biotechnology conference, Copenhagen, Denmark (*presentation*)	2005	1.6
ALW platform Molecular genetics, Lunteren (*presentation*)	2005	0.8
General courses		
Patent workshop, Senter IOP	2002	0.6
Scientific writing, CENTA	2002	1.7
Summer course, Improving teacher effectiveness, OWU	2003	1.4
VLAG PhD week	2002	1.1
WGS course career perspectives	2005	1.4
Optional courses and activities		
Organizing and participating of VLAG PhD trip Japan	2005	2.5
Preparation PhD research proposal	2002	6.0
PhD&PostDoc meetings, Microbiology	2002-2007	3.0
Research meetings, Fungal Genomics	2002-2007	3.0
Total		**40.2**

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