Phylogenetic and comparative functional analysis of the cell-separation $\alpha$-glucanase Agn1p in Schizosaccharomyces

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The post-cytokinetic separation of cells in cell-walled organisms involves enzymic processes that degrade a specific layer of the division septum and the region of the mother cell wall that edges the septum. In the fission yeast Schizosaccharomyces pombe, the 1,3-$\alpha$-glucanase Agn1p, originally identified as a mutanase-like glycoside hydrolase family 71 (GH71) enzyme, dissolves the mother cell wall around the septum edge. Our search in the genomes of completely sequenced fungi identified GH71 hydrolases in Basidiomycota, Taphrinomycotina and Pezizomycotina, but not in Saccharomycotina. The most likely Agn1p orthologues in Pezizomycotina species are not mutanases having mutanase-binding domains, but experimentally non-characterized hypothetical proteins that have no carbohydrate-binding domains. The analysis of the GH71 domains corroborated the phylogenetic relationships of the Schizosaccharomyces species determined by previous studies, but suggested a closer relationship to the Basidiomycota proteins than to the Ascomycota proteins. In the Schizosaccharomyces genus, the Agn1p proteins are structurally conserved: their GH71 domains are flanked by N-terminal secretion signals and C-terminal sequences containing the conserved block YNFNAYH/TG. The inactivation of the agn1$^{Sj}$ gene in Schizosaccharomyces japonicus, the only true dimorphic member of the genus, caused a severe cell-separation defect in its yeast phase, but had no effect on the hyphal growth and yeast-to-mycelium transition. It did not affect the mycelium-to-yeast transition either, only delaying the separation of the yeast cells arising from the fragmenting hyphae. The heterologous expression of agn1$^{Sj}$ partially rescued the separation defect of the agn1$^{D}$ cells of Schizosaccharomyces pombe. The results presented indicate that the fission yeast Agn1p 1,3-$\alpha$-glucanases of Schizosaccharomyces japonicus and Schizosaccharomyces pombe share conserved functions in the yeast phase.

INTRODUCTION

The fungal septum is a structure elaborated during cell division, simultaneously with the completion of cytokinesis, to form a physical boundary between the two progeny (reviewed by Walther & Wendland, 2003; Howell & Lew, 2012; Wloka & Bi, 2012; Mourino-Pérez, 2013). Its composition and structure vary among the larger taxonomic groups, but the major components are polysaccharides. In Aspergillus nidulans and Neurospora crassa, the major septal polysaccharide is chitin (for a review, see Mourino-Pérez, 2013). In the budding yeasts, such as Saccharomyces cerevisiae and Candida albicans, the septum is mainly composed of chitin and other polysaccharides (glucans and mannans) which are similar to those constituting the cell wall (reviewed by Howell & Lew, 2012; Walker et al., 2013). The septum of the fission yeast Schizosaccharomyces pombe contains $\beta$-glucan instead of chitin and various polysaccharides which are also present in the cell wall (for a review, see Sipiczki, 2007).

In yeasts, the septum has a trilaminar structure consisting of a primary septum sandwiched between two secondary septa. The layers differ in composition to allow the selective enzymic degradation of the primary septum without threatening the integrity of the secondary septa which will serve as the new cell wall in the daughter cells at the site of

Abbreviations: CCB, C-terminal conserved block; GH, glycoside hydrolase family.
cell separation. The primary septum of *Schizosaccharomyces pombe* is composed mainly of linear 1,3-β-glucan, whereas the secondary septa consist of 1,3-α-glucan, 1,6-branched- 1,3-β-glucan and galactomannans (Horisberger & Rouvet-Vauthey, 1985; Humbel *et al.*, 2001; Sugawara *et al.*, 2003). When the sister cells separate to become physically independent entities, they only degrade the primary septum and leave the secondary septa intact (septum cleavage or septum splitting) (Johnson *et al.*, 1973). This differential degradation of one septum layer is achieved by the activation of the Eng1p endoglucanase transported to the septum by the time of its completion (Martin-Cuadrado *et al.*, 2003). The endo-1,3-β-glucanase Eng1p is a glycoside hydrolase family 81 (GH81, EC 3.2.1.39) enzyme (http://www.cazy.org/GH81.html). Septum cleavage is essential, but not sufficient, for the separation of the sister cells. Two additional processes need to take place. The mother cell wall has to be dissolved in a narrow band around its junction with the septum (also called septum edging), in which the 1,3-α-glucanase Agn1p plays the fundamental role (Dekker *et al.*, 2004; Garcia *et al.*, 2005). Agn1p belongs to the GH71 family (EC 3.2.1.59) (http://www.cazy.org/GH71.html). In addition to the enzymic processes, a physical factor also contributes to the efficiency of cell separation. The internal turgor of the cytoplasm exerts a pressure on the secondary septa and swells them out, thereby facilitating the disruption of the primary septum and the mother cell wall (Sipiczki & Bozsik, 2000).

The cell-separation enzymes Agn1p and Eng1p are synthesized during the M phase of the cell cycle and secreted into the cell wall where they form a ring around the septum edge (Alonso-Nunéz *et al.*, 2005). The correct timing of their synthesis is ensured by specific transcription factors that act during the M phase. The key regulator is the zinc-finger protein Ace2p whose activity is essential for the transcription of both *agn1*<sup>+</sup> and *eng1*<sup>+</sup> (Alonso-Nuñez *et al.*, 2005). The production of Ace2p is controlled by the fork-head-type transcription factor Sep1p (Alonso-Nuñez *et al.*, 2005) – a central regulator of many M-phase-specific genes (Rustici *et al.*, 2004). Numerous other components of the transcriptional machinery (e.g. subunits of the Mediator and the SAGA complexes) have been shown to modulate the cell-separation process, mostly in indirect ways (Miklos *et al.*, 2008; Batta *et al.*, 2009).

In a previous study, we showed that the *sep1*<sup>+</sup>*–ace2*<sup>+</sup>–*agn1*<sup>+</sup>*–eng1*<sup>+</sup> cascade is conserved in the fission yeasts (Balazs *et al.*, 2012). The main purpose of this work was to investigate the structural and functional conservativeness of the Agn1p 1,3-α-glucanases. To this end, we identified putative Agn1p orthologues and paralogues in the genomes of completely sequenced fungi, compared their structures, and subjected their GH71 domains to phylogenetic analyses. The functional conservativeness was examined in two ways: by inactivation of the Agn1p orthologue in *Schizosaccharomyces japonicus*, the only dimorphic fission yeast, and by testing the heterologously expressed *agn1*<sup>55</sup> gene for the ability to rescue the *agn1Δ* mutation in *Schizosaccharomyces pombe*.

**METHODS**

**Identification and bioinformatic analysis of putative Agn1p orthologues.** Putative orthologues of the *Schizosaccharomyces pombe* 1,3-α-glucanase Agn1p were identified in organisms for which sequenced and annotated genomes were available in the National Center for Biotechnology Information genome sequence database (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) or in the genome database of the Broad Institute (http://www.broadinstitute.org/scientific-community/data). Searches were performed using BLASTP (Altschul *et al.*, 1997) with default parameters and SPA-C14CA.09 (Agn1p) as the query sequence. BLAST searches were also performed in the draft genome sequences of the *Taphriniomycotina* species *Saitoella commune* (http://genome.jgi.doe.gov/pages/blast.jsp?db=Saco1) and *Pneumocystis carinii* (http://pgp.cchmc.org). The most similar proteins were then extracted from the searched databases and used for a reciprocal search in the PomBase (http://www.pombase.org/) database. The proteins that gave the highest scores with Agn1p in the reciprocal search were considered its putative orthologues.

The GH71 domains were located in the proteins by scanning their sequences using the Pfam tool (http://pfam.sanger.ac.uk/search?tab= searchSequenceBlock) with the default threshold for the hidden Markov model search (*E* = 1.0). To identify conserved stretches and deletions (or insertions), pairwise BLAST B2seq (http://blast.ncbi.nlm.nih.gov/Blast.cgi) alignments and multiple CLUSTALW version 2.0 (Larkin *et al.*, 2007) and MAFFT version 6.850 (Katoh & Toh, 2008) alignments were generated. Secretory signal sequences were identified with the SignalP algorithm (Petersen *et al.*, 2011) available at http://www.cbs.dtu.dk/services/SignalP/. WebLogos (Crooks *et al.*, 2004) were generated from CLUSTALW alignments with the tool provided at http://weblogo.berkeley.edu/logo.cgi.

**Phylogenetic analysis.** For inferring phylogenetic relationships among the proteins, CLUSTAL and MAFFT multiple alignments of their GH71 domains were analysed using four methods. The neighbour-joining and maximum-parsimony trees were reconstructed with the PHYLIP version 3.67 software package (Felsenstein, 2007). In the neighbour-joining analysis, the Jones–Taylor–Thornton model of amino acid substitutions (Jones *et al.*, 1992) was used for computing distance matrices. Confidence limits were estimated from bootstrap analysis based on 1000 replications using the SEQBOOT and CONSENSE (majority-rule) programmes of the package. The maximum-likelihood tree was generated with the PhyML version 3.0 algorithm (Guindon *et al.*, 2010). For this and the Bayesian analysis, an amino acid substitution model was chosen by testing 120 models with ProtTest version 3.2.1 (Darriba *et al.*, 2011). The best model suggested by the Akaike Information Criterion (*lnL* = 16 340.30) was WAG (Whelan & Goldman, 2001). In the PhyML analysis, non-parametric bootstrap analysis was performed with 100 replicates. Bayesian inference of phylogeny was done using MrBayes version 3.2.3 (Ronquist *et al.*, 2012) with the WAG substitution model and gamma-shaped rate variation with a proportion of invariable sites. The number of discrete categories used to approximate the gamma distribution was set to 4. The Markov chain Monte Carlo processes were set so that four chains (one cold and three heated; setting a default temperature for heating the chains) were run simultaneously for 200 000 generations. Trees were sampled every 2000 generations. The mean standard deviation of split frequencies was *P* = 0.005 581 at the end of the analysis, indicating that a convergence had occurred (*P* < 0.05). The PSRF (potential scale reduction factor) values were between 1.000 and 1.004 for all parameters. The first 25 % of samples were discarded from the cold chain as burn-in and the remaining trees were used for estimating the Bayesian posterior probability of the branches. A consensus tree was derived from a total of 3002 trees. The phylogenetic trees were visualized with the TreeView (Page, 1996) and the FigTree (http://tree.bio.ed.ac.uk/) programs.
Yeast strains, media and growth conditions. The strains used in this study and their genotypes are listed in Table 1. The culture media YEA (yeast extract agar), YEL (YEA without agar), SMA (synthetic minimal agar) and EMMA (Edinburgh minimal medium agar) were described previously (Mitchison, 1970; Sipiczki & Ferency, 1978). For culturing auxotrophic strains, the minimal media were supplemented with nutrients according to the auxotrophy of the strains. To suppress the activity of the thiamine-repressible nmt1 promoter, the media were supplemented with 15 μM thiamine.

Plasmids, DNA manipulations and genetic transformation. Genomic DNA was isolated from exponential-phase yeast cultures grown in YEL with the glass bead method (Hoffman & Winston, 1987). Digestion of DNA with restriction endonucleases and ligation of DNA molecules were performed according to the instructions of the manufacturers of the enzymes used. The cloning vectors used and plasmids constructed in this study are listed in Table 1. Transformation of yeast cells of exponential-phase cultures grown in YEL with the glass bead method (Hoffman & Winston, 1987). Digestion of DNA with restriction endonucleases and ligation of DNA molecules were performed according to the instructions of the manufacturers of the enzymes used. The cloning vectors used and plasmids constructed in this study are listed in Table 1. Transformation of Escherichia coli cells, plasmid recovery from bacterial cells and basic recombinant DNA methods were performed by standard techniques described in Sambrook et al. (1989). Transformation of yeast cells of exponential-phase cultures grown in YEL was performed either with electroporation or with the lithium acetate method (Itou et al., 1983). Yeast transformants were selected and maintained on SMA plates supplemented with thiamine.

Table 1. Strains and plasmids

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<td>CCY†</td>
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<td>7-252</td>
<td>ura4&lt;sup&gt;Δ&lt;/sup&gt;-D3 mat&lt;sup&gt;Δ&lt;/sup&gt;-P2028</td>
<td>Furuya and Niki (2009)</td>
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<tr>
<td>7-256</td>
<td>agn1&lt;sup&gt;Δ&lt;/sup&gt;::kanMX6 ura4&lt;sup&gt;Δ&lt;/sup&gt;-D3 mat&lt;sup&gt;Δ&lt;/sup&gt;-P2028</td>
<td>This study</td>
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<td>0-1</td>
<td>L972 h&lt;sup&gt;-&lt;/sup&gt;, WT Schizosaccharomyces pombe var. pombe</td>
<td>U. Leupold, Bern</td>
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<td>2-1402</td>
<td>agn1::kanMX4 leu1-32 ura4-D18 ade-M210 h&lt;sup&gt;+&lt;/sup&gt; (YAB14)</td>
<td>Alonso-Nunez et al. (2005)</td>
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<td>80</td>
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<td>This study</td>
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<td>81</td>
<td>agn1::kanMX4 leu1-32 ura4-D18 ade-M210 h&lt;sup&gt;+&lt;/sup&gt; transformed with pRep42</td>
<td>This study</td>
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<td><strong>Plasmids</strong></td>
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<td>pJET1.2</td>
<td>Cloning vector, Clone Jet kit, catalogue no.: K1232</td>
<td>Fermentas</td>
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<td>pJET1.2-agn1&lt;sup&gt;Δ&lt;/sup&gt;</td>
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<td>This study</td>
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<td>pFA6a-kanMX6</td>
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<td>Bähler et al. (1998)</td>
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<td>Expression vector with high-strength nmt1 promoter and ura4&lt;sup&gt;Δ&lt;/sup&gt; marker gene</td>
<td>Maundrell (1993)</td>
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<td>pREP42</td>
<td>Expression vector with medium-strength nmt1 promoter and ura4&lt;sup&gt;Δ&lt;/sup&gt; marker gene</td>
<td>Basí et al. (1993)</td>
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<td>pREP82</td>
<td>Expression vector with low-strength nmt1 promoter and ura4&lt;sup&gt;Δ&lt;/sup&gt; marker gene</td>
<td>Basí et al. (1993)</td>
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<td>High-strength expression of agn1&lt;sup&gt;Δ&lt;/sup&gt;</td>
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<td>pREP82-agn1&lt;sup&gt;Δ&lt;/sup&gt;</td>
<td>Low-strength expression of agn1&lt;sup&gt;Δ&lt;/sup&gt;</td>
<td>This study</td>
</tr>
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</table>

*CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
†CCY: Culture Collection of Yeasts, Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia.
Cloning and heterologous expression of \textit{agn1}\textsuperscript{Sj} in \textit{Schizosaccharomyces pombe agn1}\textsuperscript{A}. The coding region of \textit{agn1}\textsuperscript{Sj} was amplified from genomic DNA of the WT 7-1 \textit{Schizosaccharomyces japonicus} with the primers Sjagn1for and Sjagn1rev (Table 2) complementary to its flanking chromosomal sequences. The PCR products obtained with the primers were cloned into pJET1.2 (Table 1) with blunt-end ligation. The cloned fragment was then excised from the plasmid (pJET1.2.agg1\textsuperscript{Sj}) and ligated into the \textit{Schizosaccharomyces pombe} expression vectors pREP2, pREP42 and pREP82 opened with the same restriction endonucleases at the thiamine-repressible \textit{nmt1}\textsuperscript{Sj} promoters. The pREP expression vectors contained full-strength (pREP2) or attenuated versions (pREP42 and pREP82) of the thiamine-repressible promoter of the \textit{Schizosaccharomyces pombe} gene \textit{nmt1}\textsuperscript{Sj} (Basi et al., 1993; Maundrell, 1993). The constructs obtained and the empty vectors were then used to transform cells of overnight YEL cultures of the uracil auxotrophic 2-1402 \textit{Schizosaccharomyces pombe} \textit{agn1}\textsuperscript{A} mutant. The transformed cultures were spread on \textit{SMA}+ adenine+ leucine plates, where only \textit{ura}+ transformants could produce colonies. To prevent the transcription of the \textit{agn1}\textsuperscript{Sj} coding sequence fused to \textit{nmt1}\textsuperscript{Sj} promoters, the medium was supplemented with 15 \textmu M thiamine inhibitory to the promoters (Basi et al., 1993; Maundrell, 1993). Heterologous expression of \textit{agn1}\textsuperscript{Sj} was induced by transferring the transformed cells into thiamine-free media. The effect of \textit{agn1}\textsuperscript{Sj} on division morphology was examined by microscopic observation in overnight cultures incubated at 30 °C.

**RESULTS AND DISCUSSION**

Identification and domain structure of fungal proteins similar to Agn1p of \textit{Schizosaccharomyces pombe}

Agn1p of \textit{Schizosaccharomyces pombe} is a 1,3-\textalpha-glucanase involved in the post-cytokinetic site-specific degradation of the band of the mother cell wall that edges the primary septum (Dekker \textit{et al.}, 2004; Garcia \textit{et al.}, 2005). It was identified as a homologue of the 1,3-\textalpha-glucanases of \textit{Trichoderma harzianum} and \textit{Penicillium purpurogenum} called mutanases (MutAp). With the advent of genome sequencing, the opportunity has arisen to search for homologues of genes and proteins in genomes of other organisms. In this study, we searched the publicly available fungal genome sequences for putative orthologues of Agn1p. The \textsc{blast} search identified a high number of proteins containing GH71 domains in all Basidiomycota and Pezizomycotina genomes, but in none of the Saccharomycotina species. GH71 is the characteristic domain of the enzymes that function as 1,3-\textalpha-glucanases (EC 3.2.1.59) (http://www.cazy.org/Glycoside-Hydrolases.html) with an endo-hyalolytic mode of enzymic activity (Fuglsang \textit{et al.}, 2000). The gene/protein identification numbers and certain basic data of the most similar proteins of the representative species are listed in Table 3.

The lack of Agn1p-like proteins in two Saccharomycotina species, \textit{Saccharomyces cerevisiae} and \textit{C. albicans}, was noticed previously by Dekker \textit{et al.} (2004) and interpreted as the consequence of the lack of 1,3-\textalpha-glucan in the cell walls of these budding yeasts. The cell wall around the neck connecting the bud with the mother cell is degraded in \textit{Saccharomyces cerevisiae} by the 1,3-\textbeta-glucanase Dse4p/
Eng1p (Baladrón et al., 2002) and certain less well-characterized hydrolases, such as Egt2p, Dse2p and Scw11p (Adams, 2004).

The overall BLAST sequence identity of the Schizosaccharomyces cryophilus Agn1p protein to its counterparts in the other species of the genus was 73, 70 and 68% for Schizosaccharomyces japonicus, Schizosaccharomyces cryophilus and Schizosaccharomyces octosporus, respectively. Within the GH71 domains, the identity was 76, 72 and 70%, respectively. The GH71 domains were flanked by short sequences on both sides. On the N-terminal side, each Agn1p protein had a secretion signal sequence of 22 aa. On the opposite side, all proteins possessed 22 aa long extensions that showed no similarity to any known domain but contained the short almost entirely conserved block \([\text{C-terminal conserved block (CCB)}]_{YNFNAY/HTG}\). In addition to the Agn1p-like protein, each species had another GH71 protein which was more similar in sequence to Agn2p – the paralogue of the Schizosaccharomyces pombe Agn1p (Dekker et al., 2004). Table 3 also lists the Agn2p proteins of the Schizosaccharomyces species. Their GH71 domains were shorter by 17 residues than those of the Agn1p proteins. The size difference can be attributed to short internal deletions (marked with vertical lines in Fig. 1). The Agn2p-like proteins were devoid of secretion signals (Fig. 1) and had slightly different CCB blocks (YNFN F/LC). The Weblogos in Fig. 1 generated from CLUSTAL alignments show the differences between the Agn1p and Agn2p CCBs.

In the subphylum Taphrinomycotina, which comprises the fission yeasts and seven other genera (James et al., 2006; Rosling et al., 2011), only two non-Schizosaccharomyces genomes are available for searching, but neither sequence is completely assembled and annotated. In the Saitoella complicata genome (Nishida et al., 2011; http://genome.jgi.doe.gov/pages/blast.jsf?db=Saico1) we found one gene coding for a GH71-containing protein (scaffold_1:1224631–1225931). This experimentally uncharacterized protein had no signal sequence, but possessed a perfect copy of the Agn1p CCB block (Fig. 1). Consistent with this, it showed significantly better sequence similarity to Agn1p

### Table 3. Identification numbers and structural features of GH71 proteins used for phylogenetic analysis

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<tr>
<th>Species</th>
<th>Database accession no. or gene/protein code</th>
<th>Length of protein (aa)</th>
<th>In protein</th>
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<td></td>
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<td>Total</td>
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<td>Saitoella complicata</td>
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<td>Schizosaccharomyces cryophilus</td>
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<td>Schizophyllum commune</td>
<td>XP_003034380.1</td>
<td>524</td>
<td>439</td>
</tr>
<tr>
<td>Serpula lacrimans</td>
<td>EGN94676.1</td>
<td>405</td>
<td>380</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Burkholderia ambibaria</td>
<td>YP_776994.1</td>
<td>455</td>
<td>393</td>
</tr>
</tbody>
</table>

http://mic.sgmjournals.org
(42%, \( E \) value: \( 7 \times 10^{-101} \)) than to Agn2p (33%, \( E \) value: \( 9 \times 10^{-64} \)) in two BLAST alignments. The search in the \( P. \) \( \text{carinii} \) genome (Hauser et al., 2010; http://pgp.cchmc.org) resulted in no hit.

The Pezizomycotina and Basidiomycota GH71 proteins also had flanking extensions on both sides of their glycoside hydrolase domains, but these extensions showed very limited similarity to those of the fission yeast proteins and varied in size and sequence. Selected examples are presented in Fig. 1. For \( \text{Trichoderma} \), we show two proteins because we found that the \( \text{Trichoderma} \) protein most similar to Agn1p was not the enzyme characterized previously as mutanase (MutAp). This finding was unexpected because the \( \text{Schizosaccharomyces pombe} \) Agn1p was not the enzyme characterized previously as mutanase (MutAp). CBM24 is a carbohydrate-binding (mutan-binding) module.

Polysaccharide-binding domains are common among fungal hydrolases, such as cellulases, xylanases, chitinases and amylases (Gilkes et al., 1991). Dekker et al. (2004) hypothesized that carbohydrate binding is essential for enzymes secreted into the environment to hydrolyse extracellular glucans as potential nutrient sources. As Agn1p acts in a
specific region of the cell wall, to which it is delivered by the
exocyst complex shortly before cell separation (Alonso-Nuñez
et al., 2005), it may not need that sort of substrate-binding
function. We also compared the Schizosaccharomyces pombe
Agn2p, the sporulation-specific parologue of Agn1p, with the
Trichoderma and Penicillium glucanases, and found
similar differences. These findings indicate that neither
Agn1p nor Agn2p are orthologues of mutanases that have
CBM24 domains. The latter enzymes are perhaps proteins
developed recently for mycoparasitism by duplication of the
genes of the GH71 -glucanases more similar to Agn1p and
Agn2p (e.g. EGR50031.1 in T. reesei) and subsequent acqui-
sition of mutanase-binding domains. The stronger similarity
of EGR50031.1 to the Schizosaccharomyces enzymes indicates
that this enzyme and its counterparts in other filamentous
fungi might be the real Agn1p and Agn2p orthologues.
However, exact functional equivalence between Agn1p and
those proteins is unlikely because no cell separation takes
place upon septation in the growing mycelium.

Phylogenetic analysis of the GH71 domains
Virtually all proteins identified in the BLAST search had
extensions upon septation in the growing mycelium.
This position is consistent with the standard phylogeny
of fungi in which Taphrinomycotina is assigned to the
monophyletic group of ascomycetous fungi (e.g. James
et al., 2006). The closer phylogenetic relationship to the
Basidiozyma proteinae may be a tree-construction artefact
attributable to the low number of Taphrinomycotina
sequences available for analysis or to different rates of
evolution of -glucanases in different fungal lineages.
Another peculiarity of the GH71 trees was the position of the Saitoella protein. It formed a lineage branching off very

![](https://www.microbiologyresearch.org/article-figures/1069/1069-fig2.png)

**Fig. 2.** Phylogenetic analysis of the GH71 domains of proteins. (a) Combined results of four methods. The phylogenetic tree
shown was inferred using the Bayesian method, and supplemented with statistical values supporting the nodes in neighbour-
joining, maximum-parsimony, maximum-likelihood and Bayesian analyses. The order of the statistical support values at the
nodes: MrBayes/neighbour joining/maximun parsimony/PhyML. Bootstrap values <50 are not shown but indicated with two
dots. d, Bifurcation different from that on the Bayesian tree; ●, protein has a secretion signal; □, protein has a CCB.
(b) Neighbour-joining tree of the Schizosaccharomyces Agn1p proteins. Statistical support values: neighbour joining/maximun
parsimony. The Burkholderia ambifaria sequence was used as outgroup. For relevant database accession numbers or gene/
protein codes of the sequences, see Table 3.
early, before the separation of the Schizosaccharomyces proteins from the Basidiomycota proteins.

As Agn1p and Agn2p are more similar to each other in amino acid sequence than to any Pezizomycotina or basidiomycete proteins, they appear to be paralogues that derived from a common ancestor by gene duplication taking place after the separation of the Schizosaccharomyces lineage from the rest of Fungi. The separation must have occurred before the split of the lineage because all extant Schizosaccharomyces species have both Agn1p and Agn2p orthologues that form separate groups on the Schizosaccharomyces branch of the phylogenetic tree. As mentioned above, we found only one GH71 gene in the partially annotated Saitoeilla complicata genome. The predicted amino acid sequence of its translation was more similar to those of the Schizosaccharomyces Agn1p proteins than to any of the Agn2 sequences. This finding could be interpreted as indicating that the Agn1p sequences have evolved at slower rates and perhaps better preserved the ancient state of GH71 hydrolases in Taphrinomycotina.

Within the Schizosaccharomyces clade, the branching order of the Agn2p proteins was consistent with the phylogenetic relationships of the four Schizosaccharomyces species established by previous analyses that indicated an early split of the genus into the lineage leading to the present-day dimorphic species Schizosaccharomyces japonicus and the lineage of the rest of the species which are not dimorphic (e.g. Sipiczki, 1995; Rhind et al., 2011; Balazs et al., 2012). This early separation was not obvious in the case of the Agn1p proteins. The basal branch was the Schizosaccharomyces japonicus protein on the neighbour-joining and maximum-parsimony trees, and the Schizosaccharomyces pombe protein on the maximum-likelihood and Bayesian trees.

This uncertainty in the branching order could be due to different evolutionary constraints exerted on Agn1p\(^{5}\) which operates in a dimorphic organism. Three of the four Schizosaccharomyces species propagate by forming yeast cells (Sipiczki, 2007; Helston et al., 2010), although certain strains of Schizosaccharomyces pombe are able to develop hypha-like structures as well under specific growth conditions (e.g. Dodgson et al., 2010). The fourth species, Schizosaccharomyces japonicus, is dimorphic, i.e. alternates between yeast and mycelial growth morphology (Sipiczki et al., 1998). Agn1p\(^{5}\) can have the same function in the yeast phase of Schizosaccharomyces japonicus as its counterpart in Schizosaccharomyces pombe, but may also have a different role in the mycelial phase which is dispensable in the non-dimorphic species. If it is indeed bifunctional, then both of its functions might have been targets of evolution.

**Inactivation of agn1\(^{5}\) and its effect on cell separation**

In the quest to identify the function(s) of Agn1p\(^{5}\), we examined the effect of the inactivation of its gene on the yeast and mycelial phases. The deletion of agn1\(^{5}\) changed drastically the growth morphology in the yeast phase. The phenotype of the deletion strains was similar to that observed in mutants defective in the transcription factors Sep1p\(^{5}\) and Ace2p\(^{5}\) (Balazs et al., 2012). The observed deficiency was much more severe than the separation defect of the Schizosaccharomyces pombe agn1\(^{A}\) mutants (Dekker et al., 2004; García et al., 2005), but not complete. Whereas the Schizosaccharomyces japonicus WT strain (7-1) mainly produced individual yeast cells without septa and cells with single septa (dividing cells) (Fig. 3a), the mutant culture (7-256) contained branching (hypha-like) chains of non-separated or partially separated cells and V-like, incompletely separated cell pairs (Fig. 3b–e). The agn1\(^{A}\) cells of Schizosaccharomyces pombe only formed V-like pairs or quadruples of cells (Fig. 3g–i). In the case of incomplete separation, the septa of the Schizosaccharomyces japonicus mutant were virtually split, but the mother cell wall was not broken down around the entire circumference of the septum edge (see sites marked with arrows in Fig. 3b, c, e). When the cells were stained with calcofluor, a fluorescence brightener of the primary septum (Sipiczki et al., 1998), the incompletely separated ends of these sister cells remained dark under the fluorescence microscope, whereas the intact septa were bright (Fig. 4b). The calcofluor-negativity indicated that the primary septum, whose degradation normally starts when the edging cell wall has been degraded, was dissolved completely. Partial cell separation was observed mainly between sister cells which had grown near the septal region, usually forming lateral extensions (branches). It is conceivable that the physical tensions generated by this growth ripped the cell wall at the septum and the rupture then triggered the degradation of the primary septum. Strong vortexing of the calcofluor-stained mutant culture corroborated this hypothesis. It increased the frequency of broken septa in the cell chains, generating cell ends with calcofluor-positive material on their ends (Fig. 4c), which then gradually faded out, whereas the intact septa remained bright. Similar observations were made previously in sep1\(^{S}\) mutants of Schizosaccharomyces pombe (Sipiczki & Bozsik, 2000) and in Schizosaccharomyces japonicus mutants defective in ace2\(^{S}\) or sep1\(^{S}\) (Balazs et al., 2012). When cultured in a liquid medium, the agn1\(^{5}\)::kanMX6 cell chains clumped together (Fig. 3d) and formed loose sediment. Supplementation of the medium with NaCl, KCl, CaCl\(_2\) or NH\(_4\)NO\(_3\) to 1 mM final concentration did not change the clumps. However, heavy vortexing reduced their size and increased the proportion of individual chains in the suspension. These findings indicated that the clumps consisted of physically entangled branched chains and groups of partially separated cells rather than aggregates of cells connected by hydrogen bonds. DAPI staining identified single nuclei in most cells of the agn1\(^{5}\)::kanMX6 chains (Fig. 4a), indicating that the inactivation of the agn1\(^{S}\) gene did not affect the synchrony of karyokinesis and cytokinesis (septation).
Cell chains of the agn1Sj::kanMX6 mutant are not hyphae

Although the agn1Sj::kanMX6 cell chains produced under conditions supporting yeast growth frequently had up to 20 cells, they could not be regarded as hyphae because their growth morphology differed from that characteristic of the hyphae of the mycelial phase. Previous studies revealed (Sipiczki et al., 1998) that the true hyphae of Schizosaccharomyces japonicus grow unipolarly, develop large vacuoles at their non-growing poles and form branches behind (on non-apical sides of) their septa. This growth pattern can be seen in Fig. 5(a). In contrast to the true hyphae, the cells of the agn1Sj::kanMX6 chains formed in the yeast phase grew bipolarily, did not contain large vacuoles and frequently formed branches (lateral extensions) on both sides of their septa (at both cell ends) (compare Figs 3e and 5a). These differences indicate that the omission of cell separation frequently occurring in the agn1Sj::kanMX6 culture is not sufficient for transition from the yeast phase to the hyphal phase.

Inactivation of agn1Sj does not affect the yeast-to-hypha transition but delays the fragmentation at the hypha-to-yeast transition

The WT Schizosaccharomyces japonicus forms yeast colonies on the surface of solid media but switches to mycelial growth (yeast-to-hypha transition) when the medium around the yeast colony becomes depleted of nitrogen-containing nutrients (Sipiczki et al., 1998). The hyphae invade the medium and grow towards fresh resources of nutrients. Upon reaching a nutrient-rich part of the substrate, they stop extending, undergo several rounds of septum formation and split the septa to convert into bipolarly growing yeast cells (hypha-to-yeast transition).

We noticed no discernible difference between the WT and the mutant in the efficiency of the yeast-to-hypha transition and the growth of the invasive hyphae. Thus, Agn1pSj is probably not involved in these processes. We found a difference only at the return to the yeast phase. Our earlier observations revealed that the hypha-to-yeast transition is a complex set of physiological and cellular events including growth arrest at the hyphal tip, extension of the cytoplasm at the expense of the large polar vacuoles, rapid multiple septation and breaking up the hypha at the
septa to produce fragments (arthrospores) that start growing at both of their ends like the yeast cells do (Sipiczki et al., 1998). Here, we compared this process in the WT and in the mutant. For this, we interrupted the nutrient gradient that maintained the hyphal growth by dropping fresh medium (YEL) on the surface of the agar medium at the growing front of the mycelium. The sudden decrease in nutrient supply stopped hyphal elongation, extended the cytoplasm within the apical cells and then launched multiple septation of these cells both in the WT and in the \( \text{agn1}\text{Sj} \) mutant. It did not form chains but only V-shape cell pairs, and rarely also quadruples and a somewhat increased percentage of septate cells. The expression of \( \text{agn1}\text{Sj} \) from the prEP plasmids drastically reduced the proportion of these morphological categories, but did not restore the standard WT morphology (Fig. 6a).

The V-shape cell pairs in the \( \text{agn1}\text{A} \) mutant were sister cells kept together by the remains of the eroded mother cell wall (see above), but the increased percentage of septate cells could be attributed either to occasional skipping of cell separation or to a delay in the onset of separation. To resolve this issue, we compared the time elapsing from the beginning of septum formation in the mother cell to the complete separation of the daughter cells by time-lapse photomicrography. We photographed 25 dividing cells at regular time intervals for each strain (examples are shown in Fig. 6b). We found that the life time of the visible septa in the WT \( \text{Schizosaccharomyces pombe} \) (0-1) culture was 20 \( \pm \) 2.00 min. In the mutant \( \text{agn1}\text{A} \) (2-1402), it was 46.25 \( \pm \) 15.06 min. The heterologous expression of \( \text{agn1}\text{Sj} \) from prEP2 (in strain 85) and from prEP82 (in strain 83) reduced the duration of the septate phase to 27.5 \( \pm \) 4.63 and 27.5 \( \pm \) 13.99 min, respectively. Thus, \( \text{Agn1p}\text{Sp} \) was active in \( \text{Schizosaccharomyces pombe} \) and could at least partially substitute the native \( \text{Agn1p}\text{Sp} \) protein.

The cell-separation deficiency of the \( \text{Schizosaccharomyces japonicus} \) mutant and the rescuing activity of the

**Heterologously expressed \( \text{agn1}\text{Sj} \) partially rescues the \( \text{agn1}\text{A} \) cell-separation defect in \( \text{Schizosaccharomyces pombe} \)**

In view of the above conclusion, \( \text{Agn1p}\text{Sj} \) and \( \text{Agn1p}\text{Sp} \) can be considered orthologous proteins performing identical functions in the yeast cells of the two species. However, the difference in the severity of the cell-separation deficiency of the mutants argues against complete functional equivalence. To further examine their functional homology, we cloned the coding region of \( \text{agn1}\text{Sp} \), fused it with the thiamine-repressible \( nmt1\text{+} \) promoters of \( \text{Schizosaccharomyces pombe} \) expression vectors (Table 1) and transformed the resulting plasmids into \( \text{Schizosaccharomyces pombe} \) \( \text{agn1}\text{A} \) cells. The transformants were selected on a minimal medium supplemented with thiamine to keep the \( nmt1\text{--agn1}\text{Sj} \) inserts of the plasmids silent. The transformants were transferred from this medium into thiamine-free liquid media to allow the expression of the \( \text{Schizosaccharomyces japonicus} \) gene in the \( \text{Schizosaccharomyces pombe} \) \( \text{agn1}\text{A} \) cells. The cell morphology of the exponential-phase cultures was then compared with the morphology of the WT and the non-transformed \( \text{agn1}\text{A} \) cells. As shown above (Fig. 3), the \( \text{Schizosaccharomyces pombe} \) mutant had a less pronounced phenotype than the \( \text{Schizosaccharomyces japonicus} \) mutant. It did not form chains but only V-shape cell pairs, and rarely also quadruples and a somewhat increased percentage of septate cells. The expression of \( \text{agn1}\text{Sj} \) from the prEP plasmids drastically reduced the proportion of these morphological categories, but did not restore the standard WT morphology (Fig. 6a).

The cell-separation deficiency of the \( \text{Schizosaccharomyces japonicus} \) mutant and the rescuing activity of the...
Schizosaccharomyces japonicus gene in the Schizosaccharomyces pombe mutant indicate that Agn1pS and Agn1pSp share homologous functions in the propagating yeast cells of these fission yeast species. However, the more severe cell defect in the Schizosaccharomyces japonicus mutant and the incomplete rescue of the Schizosaccharomyces pombe mutation by the Schizosaccharomyces japonicus gene suggest that the two proteins may also have specific functions in their native environments.

ACKNOWLEDGEMENTS

This work was supported by the Hungarian Scientific Research Fund (grant K101323). We thank Ilona Lakatos for expert technical assistance.

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Edited by: D. Mattanovich