Chapter 3

Characterization of two novel, putatively cell wall associated and GPI-anchored, \(\alpha\)-glucanotransferase enzymes of \textit{Aspergillus niger}


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Abstract

In the genome sequence of \textit{Aspergillus niger} CBS 513.88 three genes were identified with high similarity to fungal \(\alpha\)-amylases. The derived protein sequences were in two ways aberrant from all described fungal \(\alpha\)-amylases: they were predicted to be glycosylphosphatidylinositol-anchored, and some highly conserved amino acids of the \(\alpha\)-amylase family were absent. We expressed two of these enzymes in a suitable \textit{A. niger} strain and characterized the purified proteins. Both enzymes showed transglycosylation activity on donor substrates with \(\alpha\)-(1,4)-glycosidic bonds and at least five anhydroglucose units. The enzymes, designated AgtA and AgtB, produced new \(\alpha\)-(1,4)-glycosidic bonds and therefore belong to the group of the 4-\(\alpha\)-glucanotransferases (EC 2.4.1.25). Their reaction products reached a degree of polymerization of at least 30. Maltose and larger maltooligosaccharides were the most efficient acceptor substrates, although AgtA also used small nigerooligosaccharides containing \(\alpha\)-(1,3)-glycosidic bonds as acceptor substrate. An agtA knockout of \textit{A. niger} showed an increased susceptibility towards the cell wall disrupting compound Calcofluor White, indicating a cell wall integrity defect in this strain. Homologues of AgtA and AgtB are present in other fungal species with \(\alpha\)-glucans in their cell walls, but not in yeast species lacking cell wall \(\alpha\)-glucan. The possible role for these enzymes in the synthesis and/or maintenance of the fungal cell wall is discussed.
Introduction

Aspergillus niger is a filamentous ascomycete fungus with a worldwide distribution. As a saprophyte, the fungus produces and secretes a large variety of extracellular enzymes especially proteases and polysaccharide hydrolases to convert plant cell walls and storage compounds into growth substrates (see e.g. Duarte and Costa-Ferreira (1994), Martens-Uzunova et al. (2006)). This quality is exploited for the production of enzymes for the food and feed industry on a large scale. Recently, the full genome sequence of A. niger CBS 513.88 was determined and annotated (Pel et al., 2007). A high level of synteny was observed between A. niger and other sequenced aspergilli, although more extracellular hydrolytic enzymes were annotated in A. niger. A detailed full genome search showed the presence of a considerable number of previously unknown, predicted enzymes belonging to the α-amylase superfamily.

The α-amylase superfamily (Kuriki and Imanaka, 1999) comprises a large variety of enzymes that are active towards polysaccharides with α-glycosidic linkages such as starch and glycogen (MacGregor et al., 2001). Most members of this family are either involved in production of storage compounds such as glycogen or starch, or degradation of these compounds as extracellular carbon and energy sources. The tertiary structure of these enzymes is characterized by a (β/α)8 barrel containing four highly conserved amino acid regions that form the catalytic site (MacGregor et al., 2001) (see also the CAZy website at http://www.cazy.org). Based on sequence similarity, members of the α-amylase superfamily are divided over glycoside hydrolase (GH) families 13, 70 and 77. Here, we focus on family GH13, which mostly contains enzymes that perform a hydrolytic reaction, i.e. they cleave an α-glycosidic linkage using water as an acceptor molecule. The best known hydrolytic enzyme of family GH13 is α-amylase (EC 3.2.1.1), which hydrolyses the internal α-(1,4)-glycosidic bonds in starch, glycogen and malto oligosaccharides (α-(1,4) linked glucose oligomers) producing shorter malto oligosaccharides and maltose (O-α-D-Glc-(1,4)-α-D-Glc). Other GH13 family members perform a transglycosylation (or glucanotransferase) reaction in which they cleave an α-(1,4) glycosidic bond in a donor substrate and subsequently do not use water but instead another oligosaccharide as acceptor substrate to form a new α-glycosidic linkage.

A. niger produces a number of extracellular enzymes classified as members of GH13, which are involved in the degradation of starch. These include acid amylase, which is well known for its stability at low pH (Boel et al., 1990) and two almost identical α-amylase enzymes (AmyA/B) (Korman et al., 1990). We have identified several additional members of family GH13 in the A. niger genome sequence, three of which clustered together in the phylogenetic tree of the GH13 family members and showed a
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typical glycosylphosphatidylinositol (GPI) anchoring signal at the protein C-terminus. A GPI-anchor serves as a targeting signal to the cell membrane and/or the cell wall.

The cell wall of aspergilli has been shown to contain four major classes of polysaccharides: chitin, α-glucan, β-(1,3)-glucan and galactomannan (Bardalaye and Nordin, 1977; Johnston, 1965; Blumenthal and Roseman, 1957; Stagg and Feather, 1973). In addition, it contains covalently attached cell wall proteins (Bral et al., 1997). The α-glucan fraction is composed of an α-(1,3)-glucan with 3-10% α-(1,4)-glycosidic linkages (Horisberger et al., 1972; Johnston, 1965), and nigeran, a glucan with alternating α-(1,3) and α-(1,4) glycosidic bonds (Bobbitt et al., 1977). Alpha-(1,3)-Glucan synthases have been identified and functionally studied in a number of different fungi (Hochstenbach et al., 1998; Damveld et al., 2005; Beauvais et al., 2005; Reese and Doering, 2003). These enzymes are large proteins (~2400 amino acids long) consisting of three conserved domains which are predicted to be involved in the synthesis, transport and crosslinking of the α-(1,3)-glucan (Hochstenbach et al., 1998). Detailed structural studies in Schizosaccharomyces pombe have revealed that the α-glucan is a linear glucose polymer of 260 residues in length consisting of two α-(1,3)-glucan chains that are interconnected via α-(1,4) linked glucose residues (Grü n et al., 2005). A mutation in the N-terminal part of the α-(1,3)-glucan synthases (the proposed crosslinking domain) abolished the linkage between the two α-(1,3)-glucan chains indicating that this part of the protein acts as a glucanotransferase, connecting the glucan chains.

Recently, two types of putative family GH13 enzymes have been shown to play a role in fungal cell wall α-(1,3)-glucan formation. Marion et al. (2006) showed the involvement of a putative α-amylase (Amy1p) in the formation of α-(1,3)-glucan in the cell wall of Histoplasma capsulatum. In this pathogenic fungus, α-(1,3)-glucan is known to play an important role in virulence (Rappleye et al., 2004). A functional knockout of AMY1 resulted in a lack of α-(1,3)-glucan formation and decreased virulence. The second α-amylase homolog, Aah3p, was studied in S. pombe (Morita et al., 2006). A knockout strain of this GPI-anchored protein was hypersensitive towards cell wall degrading enzymes, and showed aberrant cell shape. The enzymatic activities of Amy1p and Aah3p have not been studied.

In this paper, we report the first biochemical characterization of two family GH13 enzymes putatively involved in α-(1,3)-glucan formation. We expressed and purified two GPI-anchored enzymes from A. niger, both homologues of Aah3p from S. pombe. The biochemical characterization showed that the two A. niger enzymes are family GH13 α-glucanotransferases, making them the first of their kind to be described for...
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A gene knockout of one of the enzymes in *A. niger* resulted in increased sensitivity towards Calcofluor White (CFW), a cell wall disrupting compound.

**Materials and methods**

**Bioinformatics tools**

The full genome sequence of *A. niger* strain CBS 513.88 was provided by DSM (Pel et al., 2007). A Hidden Markov model (HMM) profile was built using the HMMR package (Durbin and Eddy, 1998) based on the amino acid sequences of described α-amylases, which were retrieved from the CAZy website (http://www.CAZY.org) (Coutinho and Henrissat, 1999). The obtained profile was used to screen the *A. niger* CBS 513.88 genomic database using the WISE 2 package (Birney et al., 2004). The presence of a signal peptidase cleavage site and a GPI-attachment site were predicted by web-based search tools (http://www.cbs.dtu.dk/services/SignalP/ (Bendtsen et al., 2004) and http://mendel.imp.univie.ac.at/sat/gpi/gpi_server.html (Eisenhaber et al., 2004) respectively). The GPI-attachment prediction was confirmed by a manual comparison of the protein sequences with the consensus sequence for yeast GPI-proteins as described by De Groot et al. (2003). Amino acid sequence alignments and phylogenetic analysis were performed using MEGA3.1 (Kumar et al., 1993) and adjusted manually if necessary. Sequences from other fungal genomes were retrieved via the option genomic Blast at NCBI (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=fungi).

**Aspergillus niger strains, growth conditions and transformation**

*A. niger* strain N402 (*cspA1* derivative of ATCC9029 (Bos et al., 1988)) mRNA was used for the construction of a cDNA library. Genomic DNA of strains *A. niger* NRRL3122 and N402 was isolated and used as a template in PCR reactions. *A. niger* strain MGG029-*aamA* (Weenink et al., 2006) was used as a host for protein overexpression. This strain, derived from strain MGG029 (*prtT glaA::fleo pyrG*), is deficient in the expression of several extracellular proteases, and it has no glucoamylase gene (*glaA*) and acid amylase gene (*aamA*) resulting in very poor growth on starch (Weenink et al., 2006). Strain MA70.15 (*pyrG, ku70::amdS*) (Meyer et al., 2007) was used for the disruption of the *agtA* gene.

Aspergillus strains were grown in *Aspergillus* Minimal Medium (MM) or Complete Medium (CM) which is MM with addition of 0.1% casaminoacids and 0.5% yeast extract (Oxoid, Basingstoke, UK) (Bennett and Lasure, 1991). Cultures for protein production were grown in CMS (CM supplemented with 2% (w/v) sucrose and 1% (w/v) glucose). Spores were obtained by growing *A. niger* on CM with 2% (w/v) agar.
for 4 days and scraping off the spores with 0.9% (w/v) NaCl. Liquid cultures were inoculated with $10^6$ spores l$^{-1}$ medium, and subsequently grown at 30 °C while shaking at 280 r.p.m. Transformation of A. niger was performed as described previously (Punt and van den Hondel, 1992) using lysing enzymes (Sigma, Zwijndrecht, The Netherlands). Selection of positive clones was performed on the basis of their ability to grow successively on MM containing 15 mM CsCl and 10 mM acetamide or acrylamide as sole nitrogen source, brought about by the expression of the amdS gene (Kelly and Hynes, 1985).

Cloning procedures

All basic molecular techniques were performed according to standard procedures (Sambrook et al., 1989). E. coli TOP10 (Invitrogen, Carlsbad, U.S.A.) or DH5α (Stratagene, La Jolla, U.S.A.) were used for transformation and amplification of recombinant DNA. The primers used were obtained from Eurogentec (Seraing, Belgium) or Biolegio (Nijmegen, The Netherlands). All steps during the construction of the overexpression vectors were checked by restriction analysis, and the final constructs were checked by sequencing (GATC Biotech AG, Konstanz, Germany). Genomic DNA was isolated from A. niger N402 and NRRL3122 as described by Kolar et al. (1988).

All PCR reactions were performed with 2.5 units of Pwo DNA polymerase (Roche, Indianapolis, U.S.A.), 1x buffer and 1 mM of each dNTP in a total volume of 25 μl. A cDNA library was produced from A. niger N402 grown on MM with addition of starch as sole carbon source. Primers used are indicated in Table 1.

The overexpression vector for the transformation of A. niger was provided by Dr. J. Benen (Wageningen University, Wageningen, The Netherlands), and was produced as follows: Gene pgaII (encoding polygalacturonase II from A. niger) was cloned into pPROM-S (Benen et al., 1999) using NsiI and KpnI restriction sites. A NotI site was generated immediately downstream of the stop codon of the pgaII gene by site directed mutagenesis. The gene encoding acetamidase (amdS (Kolar et al., 1988)) was amplified by PCR with specific primers from plasmid p3SR2 (Wernars et al., 1985) and cloned in front of the pki-promoter region (Parenicova et al., 1998) using XbaI restriction sites, resulting in vector Ppki-pgaII-amdS. The construct for the overexpression of the chimaeric protein AgtA-SBD (a fusion between AgtA and the Starch Binding Domain (SBD) of A. niger glucoamylase GlaA) was made as follows: Genomic DNA of A. niger N402 was used as a template in a PCR reaction with specific primers to generate the DNA fragment encoding the SBD, including the linker region. The primers were designed to amplify nucleotide 3643 to 4149 (numbering according to glaA coding sequence EMBL ID AY250996). An ApoI restriction site was built into the forward primer to allow the subsequent cloning of the agtA gene fragment in frame with the
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SBD. The SBD-encoding fragment was cloned into vector PpkI-pgaII-amdS using NsiI and NotI restriction sites, thereby replacing the pgaII gene. The cDNA fragment encoding agtA was generated by PCR with specific primers on the cDNA library. The primers were designed to amplify the gene up to nucleotide 1766 (in gene sequence), which does not include the C-terminal GPI-anchoring part. The AgtA cDNA was cloned in frame N-terminally of the SBD using NsiI and Apal, resulting in the expression vector PpkI-agtA-SBD-amdS. Sequencing of the construct revealed one point mutation compared to the original gene sequence in the genomic database. Nucleotide 1420 (in the coding sequence of agtA) was changed from A to G, resulting in Ala474 instead of Thr474 in the derived amino acid sequence. This mutation was consistent in several independent clones and was therefore considered to be representing a strain difference between N402 and CBS 513.88. The same mutation was found in the equivalent protein sequence published by the DOE Joint Genome Institute (http://genome.jgi-psf.org/Aspni1/Aspni1.home.html).

Table 1 Primers used for the production of plasmids for overexpression and deletion of genes in A. niger. The restriction sites used for cloning are underlined.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Enzyme</th>
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<td>Primers used for construction of pΔagtA</td>
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<td></td>
</tr>
<tr>
<td>AgtAP1for</td>
<td>ATAGAGATCCGCGCGCTGTTGCTTGTCGCCAAGCTTG</td>
<td>NsiI</td>
</tr>
<tr>
<td>AgtAP2rev</td>
<td>GCCCTTGGTTGATGACGATGCCCAGTGCCTGCA</td>
<td>Apal</td>
</tr>
<tr>
<td>AgtAP3for</td>
<td>GCCCTTGGTTGATGACGATGCCCAGTGCCTGCA</td>
<td>Apal</td>
</tr>
<tr>
<td>AgtAP4rev</td>
<td>CGGGATCCGGAGTGGATAGCTGGTAAGGG</td>
<td>BamHI</td>
</tr>
<tr>
<td>Primers used for construction of PpkI-agtA-SBD- amdS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBD-fw</td>
<td>GCCCTTGGTTGATGACGATGCCCAGTGCCTGCAAGCTTG</td>
<td>NsiI, Apal</td>
</tr>
<tr>
<td>SBD-rev</td>
<td>GCCCTTGGTTGATGACGATGCCCAGTGCCTGCAAGCTTG</td>
<td>NsiI, Apal</td>
</tr>
<tr>
<td>AgtA-SBD-fw</td>
<td>GCCCTTGGTTGATGACGATGCCCAGTGCCTGCAAGCTTG</td>
<td>NsiI, Apal</td>
</tr>
<tr>
<td>AgtA-SBD-rev</td>
<td>GCCCTTGGTTGATGACGATGCCCAGTGCCTGCAAGCTTG</td>
<td>NsiI, Apal</td>
</tr>
<tr>
<td>Primers used for construction of PpkI-agtA- amdS and PpkI-agtB- amdS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AgtA-fw</td>
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<td>NsiI, Apal</td>
</tr>
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<td>AgtA-rev</td>
<td>GCCCTTGGTTGATGACGATGCCCAGTGCCTGCAAGCTTG</td>
<td>NsiI, Apal</td>
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<tr>
<td>AgtB-for</td>
<td>GCCCTTGGTTGATGACGATGCCCAGTGCCTGCAAGCTTG</td>
<td>NsiI, Apal</td>
</tr>
<tr>
<td>AgtB-rev</td>
<td>GCCCTTGGTTGATGACGATGCCCAGTGCCTGCAAGCTTG</td>
<td>NsiI, Apal</td>
</tr>
</tbody>
</table>
The constructs for overexpression of \textit{agtA} and \textit{agtB} were produced as follows: the complete gene sequences of genes \textit{agtA} and \textit{agtB} were amplified with specific primers from genomic DNA isolated from \textit{A. niger} NRRL3122 (van Dijck \textit{et al.}, 2003). The primers contained restriction sites for \textit{NsiI} and \textit{NotI}, which were used to clone the gene fragments into vector \textit{Ppki-pgaII- amdS}, thereby replacing the \textit{pgaII} gene. This resulted in the vectors \textit{Ppki-agtA- amdS} and \textit{Ppki-agtB- amdS}.

\textbf{Protein production, purification and detection procedures

Production of AgtA-SBD}

Several stable transformants were checked for their level of extracellular production of the chimaeric protein AgtA-SBD by Western blotting with polyclonal antiserum raised against purified SBD kindly provided by Dr. D. Archer and Dr. D. McKensie (University of Nottingham & IFR Norwich, UK) (Le Gal-Coeffet \textit{et al.}, 1995). SDS-PAGE and Western blots were performed according to standard protocols. Immobilon-P (Millipore, Billerica, U.S.A.) was used as blotting membrane. The untransformed strain was included as negative control while purified SBD (Van der Maarel \textit{et al.}, unpublished data) served as positive control for immunodetection. The \textit{A. niger} transformant producing highest levels of AgtA-SBD was grown in a 5 l batch fermentor (New Brunswick Scientific, Edison, U.S.A.) inoculated with 100 ml culture pregrown on Potato Dextrose Broth (Difco). The medium used for the batch fermentation consisted of the following components: 3\% (w/v) glucose, 117 mM NaNO\textsubscript{3}, 3.25 mM MgSO\textsubscript{4}.7H\textsubscript{2}O, 14.7 mM KH\textsubscript{2}PO\textsubscript{4}, 0.69 mM CaCl\textsubscript{2}.H\textsubscript{2}O, 0.5\% Yeast Extract and spore elements. Fermentor conditions were pH 4.5, a temperature of 30 °C and aeration at 1.2 l min\textsuperscript{-1}. The feed contained 20\% (w/v) glucose, 74 mM KH\textsubscript{2}PO\textsubscript{4}, 350 mM NaNO\textsubscript{3}, 1\% yeast extract and 1\% tryptone and was added at a rate of 5 ml h\textsuperscript{-1}. Three days after starting the feed the growth medium was collected by filtration over miracloth (Calbiochem, EMD Biosciences, La Jolla, CA, U.S.A.). The pH of the medium was subsequently adjusted to pH 6 with 1 M NaOH and AgtA-SBD was extracted from the medium via binding of the SBD to starch granules based on the procedure described by Paldi \textit{et al.} (2003).1 l medium was added to 13 g waxy maize starch that was prewashed with elution buffer (10 mM NaAc pH 6), and incubated for 2 h at 4 °C while shaking gently. The starch with bound proteins was collected by centrifugation (15 min at 5000 x g) and washed once with ice-cold elution buffer followed by another centrifugation step. AgtA-SBD was eluted from the starch granules by the addition of 25 ml of elution buffer per batch of 13 g starch and subsequent incubation at 40 °C for 1 h while shaking gently. After removing the starch granules by centrifugation the protein was collected from the supernatant. A second round of binding was performed to remove residual proteins and media components. The protein was concentrated and the buffer changed to 20 mM Tris-HCl pH 8 using a Centriprep YM50 column (Millipore, Bedford,
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U.S.A.). The sample was applied to an anion exchange column (ResourceQ 1 ml, Amersham Biosciences, New Jersey, U.S.A.) equilibrated with 20 mM Tris-HCl pH 8. Proteins were eluted with a NaCl gradient (0-1 M NaCl) at a flow rate of 1 ml min\(^{-1}\). AgtA-SBD was eluted as a single activity peak at 280 mM NaCl. Purification of AgtA-SBD was confirmed by Western blot analysis. Deglycosylation was performed with 850 U of Endoglycosidase H (Endo H) (New England Biolabs, Ipswich, U.S.A.) on 1.5 μg of purified protein for 20 h in a total volume of 15 μl according to the manufacturer’s instructions.

**Production of AgtA and AgtB**

After transformation of each of the plasmids Ppki-agtA-amdS and Ppki-agtB-amdS into *A. niger* MGG029-ΔaamA, 9 transformants were selected which showed the best growth on selective medium. The best transformant for overexpression of each protein was selected by growth in liquid CMS and visual inspection of protein production on SDS-PAGE. The selected transformants were grown in CMS for 3 days at 30 °C and 200 r.p.m. Mycelium was removed from the culture medium by filtration over miracloth. The medium was concentrated over a Centriprep YM-50 membrane filter and the concentrated protein was taken up in 20 mM Tris-HCl buffer pH 8. The proteins were purified via anion exchange chromatography as described above. Both AgtA and AgtB were eluted as a single activity peak at a concentration of 150 mM NaCl. At each stage of the protein purification, the protein amount was measured using the Bradford method with reagent from Bio-Rad (Hercules, U.S.A.), and purity was checked using SDS-PAGE analysis (Laemmli, 1970) and staining with Biosafe Coomassie (Bio-Rad).

**Enzymatic assays**

All oligosaccharides used were obtained from Sigma, except nigerotriose (O-α-D-Glc-(1,3)-α-D-Glc-(1,3)-D-Glc) which was purchased from Dextra laboratories (Reading, U.K.) and nigerose (O-α-D-Glc-(1,3)-α-D-Glc) which was a kind gift from Nihon Shokuhin Kako Co. Ltd. (Shizuoka, Japan). *Lactobacillus reuteri* polysaccharide was a gift from Dr. S. Kralj (University of Groningen, The Netherlands) and α-(1,3)-glucan isolated from *A. nidulans* which was a kind gift from Dr. B. J. Zonneveld (Leiden University, Leiden, The Netherlands). As soluble starch, Paselli SA2 with an average degree of polymerization (DP) of 50 (AVEBE, Foxhol, The Netherlands), was used. All reactions were performed at 37 °C. To determine the optimum pH for activity of AgtA and AgtB, 0.5 μg purified enzyme was incubated with 20 mM maltooligosaccharide (a maltooligosaccharide of DP 5) in a 20 μl reaction volume for 30 min at 11 different pH values. The reaction was buffered either by 30 mM NaAc buffer at pH 4.2 to 7.0, or KH₂PO₄/KH₂PO₄ buffer at pH 6.3 to 8.0. Subsequently, 2 μl of the reaction mixture was spotted on a Thin Layer Chromatography (TLC) plate (Silica gel 60 F₂₅₄, Merck,
Darmstadt, Germany) and, after drying, the plate was run for 6 h in 75 ml running buffer (butanol/ethanol/mQ 5/5/3 (v/v/v)) in a container of 22 x 6 x 22 cm. After running the plate was dried and sprayed with 50% sulphuric acid in methanol and left to develop for 10 min at 110 °C.

Hydrolyzing activity on potato starch was determined by the incubation of 1 μg purified enzyme with 600 μl 0.02%, 0.2% or 2% (w/v) dissolved potato starch in 50 mM NaAc buffer containing 1 mM CaCl₂. Reactions were buffered at pH 5.5 (AgtA) or pH 4.8 (AgtB) and performed in duplicate. Samples of 50 μl were taken after several time intervals up to 4 h and used for the determination of reducing ends and glucose. The formation of reducing ends was measured with the bicinchoninic acid method (Meeuwsen et al., 2000), and glucose formation was measured with the Glucose GOD-PAP assay (Roche, Mannheim, Germany). Appropriate calibration curves and negative controls were included for all assays and reactions.

Standard assay conditions for all further enzymatic reactions were as follows: 0.4 μg of purified enzyme was incubated in 20 μl 25 mM NaAc buffer pH 5.5 containing 1 mM CaCl₂ and 0.01% sodiumazide in the presence of 20 mM oligosaccharide substrate and/or 4% (w/v) soluble starch or other polysaccharide, except nigeran and α-(1,3)-glucan. Nigeran and α-(1,3)-glucan were dissolved in 1 M NaOH, after which the pH of a 1% solution was adjusted to pH 5.5 with HAc. The final concentration used in reaction mixtures was 0.5% (w/v). Reaction products were detected either by TLC (as described above) or HPLC ( Dionex) analysis. For HPLC analysis, 5 μl of the reaction mixture was diluted in 1.5 ml 90% dimethyl sulfoxide. Separation of oligosaccharides was achieved as described earlier by Kralj et al. (2004b).

Disruption of the agtA gene

The plasmid used to disrupt the agtA gene was constructed as follows. The DNA fragments flanking the agtA gene were amplified by PCR using N402 genomic DNA as template: 1.5 kb of 5' flanking DNA and 1.0 kb of 3' flanking DNA were amplified by PCR using primers AgtAP1f and AgtAP2r, AgtAP3f and AgtAP4r (Table 1), respectively. Each primer was adapted with a restriction site for further cloning. The amplified PCR fragments were digested with NotI and XbaI or XbaI and BamHI, respectively, and cloned in a three way ligation into NotI and BamHI digested pBlue-ScriptII SK to obtain plasmid pAgtAF53. Subsequently, pAgtAF53 was digested with XbaI, and ligated with the 2.7 kb XbaI fragment containing the A. oryzae pyrG gene, obtained from plasmid pAO4-13 (de Ruiter-Jacobs et al., 1989) which resulted in the agtA disruption plasmid, pΔagtA. Before transformation to MA70.15, pΔagtA was linearized with NotI. Uridine prototrophic transformants were selected by incubating protoplasts on agar plates containing MM without uridine. Transformants were purified and genomic DNA was isolated and analysed by Southern Blot analysis to identify
possible ΔagtA strains. Deletion of the agtA locus by homologous recombination was expected to result in appearance of a 4.8 kb fragment after digestion of the genomic DNA with KpnI and the loss of a 2.0 kb fragment which was expected in the wild-type. For the hybridization, the Norf-XbaI fragment (containing the 5’ flanking region of the agtA gene) was used. Independently obtained transformants were purified and strains with the expected hybridization pattern (MA71.1, MA71.3, MA71.4, MA71.7) were used for phenotypic analysis.

*Phenotypic characterization of the ΔagtA strain and AgtA/AgtB overexpression strains.*

Sensitivity towards CFW was assayed as described (Ram and Klis, 2006). Conidiospores from the control strains (MA70.15 transformed with pAO4-13 containing A. oryzae pyrG, and MGG029-ΔaamA), the ΔagtA strains (MA71.1, MA71.3, MA71.4, MA71.7), and the agtA and agtB overexpression strains were spotted on CFW-plates. All strains were also checked for their ability to grow on starch as sole carbon source by the inoculation of 3 μl of spore solution containing 10⁶, 10⁵, 10⁴ or 10³ spores on plates containing MM with either 1% (w/v) potato starch or 1% (w/v) glucose as carbon source. Colony growth was followed daily.

Table 2 Alignment of the generally conserved regions of the α-amylase family as present in *A. niger* acid amylase and α-amylase AmyA compared to homologous regions in AgtA, AgtB and AgtC from *A. niger* and the homologous protein Aah3p from *S. pombe*. Catalytic residues are underlined, generally conserved residues are indicated in bold.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Region I</th>
<th>Region II</th>
<th>Region III</th>
<th>Region IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid amylase</td>
<td>LMV/DVVPNH</td>
<td>DGLR1DSVLE</td>
<td>YCVGEVDN</td>
<td>NF1ENHD</td>
</tr>
<tr>
<td>AmyA</td>
<td>LMV/DVANH</td>
<td>DGLR1DVKH</td>
<td>YC1GEVLD</td>
<td>TF1ENHD</td>
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<td>FMTGEVLQ</td>
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<td>FTV1EGAT</td>
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<tr>
<td>Aah3p (<em>S. pombe</em>)</td>
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<td>DGLR1DAVKM</td>
<td>YSVG1VFS</td>
<td>TF1ENHD</td>
</tr>
</tbody>
</table>

Amino acid numbering ¹

| 135 – 143 | 222 – 231 | 247 – 254 | 312 – 318 |

¹Numbering according to *A. niger* acid amylase
Gene accession numbers

The full genome sequence of *A. niger* strain CBS513.88, has been deposited at the EMBL database with accession numbers AM270980-AM270998 (Pel et al., 2007). The locus tags of the genes studied here are: An09g03100 (AgtA), An12g02460 (AgtB) and An15g07800 (AgtC).

Results

Sequence analysis

The full genome sequence of *A. niger* CBS 513.88 was searched for genes encoding proteins belonging to family GH13 using a HMM profile based on known α-amylases. Apart from 3 genes encoding previously described extracellular α-amylases, 8 genes encoding for yet undescribed family GH13 proteins were identified, as well as five genes predicted to encode membrane bound α-(1,3)-glucan synthases (Pel et al., 2007).

Within the group of 8 α-amylase type proteins, three were characterized by the presence of an N-terminal signal sequence for secretion and a hydrophobic C-terminal sequence predicted to act as an attachment site for a GPI-anchor (Bendtsen et al., 2004; De Groot et al., 2003). These proteins, called AgtA, AgtB and AgtC, form the topic of this study. The predicted sites for the attachment of a GPI-anchor (the ω-sites) were largely in accordance with the consensus sequence for fungal GPI-proteins as described by De Groot et al. (2003) (Fig. 1). An exception to this consensus sequence was residue Thr543 at the ω+2 position in AgtC: an alternative ω-site could be residue Ser538 instead of Ser541 predicted by the online GPI-prediction tool (Eisenhaber et al., 2004).

Homologous enzymes with predicted GPI-anchoring sites were also identified in the available genome sequences of other aspergilli as well as in *Neurospora crassa*, *Magnaporthe grisea* and *S. pombe*. In many cases, the genes encoding the Agt homologues are located next to genes encoding predicted α-(1,3) glucan synthases. No homologous family GH13 proteins containing GPI-anchoring sites were found in the genome sequences of Saccharomycotina like *Candida albicans*, *Kluveromyces lactis* and *Saccharomyces cerevisiae*. The amino acid sequences of AgtA, AgtB and AgtC show 54-56% similarity to *A. niger* acid amylase (Boel et al., 1990). A phylogenetic analysis of the Agt proteins, compared to the *A. niger* α-amylases, the homologous proteins in *A. oryzae* and the Aah proteins in *S. pombe*, is shown in Fig 2. The Agt proteins contain most of the amino acids generally conserved in family GH13 (Table 2).

However, in all three protein sequences the highly conserved His143, which is part of conserved region I, is absent (numbering used is according to acid α-amylase of *A. niger*, unless indicated otherwise). In AgtB and AgtC, a second conserved histidine in conserved region IV is replaced by glutamate (Table 2).
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\[
A \quad [\text{NSGADC}] - [\text{GASVIETKOLF}] - [\text{GASV}] - X (4-19) - [\text{FILMVAGPSTCYWN} (10)]
\]

\[
\omega \quad \omega+1 \quad \omega+2 \quad \text{hydrophobic tail}
\]

B

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgtA</td>
<td>VTYAALRTQGAAAEALSLGIKTDAASSLGLSVAGLIVGMW</td>
</tr>
<tr>
<td>AgtB</td>
<td>GYNTSNVYSELRLAAVGGSSSAGSHSVIPSAFASLFMSVAFALAFRI</td>
</tr>
<tr>
<td>AgtC</td>
<td>KSATPSSAASSALTOSKGSETCLFGVPLGISTLVVTVAMATSYVF</td>
</tr>
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Fig. 1 GPI-anchor specific amino acid features in fungal proteins as described previously compared to the C-terminal sequence of AgtA, AgtB and AgtC. A) Consensus sequence for GPI-attachment according to De Groot *et al.* (2003), with X representing any amino acid. The \( \omega \)-site is indicated in bold. B) C-terminal ends of AgtA, AgtB and AgtC indicating the potential GPI-modification site. The \( \omega \)-site as predicted by the online tool for the prediction of a GPI-modification site (Eisenhaber *et al.*, 2004) is indicated in bold, an alternative \( \omega \)-site is indicated in bold underlined. The hydrophobic tail is underlined.

**Purification and enzyme activity of AgtA fused to a starch binding domain**

The *agtA* gene, lacking its C-terminal anchoring domain, was fused to the starch binding domain (SBD) of the *A. niger* glucoamylase gene serving as a protein tag, and the fusion construct was transformed into *A. niger* MGG029-ΔaamA resulting in 25 stable transformants. The transformant with the highest expression level of the AgtA-SBD fusion protein in the culture medium, as determined by Western blot with polyclonal antiserum raised against purified SBD (Le Gal-Coffet *et al.*, 1995), was selected for larger scale protein production. AgtA-SBD was isolated from the culture medium by binding it to waxy maize starch granules, and after further purification by anion exchange chromatography a single protein band with an apparent molecular mass of about 130 kDa was obtained (Fig. 3). This band was recognized by anti-SBD antibodies, a strong indication that this band contained the AgtA-SBD fusion protein (calculated molecular mass 73.6 kDa). Treatment with Endo H to remove N-glycosylation resulted in a small decrease of the apparent molecular mass indicating that the protein was N-glycosylated (Fig. 3). The high molecular mass of the fusion protein after Endo H treatment could be caused by heavy O-glycosylation of the linker region of the SBD (Williamson *et al.*, 1992).
Chapter 3 Two novel alpha-glucanotransferases

Fig. 2 Bootstrapped phylogenetic tree of the A. niger extracellular α-amylases (acid amylase AamA, α-amylase A and B, and putative α-amylase An04g06930) and α-glucanotransferases (AgtA/B/C), the homologous putative proteins identified in the genome of A. oryzae, and the S. pombe Aah proteins (SpAah1p to SpAah4p). The alignment and phylogenetic analysis were performed with MEGA version 3.1 using default settings. A bootstrapped tree was constructed with the neighbor-joining method using 500 replicates.

Fig. 3 SDS-PAGE analysis of purified AgtA-SBD, AgtA and AgtB in native form (N) and after treatment with Endo H (EH). The marker is indicated by M, the size of the marker proteins (in kDa) is indicated on the side.
Purified AgtA-SBD was incubated with potato starch to determine its hydrolysing activity. Hydrolysis of starch may result in the formation of glucose, maltose or longer maltooligosaccharides, the formation of which can be quantified by the measurement of the reducing ends formed during the reaction. For AgtA-SBD, a low hydrolyzing activity of 0.46 ± 0.02 \( \mu \text{mole reducing ends mg}^{-1} \text{ min}^{-1} \) was detected when incubated with 0.2\% starch, and no formation of glucose was observed. TLC analysis of the reaction mixture showed that short oligosaccharides were formed in low amounts from soluble starch (Fig. 4, lane 1). Incubation with both maltose and soluble starch resulted in the formation of more oligosaccharides than made from starch alone (Fig. 4, lane 2). No products were formed from maltose alone (result not shown).

Fig. 4 TLC analysis of reaction products of AgtA-SBD from different substrates. Enzyme (0.4 \( \mu \text{g} \)) was incubated with 20 mM oligosaccharide and/or 4\% soluble starch for 1 h at 37 °C. The arrow indicates the level where the following samples were loaded: Marker (lane M) containing a mix of maltooligosaccharides ranging from glucose (G1) to maltoheptaose (G7), unmodified soluble starch (lane S), reaction products of AgtA-SBD incubated with: soluble starch (lane 1), maltose and soluble starch (lane 2), maltopentaose (lane 3), maltoheptaose (lane 4).
When AgtA-SBD was incubated with maltopentaose or maltoheptaose (maltooligosaccharide of DP 7), a variety of oligosaccharides ranging from maltose to oligosaccharides with a degree of polymerization (DP) of at least 13 to 18, respectively, were formed (Fig. 4, lanes 3-4). This result indicated that AgtA-SBD hydrolysed starch to some extent, but mainly acted as a glucanotransferase, transferring parts of a donor oligosaccharide, which might be starch, to an acceptor substrate, e.g. maltose, thereby producing a variety of oligosaccharides of different lengths.

Production and purification of AgtA and AgtB

To rule out any effect of the incorporated SBD on the enzymatic activity of AgtA, both AgtA and AgtB were overexpressed in *A. niger* in their native form for further biochemical analysis. All selected transformants overproduced a protein with an estimated molecular mass of 85 kDa not observed in the untransformed strain. In a similar procedure of cloning and transformation, we also attempted to produce the AgtC protein. Although insertion of the overexpression construct in selected transformants was confirmed by Southern blot analysis, none of the transformants overproduced the AgtC protein. The characterization of AgtC was therefore not included in this study.

Proteins expressed in the culture medium of *A. niger* MGG029-Δ*aamA*-AgtA and of *A. niger* MGG029-Δ*aamA*-AgtB were concentrated and subsequently submitted to anion exchange chromatography, resulting in the purification of AgtA and AgtB. Both proteins had an apparent molecular mass of approximately 70 kDa, but after removal of N-glycosylation the apparent protein masses decreased to approximately 55 kDa, close to their theoretical masses (AgtA 58.8 kDa and AgtB 57.7 kDa, after removal of C-terminal end for GPI-anchoring) (Fig. 3).

Glucanotransferase activity of AgtA and AgtB on maltooligosaccharides

Both AgtA and AgtB were incubated with maltooligosaccharides ranging in size from maltose to maltohexaose (a DP 6 maltooligosaccharide) and the products were analysed by TLC. With maltopentaose or maltohexaose as substrates, both enzymes produced a range of oligosaccharides of DP 15 or larger, similar to what was observed previously for AgtA-SBD (Fig. 5, lanes 3, 4, 7 and 8). Incubation with maltopentaose at lower concentrations (2 or 10 mM) also resulted in the formation of products of DP 6 and larger (results not shown). Activity of both enzymes on the smaller maltooligosaccharides was limited (Fig. 5, lane 1, 2 and 5, 6). Neither of the enzymes produced glucose in detectable amounts. When incubated with dissolved potato starch both enzymes produced small amounts of reducing ends (hydrolysis on 2% starch 0.55 ± 0.19 μmole mg⁻¹ min⁻¹ for AgtA and 0.26 ± 0.16 μmole mg⁻¹ min⁻¹ for AgtB; the
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Hydrolysis rate was approx. 4 times lower when measured on 0.2% starch. TLC analysis of the products produced from maltoheptaose at different pH values indicated that AgtA was active between pH 4.5 and pH 6, and AgtB showed activity between pH 4 and pH 5.5 (data not shown). Glucose polymers with other types of glycosidic linkages, like dextran (a glucan polymer with α-(1,6)-glycosidic bonds), nigeran, *A. nidulans* α-(1,3) glucan, an *L. reuteri* polysaccharide containing α-(1,3)- and α-(1,6)-glycosidic bonds (Kralj et al., 2004a), and cellulose (β-(1,4)-glycosidic bonds) were tested alone or in combination with maltose as an acceptor substrate. None of these polysaccharides acted as substrates for AgtA or AgtB (data not shown).

![TLC analysis of the reaction products of AgtA (left side) and AgtB (right side) incubated with different substrates. Purified enzyme (0.4 μg) was incubated with 20 mM substrate for 1 h at 37 °C. The samples were applied at the spots where the polymeric material is visible. The marker lanes (indicated by M) contain a mix of maltooligosaccharides ranging from glucose (G1) to maltoheptaose (G7). The figure shows reaction products of AgtA/AgtB incubated with: maltotriose (lane 1/5), maltotetraose (lane 2/6), maltopentaose (lane 3/7), maltohexaose (lane 4/8).](image)

**Identification of the reaction products of AgtA and AgtB**

To study the nature of the glycosidic linkages formed as well as the maximum length of the oligosaccharide products, reaction products of AgtA and AgtB were analysed qualitatively by HPLC. AgtA and AgtB produced a similar mixture of oligosaccharides with various lengths from maltoheptaose as substrate (Fig. 6B and C). The maximum detectable product length was approximately DP 28 after one hour of incubation for...
both enzymes. A similar mixture of products was produced from maltopentaose as a substrate (results not shown). The retention time of the products was the same as the retention time of standard maltooligosaccharides (Fig. 6A), except for one small peak among the products of AgtA, which was identified as panose (4-α-isomaltosylglucose).

Fig. 6 HPLC analysis of the reaction products formed by incubation of AgtA and AgtB on maltoheptaose. A) Elution profile of a standard mixture of maltooligosaccharides containing glucose to maltoheptaose (indicated as G1- G7). B) and C) Reaction products of AgtA and AgtB respectively, after incubation of 0.4 μg purified enzyme with 20 mM maltoheptaose at 37 °C for 1 h.
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**AgtA and AgtB can use α-(1,3)-glucooligosaccharides as acceptor**

The presence of the putative GPI-anchoring sequence in both AgtA and AgtB indicate that both enzymes are possibly present in the cell wall or cell membrane. The α-glucans present in the cell wall of *A. niger* are α-(1,3)-glucan (Horisberger *et al.*, 1972) and nigeran (Bobbitt *et al.*, 1977). In the previous paragraph we showed that neither nigeran nor α-(1,3)-glucan were used as (donor) substrate by AgtA and AgtB. To investigate whether small substrates with α-(1,3)-glycosidic bonds could be used as an acceptor substrate, both enzymes were incubated with soluble starch as donor substrate, combined with glucose, maltose, nigerose or nigerotriose as acceptor substrates. Analysis of the reaction products by TLC and HPLC revealed that glucose was not used as acceptor substrate by either of the enzymes; maltose was an efficient acceptor as shown previously for AgtA-SBD (results not shown). AgtA also formed a series of oligosaccharides using either nigerose or nigerotriose as acceptor substrates, although the amount of products formed was lower than when maltose acted as acceptor substrate (Fig. 7A). AgtB did not use nigerose or nigerotriose efficiently as acceptor substrate (Fig. 7B). No activity of AgtA or AgtB was observed on as sole substrates (result not shown). These results indicated that small α-(1,3)-linked oligosaccharides can be used as acceptor substrate by AgtA, and to a very limited extent by AgtB, but only in combination with an α-(1,4)-linked donor molecule.

**ΔagtA and agtA/agtB overexpression strains are Calcofluor White hypersensitive**

To examine the consequence of the loss of the *agtA* gene in *A. niger* and to analyse the physiological role of this enzyme, a deletion mutant of the *agtA* gene was constructed. Ten randomly chosen Δ*agtA* transformants were subjected to Southern blot analysis which revealed that in 8 out of the 10 transformants the *agtA* gene was properly deleted (data not shown). Phenotypic analysis of several Δ*agtA* strains revealed that their growth rate on solid media was slightly reduced, but no changes in the morphology of the hyphae or conidia were observed. We analysed the sensitivity of the Δ*agtA* strain to the cell wall disturbing compound Calcofluor White (CFW). Hypersensitivity towards CFW has been shown to be indicative for mutants with impaired cell wall strength (Ram and Klis, 2006; Damveld *et al.*, 2005a). As shown in Fig. 8A, the Δ*agtA* strains showed an increased sensitivity towards CFW. The observed sensitivity is not as strong as for the deletion of other *A. niger* cell wall related proteins such as α-(1,3)-glucan synthaseA or the Cell wall proteinA (Damveld *et al.*, 2005b; Damveld *et al.*, 2005a). The overexpression of *agtA* and *agtB* also resulted in an increased sensitivity towards CFW (Fig. 8B). All deletion and overexpression strains were also tested for their ability
to grow on starch as sole carbon source. Deletion of \textit{agtA} and overexpression of AgtA or AgtB had no significant effect on the ability of \textit{A. niger} to grow on starch compared to the untransformed strains.

![HPLC analysis of the reaction products formed by AgtA (A) and AgtB (B) upon incubation with soluble starch and nigerotriose. G, G2 and G3 indicate peaks representing glucose, maltose and maltotriose respectively. Peaks representing products most likely containing α-(1,3) glycosidic bonds, resulting from the use of nigerotriose as acceptor substrate, are indicated with grey arrows. Enzyme (0.4 μg) was incubated with 20 mM nigerotriose and 4% soluble starch at 37 °C for 18 h.](image-url)
Alpha-glucan acting enzymes in Aspergillus niger

Fig. 8 The effect of agtA deletion (A) or agtA and agtB overexpression (B) on the susceptibility of the resulting strains towards Calcofluor White (CFW) induced cell wall stress. A) A. niger ΔagtA strains (MA71.1, MA71.3, MA71.4, MA71.7) and the control strain (MA70.15 transformed with pAO4-13), grown on complete medium containing 0, 100 or 400 μg ml⁻¹ CFW for 96 hours. B) A. niger strains overexpressing AgtA or AgtB and the parental strain (MGG029-ΔaamA) grown on complete medium containing 0 or 200 μg ml⁻¹ CFW for 72 h. The amount of spores applied per spot is indicated at the top of the figures.

Discussion

All putative GPI-anchored family GH13 enzymes identified in the genome sequences of four aspergilli, as well as N. crassa, M. grisea and S. pombe, were originally annotated as α-amylases, because of their high similarity to known extracellular fungal α-amylases. However, most of the protein sequences missed the commonly conserved His143 in region I (Jespersen et al., 1991). Mutation of this residue, which is located in the active site (Uitdehaag et al., 1999), resulted in a strongly reduced activity in several α-amylases (Nakamura et al., 1993; Chang et al., 2003), or in altered reaction specificities in other family GH13 enzymes (see e.g. Leemhuis et al. (2004), Nakamura et al. (1993)). Despite the missing His143 residue, the A. niger Agt enzymes and their
homologues are clearly members of family GH13, based on their high similarity with known proteins in this family. The second generally conserved His residue, His317 in conserved region IV, is replaced by Gln in AgtB and AgtC. Although His317 is overall highly conserved in the α-amylase family, this residue appears to be less important for the determination of the catalytic activity because some α-amylases are known which also do not possess a His in this position (Hoshiko et al., 1987; Kang et al., 2004). In conclusion, the Agt enzymes in A. niger and homologues in other fungi are highly similar to the well described fungal α-amylases, but aberrant conserved regions combined with the presence of a GPI-anchoring signal make them clearly distinguishable. We produced two of the A. niger Agt enzymes and determined their biochemical activities.

As the enzymatic activity of the Agt proteins was not known at the start of this research, it was decided to start with the production of the enzyme encoded by gene An09g03100 (AgtA) fused with a clearly recognizable tag, the SBD. This allowed isolation and purification of the protein from media via binding to starch granules, as well as recognition of the purified protein using antibodies. Subsequently, both AgtA and AgtB (encoded by gene An12g02460) were produced in their native form for further biochemical characterization. A host A. niger strain with very low α-amylase activity was chosen to strongly reduce interference by native activities when searching for the biochemical activity of the investigated enzymes. AgtA-SBD as well as native AgtA and AgtB had a very low hydrolysing activity on starch, but clearly showed α-glucanotransferase activity on maltooligosaccharides alone, and on maltooligosaccharides plus starch. We therefore propose to name the A. niger GPI-anchored family GH13 enzymes Agt (α-glucanotransferase) A and B. As the predicted protein encoded by gene An15g07800 has the same sequence characteristics, we propose to name it AgtC.

AgtA and AgtB were overproduced from their entire predicted coding sequences, which included the predicted GPI-anchoring signal. The presence of both AgtA and AgtB in the medium suggested that these proteins were not fully retained at the plasma membrane or the cell wall, but that at least part of the proteins was released into the medium. This might be an indication that the proteins are not GPI-anchored, although their C-terminal signal sequence as well as the investigation of the homologous Aah3p from S. pombe (Morita et al., 2006) suggest otherwise. Another explanation is that the enzymes had been released by endogenous phospholipase C activity, as was previously shown to occur with GPI-anchored proteins in A. niger and S. cerevisiae (Brul et al., 1997).
AgtA and AgtB produced a similar range of products consisting of maltooligosaccharides, indicating that both enzymes formed α-(1,4)-glycosidic bonds and can therefore be classified as 4-α-glucanotransferases (EC 2.4.1.25). Also a small amount of panose was produced by AgtA, indicating an ability to synthesize α-(1,6) linkages. Alternatively, panose may have been produced by a minor contamination of α-glucosidase, which is known to produce α-(1,6) linkages (Kato et al., 2002b). The enzymatic activity of AgtA and AgtB is unique among the α-glucanotransferases from bacteria as well as eukarya. The α-glucanotransferases that have been described until now usually release one glucose molecule for every transfer event (Takaha et al., 1993), but AgtA and AgtB did not produce glucose in significant amounts. Additionally, bacterial amylomaltases use very small donor and acceptor molecules (maltotriose and glucose, respectively) (Kaper et al., 2004), while AgtA and AgtB prefer longer donor molecules with a minimum length of 5 glucose residues, and maltose as smallest possible acceptor substrate. The use of the α-(1,3) linked oligosaccharides nigerose and nigerotriose as acceptor substrate by family GH13 α-glucanotransferases has not been reported before. We conclude that the A. niger α-glucanotransferases represent a new subgroup of family GH13 in view of their atypical donor and acceptor profiles and their C-terminal GPI-anchoring sequences. Based on their common putative cell wall associated location and amino acid sequences, it is expected that the closely related GPI-anchored family GH13 proteins in other fungi will show similar glucanotransferase activities, although their precise substrate and product profiles remain to be determined.

Most extracellular members of family GH13 are involved in the degradation of starch to supply energy and carbon to the cells. There are strong indications that this is not the case for AgtA and AgtB. This study describes that neither a knockout of agtA nor overexpression of AgtA or AgtB had an effect on the ability of A. niger strains to grow on starch, even if the parental strain was severely hampered in this trait. In another study, we have also shown that expression of the agtA and agtB genes is not regulated by AmyR, the general regulator for starch processing enzymes in aspergilli (Yuan et al., in preparation) (Petersen et al., 1999), and similar results were found for the homologous genes in A. nidulans (Nakamura et al., 2006). Taken together, these data indicate that the Agt proteins most likely are not involved in starch catabolism. An alternative function could be the production or modification of α-glucans in the fungal cell wall, which was suggested for one of the GPI-anchored Agt homologues in the fission yeast S. pombe, Aah3p, in a functional study (Morita et al., 2006). Deletion of aah3 resulted in a morphological defect and hypersensitivity towards cell wall degrading enzymes. The knockout could not be rescued by transformation with the aah3 gene in which the catalytic residues had been mutated, showing the importance of the enzymatic activity rather than the structural properties of the protein. Our finding of a
clear enzymatic activity for AgtA and AgtB confirms the importance of the catalytic residues for their physiological role. The proposed role for AgtA and its homologues in cell wall α-glucan production or maintenance is strengthened by the analysis of agtA knockout strains, which showed increased sensitivity towards CFW. The overexpression of AgtA and AgtB in A. niger caused a similar effect, which might be an indication that the unnaturally high amounts of these enzymes have a negative effect on cell wall strength.

The function of the Agt proteins in the fungal cell wall might be analogous to the function of GPI-anchored β-glucanosyltransferases. These enzymes, identified in several yeasts and fungi including aspergilli, play a role in the cross linking of cell wall β-glucan (Popolo et al., 1993; Mouyna et al., 2000). In A. fumigatus, the β-glucan component of the cell wall is used as a target for antifungal drugs (Beauvais and Latgé, 2001). A knockout of one of its GPI-anchored β-glucanosyltransferases, Gel2p, resulted in an altered cell wall composition, increased sensitivity for CFW, and reduced virulence (Mouyna et al., 2005). We tested the ability of AgtA and AgtB to process the two α-glucan cell wall components, nigeran and α-(1,3) glucan, but no activity was detected. Because of their poor solubility at low pH, these substrates were offered at a relatively low concentration and partly in crystalline form, which might prevent the enzymes from acting on these cell wall components in vitro. It was shown, however, that AgtA performed a transglycosylation reaction involving an α-(1,4) linked donor substrate and an α-(1,3) linked acceptor substrate. A similar reaction was described to occur in S. pombe cell walls, where two linear polysaccharide chains of α-(1,3)-glucan with several α-(1,4)-linkages at the reducing end were interconnected by a transglycosylation reaction (Grün et al., 2005). Although this process was suggested to be performed by the transferase domain of α-(1,3) glucan synthase Ags1p, a similar cross linking reaction could be performed by AgtA. This would also explain the clustering of agt and ags (α-glucan synthase) genes conserved in many Ascomycetes. Clustering of genes involved in the same metabolic pathway is well described in fungi (Keller and Hohn, 1997).

To conclude, we have studied two novel putatively GPI-anchored family GH13 enzymes of A. niger, with homologues in many other fungi. The enzymes, AgtA and AgtB, both showed a unique type of α-(1,4) glucanotransferase activity, and our functional characterization indicated that their involvement in the facilitation of growth on starch is unlikely. The characterization of a knockout of agtA suggested that this enzyme could be involved in cell wall α-glucan synthesis, which is in line with the results on a knockout of a homologous protein from S. pombe (Morita et al., 2006).
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More study is needed to confirm the proposed physiological role for these α-glucanotransferases and to identify their exact *in vivo* reaction.

**Acknowledgements**

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