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Role of the Synthase Domain of Ags1p in Cell Wall α-Glucan Biosynthesis in Fission Yeast

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The cell wall is important for maintenance of the structural integrity and morphology of fungal cells. Besides β-glucan and chitin, α-glucan is a major polysaccharide in the cell wall of many fungi. In the fission yeast *Schizosaccharomyces pombe*, cell wall α-glucan is an essential component, consisting mainly of (1,3)-α-glucan with ~10% (1,4)-linked α-glucose residues. The multidomain protein Ags1p is required for α-glucan biosynthesis and conserved among cell wall α-glucan-containing fungi. One of its domains shares amino acid sequence motifs with (1,4)-α-glucan synthases such as bacterial glycogen synthases and plant starch synthases. Whether Ags1p is involved in the synthesis of the (1,4)-α-glucan constituent of cell wall α-glucan had remained unclear. Here, we show that overexpression of Ags1p in *S. pombe* cells results in accumulation of (1,4)-α-glucan. To determine whether the synthase domain of Ags1p is responsible for this activity, we overexpressed Ags1p-E1526A, which carries a mutation in a putative catalytic residue of the synthase domain, but observed no accumulation of (1,4)-α-glucan. Compared to wild-type Ags1p, this mutant Ags1p showed a markedly-reduced ability to complement the cell lysis phenotype of the temperature-sensitive *ags1-1* mutant. Therefore, we conclude that, in *S. pombe*, the production of (1,4)-α-glucan by the synthase domain of Ags1p is important for the biosynthesis of cell wall α-glucan.
INTRODUCTION

Distinct plasma membrane-localised synthases are responsible for the production of structural polysaccharides in the fungal cell wall, mostly (1,3)-β-glucan, chitin, and α-glucan. (1,3)-β-Glucan and chitin synthases were identified first in the budding yeast *Saccharomyces cerevisiae* (Douglas et al., 1994; Bulawa et al., 1986) as integral membrane proteins with multiple transmembrane regions and a large cytoplasmic domain, which may be responsible for catalytic activity (Dijkgraaf et al., 2002; Nagahashi et al., 1995; Cos et al., 1998). Given that cell wall polysaccharides are absent in humans but crucial for maintaining the morphology and structural integrity of fungal cells, inhibitors of the synthases may function as antifungal drugs. For example, caspofungin is a (1,3)-β-glucan synthase inhibitor proven to be effective against many fungi and also safe and well tolerated in humans (Kartsonis et al., 2003). With regard to α-glucan synthase (Ags), much less is known and no inhibitors targeting this enzyme have been developed.

The α-glucan synthase Ags1p was identified in the fission yeast *S. pombe* using a temperature-sensitive mutant strain, *ags1-^1^ts*, the cells of which lyse at the restrictive temperature due to a weakened cell wall unable to withstand internal osmotic pressure (Hochstenbach et al., 1998). These observations showed that cell wall α-glucan is an essential component of the *S. pombe* cell wall. Ags1p is a multidomain protein with two probable catalytic domains predicted to reside at opposite sides of the plasma membrane, as well as a multipass transmembrane domain. This overall domain structure of Ags1p is conserved among the four Ags1p homologs of *S. pombe*, Mok11p, Mok12p, Mok13p, and (partly) Mok14p, the genes of which are expressed during sporulation (Katayama et al., 1999). Notably, this domain structure is also well conserved among Ags1p homologs in other cell wall α-glucan-containing fungi, such as several human fungal pathogens in which cell wall α-glucan accounts for ∼35% of total cell wall polysaccharides. The genome of the filamentous fungus *Aspergillus fumigatus* contains three *AGS* genes, of which *AGS1* and *AGS3* appear to be directly involved in cell wall α-glucan biosynthesis (Beauvais et al., 2005; Maubon et al., 2006). For the thermally dimorphic fungus *Histoplasma capsulatum*, the virulent yeast form contains substantial levels of cell wall α-glucan and targeting of its sole *AGS* gene by RNA interference demonstrated directly that cell wall α-glucan is important for virulence of this pathogen (Rappleye et al., 2004). In the opportunistic yeast *Cryptococcus neoformans*, inhibition of expression of its sole *AGS* gene gives rise to acapsular cells, indicating that cell wall α-glucan plays a role in anchoring the capsule, a critical virulence factor for this pathogen (Reese and Doering, 2003).

Glycogen and starch synthases are glycosyltransferases that catalyse the formation of a (1,4)-α-glucosidic bond and transfer the α-glucose moiety from UDP-glucose or
ADP-glucose to the non-reducing end of a pre-existing (1,4)-α-glucan primer. Based on amino acid sequence similarities, these synthases have been divided into two families, family 3 of glycosyltransferases (GT-3, with animal and fungal glycogen synthases) and family 5 of glycosyltransferases (GT-5, with archaeal and bacterial glycogen synthases and plant starch synthases) (Coutinho et al., 2003). Although these two families display only marginal sequence similarities, they appear to share certain structural and catalytic features (Cid et al., 2002). The recently reported crystal structures of the bacterial glycogen synthase GlgA of the bacterium Agrobacterium tumefaciens (Buschiazzo et al., 2004) and the Archaea Pyrococcus abyssi (Horcajada et al., 2006) provide a basis for our understanding of the catalytic mechanism of these synthases. Both the N- and C-terminal halves fold into a subdomain with a Rossmann-type fold, a classical structural motif characterised by a central β-sheet flanked by several α-helices. These subdomains are connected by a narrow hinge, creating a deep and wide central cleft between them. It has been proposed that, upon substrate binding, this cleft may close, bringing the nucleotide-glucose-binding motif (Lys/Arg)-X-Gly-Gly (Furukawa et al., 1993) on the N-terminal side of the cleft in close proximity to the catalytic motif Glu-X7-Glu (Cid et al., 2000) on the C-terminal side to create a functional active center (Buschiazzo et al., 2004; Horcajada et al., 2006).

In this study, we focused on the role of the putative intracellular domain of S. pombe Ags1p, denoted here as the synthase (SYN) domain. Our recent observations that cell wall α-glucan from S. pombe consists of ~10% (1,4)-linked α-glucose residues (Grün et al., 2005) prompted us to investigate whether the SYN domain is involved in the synthesis of this cell wall α-glucan constituent. Here, we show that fungal Ags SYN domains share several sequence motifs with GT-3 and GT-5 enzymes, including the Glu-X7-Glu motif, the first Glu of which is a highly conserved catalytic residue. Furthermore, we show that (1,4)-α-glucan accumulates in S. pombe cells overexpressing wild-type Ags1p, but not in S. pombe cells overexpressing the mutant Ags1p-E1526A, with the predicted catalytic residue mutated. Together, our data demonstrate that the S. pombe Ags1p SYN domain is involved in cell wall α-glucan biosynthesis by producing (1,4)-α-glucan.

RESULTS

Phylogenetic tree of the (1,4)-α-glucan synthase superfamily

Close examination of the putative intracellular domain of the S. pombe Ags1p (residues 1092–1986) revealed an N-terminal Lys-rich (19%) region (residues 1092–1144) and a C-terminal Ser-rich (15%) region (residues 1628–1986), both of which flank a domain with amino acid sequence similarities to glycogen and starch synthases, referred to
here as the SYN domain (Figure 1A). The *S. pombe* Ags1p SYN domain displays \(~26\%\) amino acid sequence identity to the *A. tumefaciens* and *P. abyssi* GlgA proteins, the three-dimensional structures of which are known. Besides the genome of *S. pombe*, the genomes of 14 other ascomycetous fungi and three basidiomycetous fungi are known to encode Ags proteins. Their Ags SYN domains show \(~70\%\) amino acid sequence identity to one another, whereas they show \(~27\%\) identity to established GT-5 synthases, the archaeal and bacterial glycogen synthases and plant synthases (data not shown). To study the phylogenetic relationship of the fungal Ags SYN domains with glycogen and starch synthases, we compared their amino acid sequences using TCoffee (Supplemental Figure S1) and ClustalW (data not shown) and generated phylogenetic trees using the neighbor-joining method (Figure 1B), as
well as FITCH, PUZZLE, and Bayesian analyses (data not shown). Although minor differences were observed in the branching pattern within the major clusters, the mutual relationships among the major clusters were similar. The fungal Ags SYN domains clustered as a monophyletic group within the (1,4)-α-glucan synthase superfamily, relating more closely to other GT-5 synthases than to GT-3 synthases (Figure 1B). Furthermore, the previously described putative catalytic residues of the glycogen synthase of Escherichia coli (EcGlgA) and human muscle glycogen synthase are highly conserved in the Ags SYN domains (Figure 2). These results suggest that the SYN domain may function as a (1,4)-α-glucan synthase.

**Ags1p overexpression causes (1,4)-α-glucan accumulation**

To investigate experimentally whether Ags1p synthesises (1,4)-α-glucan, we inserted the thiamine-repressible nmt1 promoter in front of the ags1+ ORF in the genome of S. pombe wild-type strain 972. This well characterised promoter was chosen, because it shows strong promoter activity when cells are grown in the absence of thiamine (induction conditions) (Maundrell, 1993). Furthermore, when grown in the presence of thiamine (repression conditions), cells maintain low expression levels of ags1+ sufficient for normal vegetative growth as a result of residual promoter activity.

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**Figure 2. Conservation of the Glu-X_{7}-Glu motif among fungal Ags SYN domains.** Clustal W alignment and PSIPRED secondary structure prediction for the region flanking the Glu-X_{7}-Glu motif in fungal Ags SYN domains as well as selected starch synthases and glycogen synthases. Dark-grey shading represents predicted β-sheets and light-grey shading represents predicted α-helices, except for A. tumefaciens GlgA (AtGlgA) and P. abyssi GlgA (PaGlgA), the secondary structures of which are known from the solved crystal structure. Dashes indicate identity to the amino acid of S. pombe Ags1p (SpAgs1) in the top line, and boxes indicate the position of the Glu residues of the motif. The species and synthases included are listed in supplemental Table S1. Note that the first Glu is located in a loop (neither α-helix nor β-sheet), whereas the second Glu is predicted to be part of an α-helix (i.e., α13 in AtGlgA and PaGlgA).
(Basi et al., 1993). To analyse these \( \textit{Pnmt1}-\textit{ags1}^+ \) cells, we developed a simple plate-based assay for the detection of (1,4)-\( \alpha \)-glucan accumulation. Approximately 100,000 cells were spotted onto plates with chemically defined culture medium (EMMA) with or without the addition of thiamine. Following incubation at 28°C for 3 days, the plates were developed by a 4-min exposure to iodine vapour, which stains (1,4)-\( \alpha \)-glucan, either branched (glycogen and starch) or unbranched (amylose). As a positive control, we used cells overexpressing the \( \textit{A. tumefaciens} \) gene encoding GlgA (\( \textit{AtGlgA} \)) under control of the \( \textit{nmt1} \) promoter from the pREP4 plasmid for ectopic expression in \( \textit{S. pombe} \) (Maundrell, 1993). Under repression conditions, these cells showed a yellowish staining (Figure 3A, lower panel (LOW), lane 3) similar to wild-type cells of strain 972 or cells carrying an empty pREP4 plasmid.

Figure 3. Overexpression of \( \textit{Ags1p} \) results in accumulation of material stainable by iodine vapour. Cells \( (10^5) \) were spotted onto EMMA plates without (high expression; \(-T\)) or with (low expression; \(+T\)) the addition of thiamine. After 3 days of growth at 28°C, plates were exposed to iodine vapour to stain for (1,4)-\( \alpha \)-glucan. The following cells were analysed: (A) wild-type cells (strain 972, lanes 1 and 4), cells containing pREP4 (strain AV027, lane 2) or pGlgA (strain AV069, lane 3), cells of genotype \( \textit{Pnmt1}-\textit{ags1}^+ \) (strain ND025, lane 5) or \( \textit{Pnmt1}-\textit{mok11}^+ \) (strain ND032, lane 6); (B) cells containing pREP4 (strain AV027, lanes 1 and 3), \( \textit{pAgs1} \) (strain AV028, lane 2), \( \textit{pAgs1}-\text{ATGL} \) (strain BS006, lane 4), or \( \textit{pAgs1}-\text{ASYN} \) (strain AV143, lane 5); (C) cells containing pREP4 (strain AV027, lanes 1 and 4), \( \textit{pAgs1}-\text{G696S} \) (strain AV149, lanes 2 and 5), or \( \textit{pAgs1} \) (strain AV028, lanes 3 and 6), grown at 28°C (lanes 1–3) or 36°C (lanes 4–6); (D) cells containing \( \textit{pAgs1} \) (strain AV028, lane 1), \( \textit{pAgs1}-\text{E1526A} \) (strain AV039, lane 2), \( \textit{pAgs1}-\text{E1534A} \) (strain AV040, lane 3), \( \textit{pAgs1}-\text{G1165A} \) (strain AV037, lane 4), \( \textit{pAgs1}-\text{G1165A} \) (strain AV038, lane 5), or \( \textit{pAgs1}-\text{K1422Q} \) (strain AV042, lane 6); and (E) wild-type cells (strain 972, lane 1), cells containing pREP4 (strain AV027, lane 2), \( \textit{pGSY2} \) (strain AV116, lane 3), \( \textit{pGSY2}-\Delta643 \) (strain AV117, lane 4), or \( \textit{pAgs1} \) (strain AV028, lane 5). Note that the brown staining indicates accumulation of (1,4)-\( \alpha \)-glucan. (For a full colour version, see page 205)
(Figure 3A, lanes 1 and 2). By contrast, under induction conditions cells expressing \textit{AtGlgA} showed an intense brown staining characteristic for accumulation of (1,4)-\(\alpha\)-glucan (Figure 3A, upper panel (HIGH), lane 3). Remarkably, when we tested the \textit{Pnmt1-ags1\textsuperscript{+}} cells under induction conditions, we also observed a brown staining, indicating that also these cells accumulated (1,4)-\(\alpha\)-glucan (Figure 3A, upper panel (HIGH), lane 5). This brown staining was due to \textit{ags1} overexpression because, when \textit{Pnmt1-ags1\textsuperscript{+}} cells were grown under repression conditions, the intensity of staining resembled that of control cells (Figure 3A, lower panel (LOW), lane 5). Similarly, cells overexpressing the close \textit{ags1\textsuperscript{+}} homolog \textit{mok11\textsuperscript{+}}, which is expressed normally only during sporulation (Katayama \textit{et al}., 1999; Mata \textit{et al}., 2002), also accumulated (1,4)-\(\alpha\)-glucan upon induction during vegetative growth (Figure 3A, lane 6).

To demonstrate directly that the \textit{ags1\textsuperscript{+}} ORF was responsible for the (1,4)-\(\alpha\)-glucan accumulation, we cloned it behind the \textit{nmt1} promoter in the pREP4 plasmid and analysed the ability of the resulting pAgs1 plasmid to induce (1,4)-\(\alpha\)-glucan accumulation in wild-type cells. In our plate-based assay, cells carrying plasmid pAgs1 showed a thiamine-repressible brown staining similar to that observed for \textit{Pnmt1-ags1\textsuperscript{+}} cells, indicating that both systems for \textit{ags1\textsuperscript{+}} overexpression resulted in comparable levels of (1,4)-\(\alpha\)-glucan accumulation (compare Figure 3B, lane 2 with Figure 3A, lane 5). Given that \textit{ags1\textsuperscript{+}} is an essential gene in \textit{S. pombe} (Hochstenbach \textit{et al}., 1998; Katayama \textit{et al}., 1999) and that, as a consequence, mutagenesis of the chromosomal copy could result in cell lysis, we chose the pREP4 expression system for the remainder of this study to overexpress mutant versions of Ags1p. We next analysed whether overexpression of a functional version of Ags1p is required for (1,4)-\(\alpha\)-glucan accumulation. The \textit{ags1\textsuperscript{+}T\textsuperscript{ts}} mutant strain carries a single missense mutation in its \textit{ags1} gene, encoding Ags1p-G696S, the function of which is temperature dependent (Hochstenbach \textit{et al}., 1998). To analyse the ability of this mutant Ags1p to produce (1,4)-\(\alpha\)-glucan, we induced its overexpression in wild-type cells. Overexpression of Ags1p-G696S at 28°C, when this protein is functional, resulted in (1,4)-\(\alpha\)-glucan accumulation at levels observed for overexpression of wild-type Ags1p (Figure 3C, compare lanes 2 and 3). By contrast, Ags1p-G696S overexpression at 36°C, when this protein is defective, did not result in (1,4)-\(\alpha\)-glucan accumulation (Figure 3C, lane 5). We conclude that induction of a functional Ags1p is required for accumulation of (1,4)-\(\alpha\)-glucan.

To confirm that iodine vapour was staining (1,4)-\(\alpha\)-glucan, rather than other cell components such as lipids (Palumbo and Zullo, 1987), we monitored the accumulation of (1,4)-\(\alpha\)-glucan by measuring the amount of glucose released from insoluble cell fractions after treatment with \(\alpha\)-amylase and glucoamylase, \textit{endo-} and \textit{exo-} type glucanases, respectively, specific for (1,4)-\(\alpha\)-glucan. Following the shift of \textit{Pnmt1-ags1\textsuperscript{+}} cells from EMMA medium with thiamine to EMMA medium without
thiamine, we analysed cells at regular time intervals and observed a pronounced increase in the amount of cellular (1,4)-α-glucan (Figure 4A, upper panel). In total lysates of matching cells, Ags1p overexpression levels were analysed in immunoblot analyses using an antiserum directed against a peptide of the Ags1p SYN domain (residues 1618–1631). The increasing levels of (1,4)-α-glucan correlated closely with increasing levels of Ags1p, which resolved as a specific protein band at an apparent molecular mass of ~265 kDa (Figure 4A, lower panel), consistent with the calculated molecular mass for Ags1p of 272.1 kDa. Under repression conditions, \( P_{nmt1-ags1^+} \) cells maintained background levels of amylase-sensitive glucans (Figure 4B, upper panel), whereas Ags1p levels remained undetectable in immunoblot analyses using this antiserum (Figure 4B, lower panel), as was the case for wild-type strain 972 (Figure 4A and 4B, lane 1). A similar correlation between Ags1p overexpression and (1,4)-α-glucan accumulation was observed when Ags1p overexpression was driven from plasmid pAgs1 (Figure 4C). Although induction was somewhat delayed, the maximum levels of cellular (1,4)-α-glucan were similar to those observed for \( P_{nmt1-ags1^+} \) cells (Figure 4, compare A and C). Together, these results corroborate our assertion that overexpression of Ags1p results in accumulation of (1,4)-α-glucan.

**Figure 4.** Overexpression of Ags1p results in accumulation of (1,4)-α-glucan. Cells were grown in EMMA medium without (high expression; -T) or with (low expression; +T) the addition of thiamine. At the indicated times following the start of induction, samples were taken. (Upper panels) (1,4)-α-Glucan levels of the insoluble cell fraction were measured in an assay using α-amylase and glucoamylase. (Lower panels) Ags1p levels were determined in total cell lysates by immunoblotting using anti-SYN antiserum. α-Tubulin (α-tub) served as a loading control. The following cells were analysed: (A) and (B), cells of genotype \( P_{nmt1-ags1^+} \) (strain ND025); (C) cells containing pAgs1 (strain AV028); and (D), cells containing pREP4 (strain AV027).
The intracellular domain of Ags1p is responsible for (1,4)-α-glucan accumulation

In addition to the SYN domain, Ags1p contains a putative extracellular domain predicted to exhibit transglycosylase activity (Hochstenbach et al., 1998), denoted here as the TGL domain. To explore the question of which domain was responsible for the observed (1,4)-α-glucan accumulation, the SYN domain or the TGL domain, we deleted each domain separately and replaced them with a Myc epitope tag. First, we overexpressed Ags1p-ΔTGL (lacking residues 113–724) and observed induction of a protein with an apparent molecular mass of ~211 kDa, consistent with a calculated molecular mass of 203.6 kDa (Figure 5, compare lanes 5 and 6). Clearly, Ags1p-ΔTGL was able to synthesise (1,4)-α-glucan as determined in our plate-based assay (Figure 3B, lane 4). Next, we overexpressed Ags1p-ΔSYN (lacking residues 1118–1689 including the SYN epitope formed by residues 1618–1631) and observed the appearance of an ~216 kDa protein, consistent with a calculated molecular mass of 209.5 kDa (Figure 5, lanes 7 and 8). Importantly, overexpression of Ags1p-ΔSYN did not result in accumulation of (1,4)-α-glucan (Figure 3B, lane 5). Together, these results demonstrate that the putative extracellular domain is not responsible for (1,4)-α-glucan biosynthesis.

To investigate whether the SYN domain is involved in the synthesis of (1,4)-α-glucan, we introduced mutations in several conserved residues of Ags1p. First, we examined the role of a putative catalytic residue (Glu1526) that is conserved among all members of the (1,4)-α-glucan synthase superfamily and that represents the first Glu in the Glu-X-Glu motif (Figure 2). Replacement of this Glu with Ala to create Ags1p-E1526A resulted in a complete reduction of brown straining in our plate-based assay (Figure 3D, lane 2). Furthermore, no accumulation of (1,4)-α-glucan was detected in our amylase assay, despite the overexpression of this mutant Ags1.
protein (Figure 6, lane 3). Similarly, substitution of the second Glu in the Glu-X-Glu motif or the Lys or first Gly in the putative nucleotide-glucose-binding motif (Lys/Arg)-X-Gly-Gly reduced the brown staining in the plate-based assay (Figure 3D, lanes 3–5) as well as (1,4)-α-glucan levels in the amylase assay (Figure 6, lanes 4–6), despite proper overexpression of the mutant Ags1 proteins. Interestingly, replacement of Gln for Ags1p Lys1422, whose equivalent (Lys277) in EcGlgA was proposed to function as a catalytic residue (Furukawa et al., 1994), only modestly affected iodine staining in the plate-based assay (Figure 3D, lane 6), reducing (1,4)-α-glucan accumulation by only ~50% during overexpression (Figure 6, lane 7). In conclusion, these data support our hypothesis that the SYN domain of Ags1p is responsible for the synthesis of (1,4)-α-glucan.

Given that many members of the (1,4)-α-glucan synthase superfamily are dependent on a primer to initiate synthesis, we addressed whether such a primer is present in vegetatively grown S. pombe cells. We tested glycogen synthase II from S. cerevisiae (ScGsy2p) as a reporter, which normally elongates (1,4)-α-glucan primers generated by glycogenin but lacks the ability to initiate de novo (1,4)-α-glucan synthesis (Ugalde et al., 2003; Cheng et al., 1995). Overexpression of wild-type ScGsy2p in S. pombe cells resulted in a brown staining, whereas low expression did not (Figure 3E, lane 3). When we analysed a hyperactive form of ScGsy2p, ScGsy2p-Δ643, which lacks the C-terminal phosphorylation sites for negative control of its synthase activity, brown staining was obtained even when expression levels were low (Figure 3E, lane 4). These results demonstrate that ScGsy2p is able to synthesise (1,4)-α-glucan, indicating that S. pombe cells contain a primer for (1,4)-α-glucan biosynthesis.

Figure 6. Replacement of conserved Ags1p SYN domain residues reduces accumulation of (1,4)-α-glucan. Cells were grown in EMMA medium without the addition of thiamine (high expression) for 20 h. (Upper panel) (1,4)-α-Glucan levels of the insoluble cell fraction were measured in an assay using α-amylase and glucoamylase. (Lower panel) Ags1p levels were determined in total cell lysates by immunoblotting using anti-SYN antiserum. α-Tubulin (α-tub) served as a loading control. The following cells were analysed: cells containing pREP4 (strain AV027, lane 1), pAgs1 (strain AV028, lane 2), pAgs1-E1526A (strain AV039, lane 3), pAgs1-E1534A (strain AV040, lane 4), pAgs1-L1634 (strain AV037, lane 5), pAgs1-G165A (strain AV038, lane 6), or pAgs1-K1422Q (strain AV042, lane 7).
(1,4)-α-Glucan biosynthesis is an important function of Ags1p

To determine whether (1,4)-α-glucan biosynthesis is important for Ags1p function in vivo, we assessed the ability of mutant Ags1 proteins to complement the cell lysis and β-glucanase-hypersensitivity phenotypes of the mutant strain ags1-1ts (Hochstenbach et al., 1998). This temperature-sensitive strain grew well at a permissive temperature of 28°C, but unlike wild-type strain 972, it was unable to grow at a restrictive temperature of 36°C (Figure 7A, compare rows 1 and 4). By contrast, cells of the ags1-1ts strain carrying plasmid pAgs1 were able to grow at 36°C in the presence of thiamine, conditions that repress ags1 expression to low, wild-type like levels (Figure 7A, row 6). Similarly, in a β-glucanase-hypersensitivity assay, in which the ags1-1ts mutant was grown at 37°C for only 4 h to create a weakened cell wall structure at areas of new growth, plasmid-derived Ags1p was able to protect the ags1-1ts mutant from lysis after exposure to purified β-glucanase (Table 1). Together, these data show that wild-type Ags1p can complement the cell lysis and β-glucanase-hypersensitivity phenotypes of ags1-1ts. We demonstrated that the temperature-sensitive version of Ags1p, (Ags1p-G696S; used as negative control) failed to complement the cell lysis phenotype of ags1-1ts (Figure 7B). Furthermore, neither Ags1p-ΔTGL nor Ags1p-ΔSYN were able to complement the lysis phenotype (Figure 7C), demonstrating that both domains are essential for Ags1p function in vivo.
Ags1p-E1534A, Ags1p-G1165A, Ags1p-K1422Q, and, to a lesser extent, Ags1p-K1163Q were found to be able to complement the cell lysis and β-glucanase-hypersensitivity phenotypes of ags1-1ts (Figure 7D and Table 1). Importantly, ags1-1ts cells expressing Ags1p with a mutation in the putative catalytic Glu residue of the SYN domain (Ags1p-E1526A) were markedly reduced in their ability to grow at 36°C (Figure 7D, row 3) and to protect the ags1-1ts mutant from lysis after exposure to β-glucanase (Table 1). From these experiments, we conclude that Glu1526 is important for Ags1p function in vivo.

DISCUSSION

In this study, we integrated the SYN domain of fungal α-glucan synthases into the superfamily of (1,4)-α-glucan synthases, which already included glycogen and starch synthases. First, we demonstrated that Ags SYN domains display striking amino acid sequence similarities to archaeal and bacterial glycogen synthases and plant starch synthases, all belonging to the glycosyltransferase family GT-5 (Figure 2 and Supplemental Figure S1). Second, we showed that Ags SYN domains form a monophyletic group that clusters within the (1,4)-α-glucan synthase superfamily (Figure 1B), suggesting that a hypothetical ags1 ancestor gene existed before the divergence of the Ascomycota and Basidiomycota. Third, we demonstrated that overexpression of Ags1p in S. pombe cells resulted in accumulation of (1,4)-α-glucan, as detected by brown staining using iodine vapour (Figure 3) and in a quantitative amylase assay (Figure 4). Fourth, overexpression of Ags1p lacking the TGL domain but retaining the SYN domain induced accumulation of (1,4)-α-glucan, whereas Ags1p without the SYN domain did not (Figure 3). Fifth, replacement of a highly conserved and putative catalytic residue in the SYN domain (Ags1p-E1526A), the first Glu in the Glu-X₇-Glu motif, abrogated (1,4)-α-glucan synthesis. Similarly, mutations in the putative nucleotide-glucose-binding motif, K1163Q and G1165A, resulted in a major decrease in (1,4)-α-glucan accumulation upon overexpression (Figures 3 and 6). Finally, synthesis of (1,4)-α-glucan was found to be an important function of Ags1p in vivo, because cells dependent on Ags1p-E1526A grew slower than cells dependent on wild-type Ags1p and their cell wall structure was weakened dramatically (Figure 7 and Table 1). On the basis of these results, we conclude that, of the two probable catalytic domains of the S. pombe Ags1p, the SYN domain is responsible for the production of the (1,4)-α-glucan constituent of cell wall α-glucan.

The Glu-X₇-Glu motif is conserved among archaeal and animal glycogen synthases, as well as fungal Ags SYN domains, whereas in bacterial glycogen synthases and plant starch synthases only the Glu equivalent to the first Glu of the Glu-X₇-Glu motif is conserved (Figure 2). Substitution of this Glu with Ala in EcGlgA (E377A)
resulted in a 10,000-fold reduction of the catalytic activity without affecting its ability to bind the ADP-glucose or glycogen substrates, demonstrating that this residue is involved in catalysis rather than substrate binding (Yep et al., 2004). However, in the glycosyltransferase Gpi3p of S. cerevisiae and S. pombe, the second Glu of the Glu-X-Glu motif is of greater importance than the first for in vivo function (Kostova et al., 2003). To address the question of which of the two Glu of the Glu-X-Glu motif is more important for the S. pombe Ags1p SYN domain, we mutagenised them individually. Replacement of the first Glu in Ags1p-E1526A abrogated (1,4)-α-glucan production (Figures 3 and 6) and dramatically reduced Ags1p function in vivo (Figure 7 and Table 1). By contrast, replacement of the second Glu in Ags1p-E1534A reduced (1,4)-a-glucan accumulation upon overexpression but did not result in a decrease in the function of Ags1p in vivo. Therefore, it appears that the first Glu of the Glu-X-Glu motif is of greater importance than the second for the S. pombe Ags1p SYN domain. Attempts to definitively answer this question were thus far unsuccessful because we were unable to generate ags1Δ strains expressing mutant versions of Ags1p from plasmid. For example, when we allowed ags1Δ/ags1Δ diploid cells carrying plasmid pAgs1 to sporulate, the ags1Δ ascospores lysed during germination (apart from a few exceptions), presumably because Ags1p plays a critical role during germination and because the nmt1 promoter of pAgs1 failed to supply proper levels of Ags1p during this process.

Besides the catalytic Glu-X-Glu motif, the nucleotide-glucose-binding motif (Lys/Arg)-X-Gly-Gly is conserved among all members of the (1,4)-α-glucan synthase superfamily, including the Ags SYN domains. Replacement of the equivalent residues, Lys1163 and Gly1165, showed that they play a role in (1,4)-α-glucan production by the S. pombe Ags1p (Figures 3 and 6). However, we found no evidence for a catalytic role of Ags1p-Lys1422. The equivalent Lys in E. coli GlgA (Lys277) was predicted to be

| Genotype* | Strain | Cell Survival (% of initial OD<sub>595</sub>) |
|-----------|--------|---------------------------------|
|           |        | untreated | treated    |
| Wild type | 972    | 100 ± 3 | 89 ± 2    |
| ags1–1<sup>T</sup> | FH021 | 99 ± 1 | 19 ± 3    |
| ags1–1<sup>T</sup> [pREP4] | AV039 | 100 ± 3 | 18 ± 3    |
| ags1–1<sup>T</sup> [pAgs1] | AV060 | 99 ± 5 | 72 ± 6    |
| ags1–1<sup>T</sup> [pAgs1-E1526A] | AV064 | 100 ± 1 | 22 ± 2    |
| ags1–1<sup>T</sup> [pAgs1-E1534A] | AV065 | 100 ± 1 | 56 ± 5    |
| ags1–1<sup>T</sup> [pAgs1-K1163Q] | AV062 | 99 ± 1 | 24 ± 7    |
| ags1–1<sup>T</sup> [pAgs1-G1165A] | AV063 | 100 ± 1 | 52 ± 5    |
| ags1–1<sup>T</sup> [pAgs1-K1422Q] | AV066 | 96 ± 1 | 63 ± 18    |

*Complete genotypes and plasmid descriptions are provided in Tables 2 and 3, respectively.
involved in catalysis (Furukawa et al., 1994), but our results would be consistent with more recent data on the crystal structure of AtGlgA, which suggested that this residue has a more distant effect on catalysis (Buschiazzo et al., 2004). Definitive identification of the catalytic residues of the Ags1p SYN domain will have to await the development of an in vitro assay for Ags1p activity. To our knowledge, no such assay has been described, and, despite numerous attempts, we were unsuccessful in developing such an assay, either with isolated membranes containing native Ags1p or with recombinant versions of the Ags1p SYN domain. Nonetheless, the results presented here suggest that the catalytic mechanism for the S. pombe Ags1p SYN domain appears to be similar to that of other members of the (1,4)-α-glucan synthase superfamily.

Production of (1,4)-α-glucan is hypothesised to require two steps: initiation and elongation. In animal and yeast cells, glycogen synthesis is initiated by autoglucosylation of the protein glycogenin, which catalyses the transfer of glucose residues from UDP-glucose onto one of its tyrosine residues. The produced (1,4)-α-glucan oligosaccharide is then used as a primer for elongation by glycogen synthase (Lomako et al., 2004). For plant starch biosynthesis, the nature of a relevant primer remains elusive (Ball and Morell, 2003). Also for bacterial glycogen synthases, the requirement for a primer is controversial: E. coli GlgA appears to require a (1,4)-α-glucan oligosaccharide as primer (Fox et al., 1976), whereas A. tumefaciens GlgA appears to be involved in both initiation and elongation of glycogen, without the need for a primer (Ugalde et al., 2003). In this study, we have shown for the first time that S. pombe cells contain a primer for (1,4)-α-glucan biosynthesis (Figure 3). However, the S. pombe genome does not encode a readily identifiable glycogenin-like protein. Further studies will be required to determine the nature of this primer and its potential relevance to (1,4)-α-glucan biosynthesis by Ags1p.

Our previous finding that cell wall α-glucan is composed of two distinct constituents, (1,3)-α-glucan and (1,4)-α-glucan oligosaccharides (Grün et al., 2005), combined with our present finding that the SYN domain of Ags1p is able to synthesise the latter constituent, raises the question of which enzyme or enzyme domain produces the (1,3)-α-glucan constituent. We cannot exclude the unlikely possibility that the SYN domain might be capable of catalysing two distinct reactions, synthesising not only (1,4)-α-glucan but also (1,3)-α-glucan. Also, the TGL domain of Ags1p seems to be an improbable candidate for (1,3)-α-glucan synthesis, because it has sequence similarities to bacterial and fungal amylases of the glycoside hydrolase family GH-13, enzymes known to hydrolyse or transglycosylate (1,4)-α-glucan. Therefore, we hypothesise that an unidentified enzyme may be responsible for synthesis of the (1,3)-α-glucan constituent. If so, this would require a further refinement of our speculative model for cell wall α-glucan
biosynthesis (Hochstenbach et al., 1998; Grün et al., 2005). In this refined model, the (1,4)-α-glucan oligosaccharides of cell wall α-glucan would be produced by the Ags1p SYN domain. The degree of polymerisation of the (1,4)-α-glucan produced by Ags1p during overexpression appears to be between 10 and 40, based on the red-brown colour of the cells observed after exposure to iodine vapour (Bailey and Whelan, 1961). Interestingly, this size range includes the estimated length of the (1,4)-α-glucan oligosaccharides observed in cell wall α-glucan, which are predicted to consist of ~12 (1,4)-linked α-glucose residues (Grün et al., 2005). The (1,4)-α-glucan oligosaccharides might serve as a primer for the unknown (1,3)-α-glucan synthase for elongation. The resulting building block would then be transported across the membrane via the putative pore domain of Ags1p and be coupled to another building block by the TGL domain, thereby forming mature cell wall α-glucan.

On the basis of the strict conservation of the SYN domain among fungal Ags1 proteins, we predict that, not only S. pombe, but indeed all fungi expressing an Ags1p homolog may contain (1,4)-linked α-glucose residues coupled to cell wall (1,3)-α-glucan. Although the presence of (1,4)-α-glycosidic linkages was noticed in cell-wall α-glucan fractions from both ascomycetous and basidiomycetous fungi, such as the filamentous fungus Aspergillus niger (Johnston, 1965) and the medically important pathogen C. neoformans (James et al., 1990), additional work will be required to demonstrate that these linkages, rather than representing a contamination of glycogen, are linked covalently to (1,3)-α-glucan. We hope that our present finding may contribute to a rational design for antifungal drugs directed against Ags1p.

**EXPERIMENTAL PROCEDURES**

Strains and culture media

E. coli strain DH5α (Invitrogen) was used for all plasmid isolations. The kanMX6-3nmt1 cassette (Bähler et al., 1998) was integrated in front of the ags1 or mok11 open reading frame (ORF) at its chromosomal location in S. pombe wild-type strain 972. S. pombe plasmid transformations were performed using strains with genotypes h+ ura4-D18 or h+ ags1-1ts ura4-D18 using a lithium acetate method at pH 4.9 (Ito et al., 1983). Cells were grown in YEA medium (Hochstenbach et al., 1998) or EMM2 medium (Moreno et al., 1991) supplemented with 250 mg/l of adenine sulfate (EMMA). Following plasmid transformation, expression of cloned genes was repressed by growing the cells on EMMA plates supplemented with 100 μM thiamine at 28°C (or at 21°C for ags1-1ts strains) for 3–5 days. For overexpression experiments, cells were grown overnight at 28°C in EMMA medium containing 10 μM thiamine, washed twice with EMMA medium (which lacks thiamine), and grown at 28°C in EMMA medium for 6–24 h. The S. pombe strains used in this study are listed in Table 2.
Sequence alignments, phylogenetic analyses, and secondary structure prediction

The amino acid sequences used in this study are listed in Supplemental Table S1. Sequencing projects used to obtain protein sequences were the Fungal Genome Initiative, Broad Institute of Harvard and MIT (http://www.broad.mit.edu); the United States Department of Energy Joint Genome Institute (http://www.jgi.doe.gov); The Institute for Genomic Research (http://www.tigr.org); and Genoscope (http://www.genoscope.cns.fr). The 79 selected sequences were aligned using the algorithms TCoffee (Notredame et al., 2000) and ClustalW (Thompson et al., 1994). The resulting multiple sequence alignments showed only minor differences between them, and the TCoffee alignment was chosen for phylogeny reconstruction based on the number of identities and conserved residues (Supplemental Figure S1). After removal of all positions containing gaps in >50% of the sequences using the program LISTPOS (J. A. M. Leunissen, unpublished), phylogenetic relationships were reconstructed by using the neighbor-joining method (Saitou and Nei, 1987), as well as FITCH (Fitch and Margoliash, 1967), TREE-PUZEL (Strimmer and von Haeseler, 1996), and Bayesian (Ronquist and Huelsenbeck, 2003) analyses. For the neighbor-joining and FITCH methods, the programs NEIGHBOR and FITCH from the PHYLIP package (Felsenstein, 2002) were used, respectively, and the distance matrices for these methods were calculated with the program PROTDIST using the Jones-Taylor-Thornton model.

Table 2. S. pombe strains used in this study

| Strain | Genotype | Source               |
|--------|----------|----------------------|
| 972    | h-       | P. Nurse             |
| AV027  | h- ura4-D18 [pREP4] | This study           |
| AV028  | h- ura4-D18 [pAV011] | This study           |
| AV037  | h- ura4-D18 [pAV035] | This study           |
| AV038  | h- ura4-D18 [pAV036] | This study           |
| AV039  | h- ura4-D18 [pAV037] | This study           |
| AV040  | h- ura4-D18 [pAV038] | This study           |
| AV042  | h- ura4-D18 [pAV040] | This study           |
| AV059  | h- ags1-1 ura4-D18 [pREP4] | This study           |
| AV060  | h- ags1-1 ura4-D18 [pAV011] | This study           |
| AV062  | h- ags1-1 ura4-D18 [pAV035] | This study           |
| AV063  | h- ags1-1 ura4-D18 [pAV036] | This study           |
| AV064  | h- ags1-1 ura4-D18 [pAV037] | This study           |
| AV065  | h- ags1-1 ura4-D18 [pAV038] | This study           |
| AV066  | h- ags1-1 ura4-D18 [pAV040] | This study           |
| AV069  | h- ura4-D18 [pAV066] | This study           |
| AV116  | h- ura4-D18 [pAV111] | This study           |
| AV117  | h- ura4-D18 [pAV112] | This study           |
| AV143  | h- ura4-D18 [pAV128] | This study           |
| AV145  | h- ags1-1 ura4-D18 [pAV128] | This study           |
| AV149  | h- ura4-D18 [pAV137] | This study           |
| AV153  | h- ags1-1 ura4-D18 [pAV137] | This study           |
| BS006  | h- ura4-D18 [pBS010] | This study           |
| BS016  | h- ags1-1 ura4-D18 [pBS010] | This study           |
| FH021  | h- ags1-1 (Hochstenbach et al., 1998) | This study           |
| ND025  | h- ags1::Pmnt1-kanMX6 | This study           |
| ND032  | h- mok1::Pmnt1-kanMX6 | This study           |
| ND214  | h- ags1-1::Pmnt1-kanMX6 | This study           |
TREE-PUZZLE calculations were performed with the program TREE-PUZZLE, and Bayesian analysis was performed with the program MrBayes for 1,000,000 generations using a mixed model. The unrooted trees were rooted by the midpoint method using the program RETREE from the PHYLIP package and the resulting rooted trees were converted to Scalable Vector Graphics format using SVGTREE (Alako et al., 2006). Neighbor-joining bootstrapping was performed by generating 1000 random bootstrap samples with the programs SEQBOOT, PROTDIST, and NEIGHBOR; trees generated from these samples were analysed using the extended majority consensus method as implemented in the program CONSENSE from the PHYLIP package. To calculate the percentage of identities in pairwise amino acid sequence alignments, the program GAP from the Wisconsin Package (version 10.3; Accelrys, San Diego, CA) was used. Secondary structure predictions resulted from the program PSIPRED (version 2.5) (McGuffin et al., 2000).

Construction of plasmids

All PCR amplifications were performed using Phusion™ polymerase (Finnzymes). The primers used in this study are listed in Supplemental Table S2. The ORF of ags1 (GenBank™ accession number AF061180) was cloned between the MluNI and BamHI restriction enzyme sites of pREP4 (Maundrell, 1993), using three fragments generated by PCR and two fragments subcloned from cosmid c17A7. Then, the Smal site in the multiple cloning site of the resulting plasmid was removed, producing pAV011. The ORF of the A. tumefaciens glycogen synthase gene was amplified from plasmid pBG19 (kindly provided by R. Ugalde) (Ugalde et al., 2003) by PCR using primers AV098A and AV099A, whereas the ORF of the S. cerevisiae glycogen synthase gene GSY2 or its hyperactive version was amplified from genomic DNA of S. cerevisiae strain BY4741 using primers AV171 and AV172 or AV171 and AV173, respectively. These ORFs were cloned between the XhoI and BglII sites of the pREP4-based vector pAdH006 (Dekker et al., 2006), generating pAV066, pAV111, or pAV112, respectively. To create pAgs1-ΔTGL, the fragment between the XhoI and SpeI sites in pAV011 was replaced with the Myc epitope tag-encoding hybridisation product of oligonucleotides BS013 and BS014, producing pBS010. To create pAgs1-ΔSYN, a KpnI site was introduced in the ags1 ORF of pAV011 through introduction of the silent mutation C3351A following the QuickChange™ site-directed mutagenesis protocol (Stratagene) using primers AV041 and AV042 (K1163Q),
AV043 and AV044 (G1165A), AV045 and AV046 (E1526A), AV047 and AV048 (E1534A), or AV051 and AV052 (K1422Q). The Smal-SalI fragment of pAV011 was replaced with the mutated Smal-SalI fragments, generating pAV035, pAV036, pAV037, pAV038, or pAV040, respectively. To introduce the temperature-sensitive mutation (G696S) into the gene fragment encoding the TGL domain, the XhoI-EcoRI fragment was cloned into the pUC18-based vector pAdH100, and site-directed mutagenesis was performed using primers BS011 and BS012. The XhoI-SpeI fragment of pAV011 was replaced with the mutated XhoI-SpeI fragment, generating pAV137. All constructs used in this study were sequenced in both directions using a series of overlapping PCR amplification products (BigDye Terminator sequencing kit, Applied Biosystems). The plasmids used in this study are listed in Table 3.

### Plate-based assay for (1,4)-α-glucan accumulation

Cells were grown in EMMA medium containing 10 μM thiamine at 28°C to an optical density at 595-nm wavelength (OD 595) of 1.0–1.5, washed and taken up in MQ-H₂O to a final concentration of 2.5 × 10⁷ cells/ml. Four microliters of cell suspension (1 × 10⁵ cells) were spotted onto EMMA plates supplemented with or without 10 μM thiamine. Plates were incubated at 28°C for 3 days and exposed to iodine vapour for 4 min.

### (1,4)-α-Glucan determination

Cells (10⁹) were taken up in 5 mM sodium azide, 20 mM Tris•HCl (pH 7.6) broken with acid-washed glass beads using Fastprep 120 (Bio101, Inc) at speed 6 for five intervals of 15 sec, and centrifuged at 16,000 × g at 4°C for 10 min. Cell pellets were resuspended in 2% (w/v) SDS, 100 mM EDTA, 20 mM DTT, and 50 mM Tris (pH 7.6), boiled for 10 min, and centrifuged again. The resulting pellets were washed twice with MQ-H₂O and once with 10 mM sodium acetate (pH 5.6) and were divided equally over four

| Number | Name           | Description                   | Source          |
|--------|----------------|-------------------------------|-----------------|
| pREP4  | pAgs1          | S. pombe expression plasmid   | (Maundrell, 1993) |
| pAV011 | pAgs1          | pREP4 with ags1               | This study      |
| pAV035 | pAgs1-K1163Q   | pAV011 with ags1-A3487C       | This study      |
| pAV036 | pAgs1-G1165A   | pAV011 with ags1-G3494C       | This study      |
| pAV037 | pAgs1-E1526A   | pAV011 with ags1-A4577C, G4578T | This study |
| pAV038 | pAgs1-E1534A   | pAV011 with ags1-A4601C       | This study      |
| pAV040 | pAgs1-K1422Q   | pAV011 with ags1-A4264C       | This study      |
| pAV066 | pGlgA          | pREP4 with A. tumefaciens glgA | This study      |
| pAV111 | pGsy2          | pREP4 with S. cerevisiae GSY2 | This study      |
| pAV112 | pGsy2-Δ643     | pREP4 with S. cerevisiae GSY2 (1–1929) | This study |
| pAV127 | pAgs1-KpnI     | pAV011 with ags1-C3351A       | This study      |
| pAV128 | pAgs1-ΔSYN     | pAV011 with ags1-Δ(3354–5068) | This study |
| pAV137 | pAgs1-G696S    | pAV011 with ags1-G2086A       | This study      |
| pBS010 | pAgs1-ATGL     | pAV011 with ags1-Δ(337–2172)  | This study      |

Table 3. Plasmids used in this study
fractions. Fractions were resuspended in 200 μl 10 mM sodium acetate (pH 5.6) and incubated at 37°C for 2.5 h in the presence of 1.8 units of α-amylase (Roche Applied Science; 102814) and 6.0 milliunits of glucoamylase (Roche Applied Science; 1202332), 0.1 unit of Zymolyase-100T (Seikagaku Corp.; 120493), all three enzymes, or buffer only. In a control experiment, we confirmed that the amount of enzyme added was sufficient for complete digestion. After digestion, supernatants were collected and the amount of liberated reducing ends was measured using a colorimetric assay (Lever, 1972). The reducing ends were converted to glucose equivalents, the background (buffer-only fraction) was subtracted, and the glucose equivalents/10⁹ cells were corrected for the β-glucan amounts (Zymolyase-100T digestion). Digestion of (1,4)-α-glucan was checked visually for completion by resuspending the remaining pellets in 100 μl of MQ-H₂O and adding 900 μl of a potassium triiodide reagent prepared from 0.01% (w/v) iodine and 0.1% (w/v) potassium iodide in 5 M calcium chloride.

**Immunoblotting**

Cells (2 × 10⁷) were taken up in 10% (w/v) of trichloroacetic acid, broken with acid-washed glass beads using Fastprep 120 at speed 6 for three intervals of 15 sec, incubated on ice for 10 min, and centrifuged at 16,000 × g at 4°C for 15 min. The resulting pellets were washed with ice-cold acetone, resuspended in 200 μl of SDS sample buffer containing 20 mM DTT, and incubated at 37°C for 10 min. Total cell lysates were separated on an SDS/6% polyacrylamide gel (or on an SDS/8% polyacrylamide gel for the α-tubulin control) under reducing conditions, blotted onto nitrocellulose membranes (0.45 μm; Schleicher & Schuell), and probed with an anti-α-tubulin antibody (Sigma-Aldrich; T-5168), mouse anti-Myc tag monoclonal antibody 9B11 (Cell Signaling Technology; 2276) or anti-SYN antiserum SN269. This anti-SYN antiserum was generated in rabbits immunised with peptide H₂N-CSQKYGRNSR5SR5S-CONH₂ (based on residues 1618–1631 of *S. pombe* Ags1p) coupled to keyhole limpet hemocyanin. Blots were incubated with horseradish peroxidase-conjugated goat-anti-mouse IgG (BioRad) or horseradish peroxidase-conjugated goat-anti-rabbit IgG (BioRad) and developed by chemiluminescence (ECL kit, Amersham Bioscience).

**Complementation assay**

Cells were grown in EMMA medium containing 100 μM thiamine at 21°C to an OD₅₉₅ of ~1.0, washed, and taken up in MQ-H₂O to a final concentration of 2.5 × 10⁷ cells/ml. Four microliters of cell suspension were spotted in 10-fold serial dilutions onto EMMA plates containing 100 μM thiamine. Plates were incubated at 28°C or 36°C for 3–5 days.
β-Glucanase hypersensitivity assay

Cells were grown in EMMA medium containing 100 μM thiamine (repression conditions) at 21°C to an OD595 of 1.0–3.0, diluted with fresh EMMA medium containing 100 μM thiamine to an OD595 of 0.2, and incubated at 37°C for 4 h. Cells were washed once with 1 mM 2-mercaptoethanol, 10 mM Tris•HCl (pH 7.6) (digestion buffer) and taken up at an OD595 of 0.2 in digestion buffer containing 0.4 units/ml of Zymolyase-20T (Seikagaku; 120491). After a 1-h incubation at 28°C under continuous shaking, cell lysis was monitored by measurement of the optical density at 595 nm.

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### SUPPLEMENTAL DATA

Supplemental Table S1. Amino acid sequences used in this study

| Synopsis | Species | Accession number of GenBank or sequencing project | Synopsis | Species | Accession number of GenBank or sequencing project |
|----------|---------|-----------------------------------------------|----------|---------|-----------------------------------------------|
| AbAg1A   | Aspergillus niger | AA999324 | LpGAg1A | Lactobacillus plantarum | NP_785807 |
| AbAg1A   | Aspergillus fumigatus | AAL26129 | MgAg1A | Magnaporthe grisea | EAA49021 |
| AbAg2A   | Aspergillus niger | AAI18964 | MgAg1A | Magnaporthe grisea | EAA49024 |
| AbAg3   | Aspergillus fumigatus | EAL90874 | MteGAg1A | Mycoplasma mobile | YP_016099 |
| AbGy1    | Aspergillus fumigatus | EAL86056 | MteGy1 | Mus musculus (house mouse, muscle) | Q9ZIE4 |
| AbGy1A   | Ashbya gossypii | AAS50373 | NcAg1 | Neurospora crassa | EAA38439 |
| AbGy1A   | Aspergillus nidulans | EAA63275 | NcAg2 | Neurospora crassa | EAA38536 |
| AbGy1A   | Aspergillus nidulans | EAA58394 | NtGy1 | Neurospora crassa | EAA95870 |
| AbGy1A   | Aspergillus nidulans | EAA58163 | NpCg1A | Nostoc punctiforme | ZP_09106629 |
| AbAg1A   | Aspergillus oryzae | BAE58541 | OsGy1 | Orgyia sativa (rice) | AAL9872 |
| AbAg2A   | Aspergillus oryzae | BAE59959 | OsSII | Orgyia sativa (rice) | BAD90992 |
| AbAg3A   | Aspergillus oryzae | BAE66017 | PsAg1 | Podospora anserina | contig_128 (GS) |
| AbGy1A   | Aspergillus oryzae | BAE6422 | PnGAg1A | Pyrococcus abyssi | CAB49000 |
| AtAg1A   | Agrobacterium tumefaciens | NP_534560 | PhAg1A | Paracoccus halodurans | AAV52833 |
| BiGy1    | Botrytis cinerea | BC1G_07871.1 | PlAg1 | Pseudomonas aeruginosa | e_pep02.11.4.1 (JGI) |
| BiGy1   | Brachypodium japonicum | Q9AF666 | PpCg1A | Pyrococcus furiosus | AAL32164 |
| BiGy1A   | Bacillus subtilis | P90125 | PnCg1A | Pyrococcus horikoshii | NP_143087 |
| CcAg1    | Coprinus cinereus | CC1G_12015.1 | PnGc1A | Pseudoalteromonas marinus | AAQ00997 |
| CcGy1A   | Coprinus cinereus | CC1G_01973.1 | PnGBSS | Psia sativa (pea) | Q43092 |
| CcAg1    | Chaetomium globosum | EAK06047 | PoSS | Psia sativa (pea) | CAAN1269 |
| CcGy1    | Chaetomium globosum | EAK86497 | PoGBSS | Phasus vulgaris (kidney bean) | BAA0346 |
| CcAg1    | Coccidioides immitis | CIMG_00204.2 | PoSS | Phasus vulgaris (kidney bean) | BAD18846 |
| CcGy1    | Coccidioides immitis | CIMG_00454.2 | SgGy1 | Saccharomyces cerevisiae | NP_16670 |
| CmAg1A   | Cryptococcus neoformans Serotype A | CNAG_03120.1 | SgGy2 | Saccharomyces cerevisiae | NP_013339 |
| CmGy1A   | Cryptococcus neoformans Serotype A | CNAG_04621.1 | SmGlAg1 | Saccharomyces cerevisiae | NAAS9186 |
| CmAg1A   | Cryptococcus neoformans Serotype B | supercontig 1.5 | SpAg1 | Schizosaccharomyces pombe | AAC31430 |
| CmGy1A   | Cryptococcus neoformans Serotype B | supercontig 1.2 | SpMk11 | Schizosaccharomyces pombe | CAB90796 |
| CmAg1A   | Cryptococcus neoformans Serotype D | AA44814 | SpMk12 | Schizosaccharomyces pombe | CAB37503 |
| CmGy1A   | Cryptococcus neoformans Serotype D | AA42599 | SpMk13 | Schizosaccharomyces pombe | CAB38509 |
| CmAg1A   | Coccidioides posadae | S1a09557 (TIGR) | SpMk14 | Schizosaccharomyces pombe | CAB40008 |
| CmGy1A   | Coccidioides posadae | S2a07351 (TIGR) | SrAg1 | Sclerotinia sclerotiorum | SS1G_03583.1 (BI) |
| CmGlAg1 | Clostridium thermocellum | ZP_00550841 | SpGlAg2 | Synechocystis sp. | NP_441947 |
| CmGlAg1 | Clostridium thermocellum | ZP_00550841 | SpGlAg2 | Synechocystis sp. | NP_441947 |
| CmGlAg1 | Clostridium thermocellum | ZP_00550841 | SpGlAg2 | Synechocystis sp. | NP_441947 |
| CmGlAg1 | Clostridium thermocellum | ZP_00550841 | SpGlAg2 | Synechocystis sp. | NP_441947 |
| CmGlAg1 | Clostridium thermocellum | ZP_00550841 | SpGlAg2 | Synechocystis sp. | NP_441947 |
| CmGlAg1 | Clostridium thermocellum | ZP_00550841 | SpGlAg2 | Synechocystis sp. | NP_441947 |
| CmGlAg1 | Clostridium thermocellum | ZP_00550841 | SpGlAg2 | Synechocystis sp. | NP_441947 |
| CmGlAg1 | Clostridium thermocellum | ZP_00550841 | SpGlAg2 | Synechocystis sp. | NP_441947 |

*Synopsis comprises a species abbreviation followed by an abbreviation of the type of (1,4)-a-glucan synthase with, if multiple homologs are known, a serial number or letter.*

*Sequencing projects used to obtain Ags and Gsy sequence data are indicated as follows: BI, Broad Institute of Harvard and MIT; TIGR, The Institute for Genomic Research; GS, Genoscope; and JGI, the United States Department of Energy Joint Genome Institute.*
### Supplemental Table S2. Primers used in this study

| Primer | Sequence | Purpose |
|--------|----------|---------|
| AV041  | 5’–CGGATCCGAAAATTCGCCGATCAAAATGGGACTTGGAATCTGCTG | To mutate ags1, K136Q; FW |
| AV042  | 5’–CACCAGACTGCCAACATGTCGGAATTTCTTCAATTGCG | To mutate ags1, K136Q; REV |
| AV043  | 5’–GGAAATGTCGTCATCAAAATGGGACTTGGAATCTGCTG | To mutate ags1, G1165A; FW |
| AV044  | 5’–CCTATACACAGTGGCAGGCTGTTGTGATTCG | To mutate ags1, G1165A; REV |
| AV045  | 5’–GCTTCTGATTCAGTCCTTCCTTATTCGTTTATTCG | To delete Chol-Smel fragment of ags1; FW |
| AV046  | 5’–GCCAATACCATCAGATCTTACAGGATGCGAGCATTCAG | To delete Chol-Smel fragment of ags1; REV |
| AV047  | 5’–GCTTCTAGTGTGCTGGATTGGAATTCGAGGAATGGGGG | To amplify gsy1, K1354A; FW |
| AV048  | 5’–CCCTAATACCTTAAAGATCGACGACTGACTAAAA | To amplify gsy1, K1354A; REV |
| AV049  | 5’–GAAGATACGTGATAGTATCTCTGCTGATTCAAGATGGTCTC | To amplify A. tumefaciens glgA; FW |
| AV050  | 5’–TCTCTTATACATTAGGATTGGCTTTACGATGGGCTT | To amplify A. tumefaciens glgA; REV |
| AV071  | 5’–GATCTCTGACAGCTGCATTAGTATGATCA | To amplify pREP4 SmuI site following cloning of ags1; FW |
| AV072  | 5’–TGCAATCATTGACAGCTGCATTAGTATGATCA | To amplify pREP4 SmuI site following cloning of ags1; REV |
| AV076  | 5’–GGGCAATACCTTGAAGCTCTCTCTGCTGTTATTCG | To delete XhoI-Smfl fragment of ags1; FW |
| AV077  | 5’–GGGCAATACCTTGAAGCTCTCTCTGCTGTTATTCG | To delete XhoI-Smfl fragment of ags1; REV |
| AV099A | 5’–AGAGACTGCTGATAGTATCTCTGCTGATTCAAGATGGTCTC | To amplify A. tumefaciens glgA; FW |
| AV099B | 5’–TCTCTTATACATTAGGATTGGCTTTACGATGGGCTT | To amplify A. tumefaciens glgA; REV |
| AV171  | 5’–AGAGACTGCTGATAGTATCTCTGCTGATTCAAGATGGTCTC | To amplify S. cerevisiae GSY2 and GSY2–Δ643; FW |
| AV172  | 5’–AGAGACTGCTGATAGTATCTCTGCTGATTCAAGATGGTCTC | To amplify S. cerevisiae GSY2; REV |
| AV173  | 5’–AGAGACTGCTGATAGTATCTCTGCTGATTCAAGATGGTCTC | To amplify S. cerevisiae GSY2–Δ643; REV |
| AV219  | 5’–GGGCAATACCTTGAAGCTCTCTCTGCTGTTATTCG | To delete XhoI-Smfl fragment of ags1; FW |
| AV220  | 5’–GGGCAATACCTTGAAGCTCTCTCTGCTGTTATTCG | To delete XhoI-Smfl fragment of ags1; REV |
| AV221  | 5’–GGGCAATACCTTGAAGCTCTCTCTGCTGTTATTCG | To delete XhoI-Smfl fragment of ags1; FW |
| AV222  | 5’–GGGCAATACCTTGAAGCTCTCTCTGCTGTTATTCG | To delete XhoI-Smfl fragment of ags1; REV |
| BS011  | 5’–GGGCAATACCTTGAAGCTCTCTCTGCTGTTATTCG | To amplify gsy1, G1396S; FW |
| BS012  | 5’–GGGCAATACCTTGAAGCTCTCTCTGCTGTTATTCG | To amplify gsy1, G1396S; REV |
| BS013  | 5’–GGGCAATACCTTGAAGCTCTCTCTGCTGTTATTCG | To amplify gsy1, K1163Q; FW |
| BS014  | 5’–GGGCAATACCTTGAAGCTCTCTCTGCTGTTATTCG | To amplify gsy1, K1163Q; REV |
| ND122  | 5’–GGGCAATACCTTGAAGCTCTCTCTGCTGTTATTCG | To integrate Pm1 in front of mok12; FW |
| ND123  | 5’–GGGCAATACCTTGAAGCTCTCTCTGCTGTTATTCG | To integrate Pm1 in front of mok12; REV |
| ND138  | 5’–GGGCAATACCTTGAAGCTCTCTCTGCTGTTATTCG | To integrate Pm1 in front of mok12; FW |
| ND139  | 5’–GGGCAATACCTTGAAGCTCTCTCTGCTGTTATTCG | To integrate Pm1 in front of mok12; REV |
| RS054  | 5’–GGGCAATACCTTGAAGCTCTCTCTGCTGTTATTCG | To integrate Pm1 in front of mok12; FW |
| SE003  | 5’–GGGCAATACCTTGAAGCTCTCTCTGCTGTTATTCG | To integrate Pm1 in front of mok12; REV |
| SE004  | 5’–GGGCAATACCTTGAAGCTCTCTCTGCTGTTATTCG | To integrate Pm1 in front of mok12; FW |
| SE007  | 5’–GGGCAATACCTTGAAGCTCTCTCTGCTGTTATTCG | To integrate Pm1 in front of mok12; REV |
| SE008  | 5’–GGGCAATACCTTGAAGCTCTCTCTGCTGTTATTCG | To integrate Pm1 in front of mok12; FW |

### Supplemental Figure S1. Multiple sequence alignment of members of the (1,4)-α-glucan synthase superfamily.

A TCoffee alignment was constructed for fungal Ags SYN domains (indicated as Ags), archaeal and bacterial glycogen synthases (GlgA), plant granule-bound starch synthases (GBSS) and soluble starch synthases (SS), and fungal and animal glycogen synthases (Gsy and Gys, respectively). Species and synthases included are listed in Supplemental Table S1. The (Lys/Arg)-X-Gly-Gly motif at the N-terminal end of the alignment and the Glu-X-glutamyl motif at its C-terminal end are indicated. Boxes indicate sequence identities among the proteins shown, grey shading indicates sequence similarities to the S. pombe Ags1 SYN domain shown in the top line, and dashes indicate gaps in the alignment.
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