

Chapter

1

The fungal cell wall

Introduction

The variety of fungal species in nature is tremendous: 1.5 million species have been estimated to exist, of which approximately 70,000 have been described (Hawksworth, 1991). Briefly, a few general aspects of fungi will be discussed. Many fungi are important for life in that they live in symbiosis with plants or animals. Others live from plant materials, such as wood and leaf litter, or from dead animals and animal excrements. Therefore, they contribute significantly to the decomposition of organic material. A number of fungi are also of industrial importance, such as *Saccharomyces cerevisiae*, used for instance in brewing and bakery industries and for the production of heterologous proteins. Other fungi are used in food industry, such as the fruiting bodies of *Lentinus edodes* (shiitake), *Pleurotus ostreatus* (oyster mushroom), and the cultivated mushroom *Agaricus bisporus*. Unfortunately, a number of fungi causes devastating diseases in plants or animals. *Ophiostoma ulmi*, for example, causes the Dutch elm disease (Hintz, 1999), whereas *Cryphonectria parasitica* caused the chestnut blight, which brought the American chestnut population almost to extinction (Chasan, 1992). Fungi that can infect humans include *Candida albicans* (Mitchell, 1998), *Aspergillus fumigatus* (Latgé, 2001), and *Cryptococcus neoformans* (Thomas & Schwartz, 2001).

The cell wall is essential for fungal survival and its composition is unique to fungi, and therefore it forms an ideal target for the development of (novel) antifungal drugs. For this purpose, understanding of the cell-wall architecture as well as of the biochemical processes involved in cell-wall assembly are essential. Budding yeast *S. cerevisiae* and fission yeast *Schizosaccharomyces pombe* are unicellular fungi that have been used as model organisms to study eukaryotic processes, such as cell division and cell morphogenesis (Botstein *et al.*, 1997; Hayles & Nurse, 2001). Also fungus-specific aspects such as the assembly of the cell wall are preferably studied in these yeasts, because their genome sequences are known (Goffeau *et al.*, 1997; Wood *et al.*, 2002) and because of their genetic tractability.

Cell-wall architecture

The fungal cell is encapsulated by an extracellular matrix, the cell wall, which protects it from osmotic pressure and environmental stress, and determines cell shape. The cell wall has been described on one hand as a rigid layer of glycoproteins and polysaccharides, and on the other hand as a dynamic structure flexible enough to cope with cell growth. The cell walls of most fungi consist of five major components: (1→3)- β -glucan, (1→6)- β -glucan, (1→3)- α -glucan, chitin, and glycoproteins (**Fig. 1**). Of all fungi, the cell wall of *S. cerevisiae* has best been studied with regard to its structure and biosynthesis. It is composed of (1→3)- β -glucan, that forms an alkali-soluble fraction (20% of total cell wall) or a chitin-linked, alkali-insoluble fraction (35%). Furthermore, (1→6)- β -glucan (5%), chitin (2%), and mannoproteins (40%) are present (Klis *et al.*, 1997). Interestingly,

this yeast lacks α -glucan. By using electron microscopy, it was shown that the cell-wall components are organized in a layered structure in which (1 \rightarrow 3)- β -glucan forms densely interwoven microfibrils present as the innermost layer, followed by (1 \rightarrow 6)- β -glucan and mannoproteins (Osumi, 1998; Cabib *et al.*, 1982). Most chitin, approximately 90%, is found in the region of the bud scars which remain after cell separation, whereas the remainder of the chitin is dispersed over the lateral walls. A covalent linkage between chitin and β -glucan was first suggested by Hartland *et al.* (1994), who based their conclusion on the insolubility of part of the β -glucan under alkaline conditions. Treatment with chitinase or disruption of the chitin synthase 3 gene led to solubilization of their preparation. Later, the covalent linkage between chitin, (1 \rightarrow 3)- β -glucan, (1 \rightarrow 6)- β -glucan and mannoproteins was demonstrated by the group of Cabib who digested cell walls with (1 \rightarrow 3)- β -glucanase and chitinase and analyzed the products by chemical analyses methods, mass spectrometry, and NMR spectroscopy (Kollár *et al.*, 1995; Kollár *et al.*, 1997; Ovalle & Lipke, 1998). They concluded that the mannoproteins are attached to (1 \rightarrow 6)- β -glucan via a glycosylphosphatidylinositol anchor. The (1 \rightarrow 6)- β -glucan contains (1 \rightarrow 3)-linked branches to which the reducing end of chitin may be connected via a (1 \rightarrow 2) or (1 \rightarrow 4) linkage. Finally, the reducing end of the (1 \rightarrow 6)- β -glucan is linked to a non-reducing end of (1 \rightarrow 3)- β -glucan through an as yet unknown linkage.

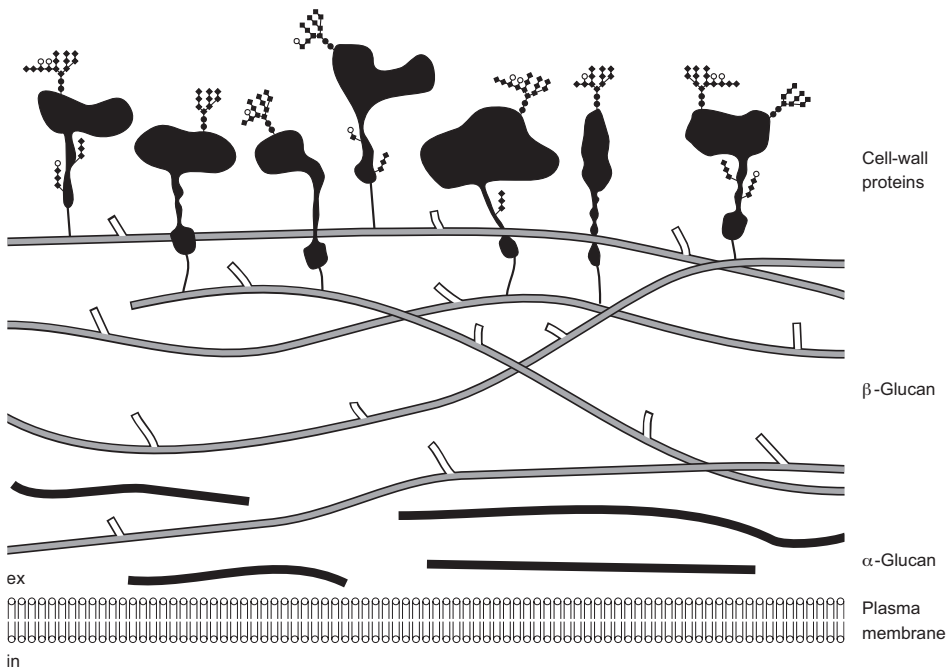


Fig. 1. Schematic representation of the fungal cell wall. The cell wall is composed of an outer layer of glycoproteins and inner layers of polysaccharides. Note that the composition of the cell wall (e.g., presence of α -glucan and chitin) varies between different fungal species.

A similar, layered cell-wall architecture was found in other fungi such as *C. albicans* (Chaffin *et al.*, 1998), *A. fumigatus* (Fontaine *et al.*, 2000), and fission yeast *S. pombe* (Kopecká *et al.*, 1995). Importantly, the cell walls of the latter two fungi differ from that of *S. cerevisiae* in that they contain galactomannan instead of mannan, and (1→3)- α -glucan (Bernard & Latgé, 2001; Manners & Meyer, 1977). Although it was shown that (1→3)- α -glucan forms a network of microfibrils in fission-yeast cell walls (Kopecká *et al.*, 1995), it is as yet unknown whether α -glucan is part of a β -glucan-chitin network.

Cell-wall polysaccharides

(1→3)- β -Glucan

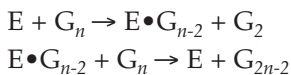
Because of its predominant presence, (1→3)- β -glucan has best been studied of all fungal cell-wall polysaccharides with regard to chemical structure, physico-chemical properties, and biosynthesis. (1→3)- β -Glucan is present in the cell walls of almost all fungi, except of the hyphal walls of Zygomycetes (Barreto-Bergter & Gorin, 1983; Wessels & Sietsma, 1982; Bartnicki-García, 1968). The presence of β -glucan in the cell wall of *S. cerevisiae* has been established as early as 1950 (Bell & Northcote, 1950), but it took another sixteen years before its chemical structure was determined, namely a (1→3)- β -glucan with occasional (1→6)-linked branches (Manners & Patterson, 1966). The degree of polymerization was estimated to be approximately 1500 (Manners & Masson, 1969; Fleet & Manners, 1976). The glucan is only slightly crystalline in the lateral wall, which is probably due to branching (Kreger & Kopecká, 1975). Indeed, treatment of hyphal walls with hot 2% hydrochloric acid (HCl), which may have caused hydrolysis of intrachain glycosidic linkages and glycosidic linkages of branching points, resulted in an increase in crystallinity (Jelsma & Kreger, 1975). X-ray crystallography of HCl-treated (1→3)- β -glucan revealed that it forms a six-fold, parallel triple helix that is stabilized via 2-OH•••O-2 hydrogen bonds (Jelsma & Kreger, 1975; Chandrasekaran, 1997). Gawronski *et al.* (1999) investigated the solution structure of β -glucan by small-angle X-ray scattering and found that a β -glucan composed of a (1→3)-linked main chain with occasional branching at C-6 is composed of triple helices that tend to aggregate as trimers in aqueous solution. A β -glucan that consisted of a (1→3)-linked backbone with branches of only single glucose residues (1→6)-linked to the main chain consisted of a single triple helix (Gawronski *et al.*, 1999), indicating that the type of branching influences aggregation of individual β -glucan polymers.

(1→3)- β -Glucan synthase is a plasma-membrane localized enzyme that catalyzes the intracellular synthesis of (1→3)- β -glucan and that is thought to facilitate extrusion of the newly synthesized (1→3)- β -glucan through the plasma membrane into the extracellular space via a putative membrane-spanning pore (**Fig. 2A**) (Inoue *et al.*, 1996). Purifying (1→3)- β -glucan synthases were achieved by a technique called 'product entrapment'. Product entrapment is based on the affinity of the enzyme to its reaction product and has been successfully applied in the purification of many processive enzymes from crude cell

membranes, such as chitin synthase (Kang *et al.*, 1984), cellulose synthase (Wong *et al.*, 1990), and glycogen synthase (Zhang *et al.*, 1993). Several groups demonstrated that also (1→3)-β-glucan synthases can be purified via product entrapment (Awald *et al.*, 1993; Inoue *et al.*, 1995; Kelly *et al.*, 1996). Two genes, each encoding a putative (1→3)-β-glucan synthase were cloned, called *FKS1* (*GSC1*) and *FKS2* (*GSC2*), of which the first is expressed during vegetative growth and the latter during sporulation (Douglas *et al.*, 1994a; Mazur *et al.*, 1995). However, disruption of either *FKS1* or *FKS2* did not result in a lethal phenotype during vegetative growth, but the double mutant was inviable (Inoue *et al.*, (1995), indicating that these genes can take over each other's function. *In vitro* studies showed that the glucan synthases require UDP-Glc as a donor and that activity is stimulated by the addition of GTP (Shematek *et al.*, 1980). Cabib *et al.* found that a small GTPase that is associated with (1→3)-β-glucan synthase is essential for synthase activity (Mol *et al.*, 1994). This GTPase was later identified as Rho1p. A temperature-sensitive *rho1* mutant was constructed that resulted in a decrease in (1→3)-β-glucan synthase activity (Yamochi *et al.*, 1994). The activity could be restored by the addition of purified or recombinant wild-type Rho1p (Drgonová *et al.*, 1996). This and other studies indicate that Rho1p is a regulatory subunit of Fks1p (Qadota *et al.*, 1996; Mazur & Baginsky, 1996).

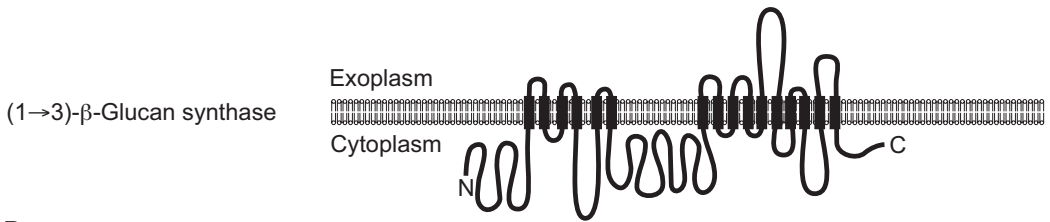
Genes homologous to *FKS1* and *FKS2* are well conserved among other fungi, such as the human pathogenic yeasts *A. fumigatus* (Kelly *et al.*, 1996), *C. albicans* (Mio *et al.*, 1997), *C. neoformans* (Thompson *et al.*, 1999), and *Paracoccidioides brasiliensis* (Pereira *et al.*, 2000), and fission yeast *S. pombe* (Ishiguro *et al.*, 1997). In *S. pombe*, like in *S. cerevisiae*, a GTPase, Rho1p, was identified that is involved in (1→3)-β-glucan synthase activation and that regulates cell morphogenesis (Arellano *et al.*, 1996). (1→3)-β-Glucan synthase activity can be stimulated by GTP in most fungi studied, indicating that the stimulatory role of Rho1p is conserved. The only exceptions described to date are the Oomycetes *Phytophthora sojae* and *Achlya ambisexualis* that both do not display activation of (1→3)-β-glucan synthase upon addition of GTP (Antelo *et al.*, 1998).

As described above, (1→3)-β-glucan is a predominantly (1→3)-linked β-glucan containing approximately 3% of (1→6)-linked branches. Since (1→3)-β-glucan synthase is a plasma-membrane protein, it is likely that the branching occurs extracellularly rather than intracellularly. Hartland *et al.* (1991) have isolated a transglycosylase from the cell walls of *C. albicans* that may be involved in the branching of linear (1→3)-β-glucan. In an *in vitro* study, the authors showed that this transglycosylase cleaves laminaribiose from the reducing end of a linear (1→3)-β-glucan. The remaining (1→3)-β-glucan is then transferred to a second (1→3)-β-glucan where it is linked via a (1→6)-linked branch point, according to:

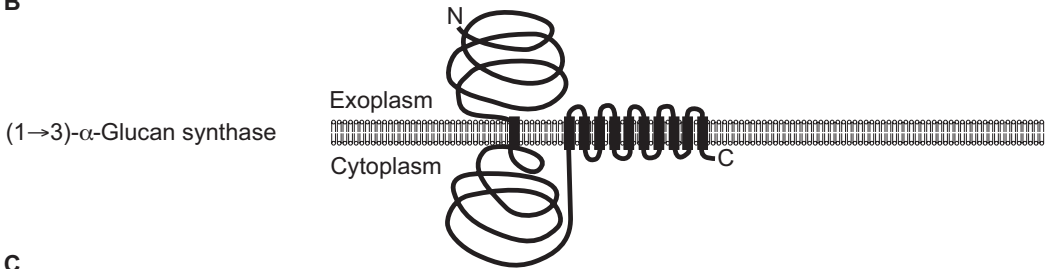


were E = enzyme; G = substrate; n = degree of substitution

A



B



C



D

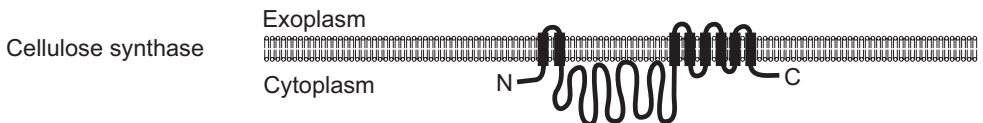


Fig. 2. Topology of a number of polysaccharide transmembrane synthases. (A) (1→3)-β-Glucan synthase Fks1p from *S. cerevisiae* (Kurtz & Rex, 2001); (B) (1→3)-α-glucan synthase Ags1p from *S. pombe* (Hochstenbach *et al.*, 1998); (C) chitin synthase Chs1p from *S. cerevisiae* (Bulawa *et al.*, 1986); (D) plant cellulose synthase I (Brown & Saxena, 2000). Enzyme topologies are based on hydropathy plots.

(1→6)-β-Glucan

A second type of β-glucan found in a large number of fungi is a highly-branched polysaccharide consisting of a (1→6)-linked backbone with (1→3)-linked side branches (Barreto-Bergter & Gorin, 1983). Depending on the fungal species, this polysaccharide is present in the cell wall from 5% (w/w) of total cell wall in *S. cerevisiae* to 20% in *C. albicans* (Klis *et al.*, 2001). In *S. cerevisiae*, it consists of on average 140 glucose residues and connects (1→3)-β-glucan and chitin with cell-wall proteins (Kollár *et al.*, 1997; Klis *et al.*, 1997; Kapteyn *et al.*, 1999). In an effort to identify genes that are involved in (1→6)-β-glucan synthesis, mutant *S. cerevisiae* strains were screened on decreased (1→6)-β-glucan levels. The screens involved the use of K1 killer toxin, which displays a lectin-like affinity for (1→6)-linked β-glucose

that upon binding to the polysaccharide permeabilizes the plasma membrane and kills the yeast (Boone *et al.*, 1990). Mutant strains defective in (1→6)- β -glucan synthesis are K1 killer-toxin resistant, the so-called *kre* mutants, and by using these strains, genes associated with (1→6)- β -glucan biosynthesis can be identified (Shahinian & Bussey, 2000). Disruption of the *KRE5* gene, for example, led to an aberrant cell wall and very slow cell growth. Analysis of its cell wall showed that (1→6)- β -glucan was completely lacking in the *kre5* Δ null mutant. Based on *KRE5* sequence analysis and immunostaining, it was demonstrated that Kre5p is localized to the endoplasmic reticulum (Meaden *et al.*, 1990; Levinson *et al.*, 2002). The function of Kre5p remains unknown, but it has sequence similarity to UDP-glucose: glycoprotein glucosyltransferases, suggesting that it might be a glucosyltransferase involved in (1→6)- β -glucan synthesis (Parodi, 1999). Similarly, several other *KRE* genes have been identified in *S. cerevisiae*, but presently, gene(s) encoding a (1→6)- β -glucan synthase have as yet to be identified.

(1→3)- α -Glucan

Although absent in *S. cerevisiae* and *C. albicans*, (1→3)- α -glucan is found in a large number of fungi, mainly Ascomycetes and Basidiomycetes, where it is present at levels of 9 to 46% of the cell wall, which even reaches 88% in wall material of certain fruiting bodies (Table I). The chemical structure of α -glucan varies among the different fungi from polysaccharides consisting solely of (1→3)-linked α -glucose, to polysaccharides containing small percentages of (1→4)-linked residues, to nigeran-type polysaccharides in which alternating (1→3) and (1→4) linkages occur (Table I). Of the α -glucans consisting mainly of (1→3)-linked residues, degrees of polymerization vary between approximately 60 and 3500 (Table I). Crystallographic data show that (1→3)- α -glucan forms a two-fold single helix, which is stabilized by 2-OH•••O-4 hydrogen bonds (Jelsma & Kreger, 1979; Ogawa *et al.*, 1981). It has a sheet-like packing arrangement that is also observed for cellulose, suggesting that (1→3)- α -glucan is a structural polysaccharide (Chandrasekaran, 1997). However, crystals of good quality could only be obtained after chemical treatment of (1→3)- α -glucan with 2% hydrochloric acid at 60 °C (Jelsma & Kreger, 1979) or by per-*O*-acetylation prior to crystallization followed by deacetylation (Ogawa *et al.*, 1981).

Despite the large numbers of fungi that produce α -glucan, little is known about its function and its biosynthesis. In contrast to (1→3)- β -glucan, which is accepted to be essential for cell morphology and integrity, the function of α -glucan is controversial. In *Aspergillus nidulans*, for instance, complete inhibition of α -glucan synthase did not affect hyphal outgrowth, and it was suggested that in this fungus, α -glucan may function as a reserve carbohydrate (Zonneveld, 1973; Zonneveld, 1972). Similar results were obtained by inhibiting α -glucan synthase in regenerating protoplasts of *Schizophyllum commune* (Sietsma & Wessels, 1988). In contrast, Kopecká *et al.* (1995) showed that in fission yeast not only (1→3)- β -glucan, but also (1→3)- α -glucan may contribute to the rigidity of the cell. By using electron microscopy, the authors showed that α -glucan is present as a fibrillar layer, which is covered by layers of β -glucans. After removal of the β -glucans, the original cylindrical

Table I-A. Ascomycetous fungi whose cell walls contain (1→3)- α -glucan¹

Species	Taxonomic class	Wall dry weight (%)	Type of linkage	DP ²	References
Subphylum: Euascomycetes					
<i>Acroscyphus sphaerophoroides</i>	Lecanoromycetes		(1→3), (1→4) (2:3), (1→6) 6%		Takeda <i>et al.</i> (1972)
<i>Alectoria sarmentosa</i>	Lecanoromycetes		(1→3), (1→4) (11:9)		Takeda <i>et al.</i> (1972)
<i>Alectoria sulcata</i>	Lecanoromycetes		(1→3), (1→4) (11:9)		Takeda <i>et al.</i> (1972)
<i>Aspergillus flavipes</i>	Eurotiomycetes	16-32	(1→3)		Leal <i>et al.</i> (1992)
<i>Aspergillus flavus</i>	Eurotiomycetes		(1→3)		Seo <i>et al.</i> (1999)
<i>Aspergillus fumigatus</i>	Eurotiomycetes		(1→3)		Bernard & Latgé (2001)
<i>Aspergillus nidulans</i>	Eurotiomycetes	22	(1→3)		Borgia & Dodge (1992) Bull (1970) Polacheck & Rosenberger (1977)
<i>Aspergillus niger</i> var. unknown)	Eurotiomycetes	20-30	(1→3), some (1→4) (1→3), (1→4)	330-700	Bobbit <i>et al.</i> (1977) Hasegawa <i>et al.</i> (1969) Horisberger <i>et al.</i> (1972) Johnston (1965) Bobbit <i>et al.</i> (1977)
<i>Aspergillus niger</i> var. <i>awamori</i>	Eurotiomycetes	9	(1→3), some (1→4) (1→3), (1→4)		Bobbit <i>et al.</i> (1977)
<i>Aspergillus ochraceus</i>	Eurotiomycetes	16-32	(1→3)		Leal <i>et al.</i> (1992)
<i>Blastomyces dermatitidis</i> ³	Eurotiomycetes	34.5	(1→3) (yeast form)		Hogan & Klein (1994) Kanetsuna & Carbonell (1971) Klein (1997)
<i>Cetraria islandica</i>	Lecanoromycetes		(1→3), (1→4) (55:45) (1→3), (1→4) (2:1)	35-50 12000	Peat <i>et al.</i> (1961) Fleming & Manners (1966) Olafsdottir <i>et al.</i> (1999)
<i>Cetraria richardsonii</i>	Lecanoromycetes		(1→3), (1→4) (11:9)		Nishikawa <i>et al.</i> (1969)
<i>Cladosporium herbarum</i>	Dothideomycetes	33	(1→3), (1→4) (1:2)		Miyazaki & Naoi (1974)
<i>Elsinoe leucospila</i>	Dothideomycetes		(1→4), (1→3) (5:2)		Misaki <i>et al.</i> (1978) Tsumuraya <i>et al.</i> (1978)
<i>Eupenicillium crustaceum</i>	Eurotiomycetes	16-32	(1→3)		Leal <i>et al.</i> (1992)
<i>Evernia prunastri</i>	Lecanoromycetes		(1→3), (1→4) (4:1) (1→3), (1→4) (3:2)	110-210 60-160	Stefanovich (1969) Takeda <i>et al.</i> (1972)
<i>Fusarium oxysporum</i>	Sordariomycetes		(1→3)		Schoffelmeyer <i>et al.</i> (1999)
<i>Fusicoccum amygdali</i>	Dothideomycetes		(1→3), (1→4) (112:3)	600	Buck & Obaidah (1971) Obaidah & Buck (1971)
<i>Histoplasma capsulatum</i> ⁴	Eurotiomycetes	46.5	(1→3) (yeast form)		Eissenberg <i>et al.</i> (1991) Eissenberg <i>et al.</i> (1996) Kanetsuna <i>et al.</i> (1974) Klimpel & Goldman (1988)
<i>Histoplasma farciminosum</i> ⁵	Eurotiomycetes	13.5	(1→3) (yeast form)		San-Blas & Carbonell (1974)
<i>Letharia vulpina</i>	Lecanoromycetes		(1→3), (1→4) (11:9 and 3:2)		Iacomini <i>et al.</i> (1988)
<i>Neurospora crassa</i>	Sordariomycetes	14	(1→3), (1→4) (10%)		Cardemil & Pincheira (1979) Marshall <i>et al.</i> (1997)
<i>Paracoccidioides brasiliensis</i>	Eurotiomycetes	45	(1→3) (yeast form)		Kanetsuna & Carbonell (1970) San-Blas & San-Blas (1977) San-Blas & Vernet (1977) San-Blas <i>et al.</i> (1977) San-Blas <i>et al.</i> (1997)
<i>Parmelia caperata</i>	Lecanoromycetes		nigeran type ⁶	100-130	Takeda <i>et al.</i> (1970)
<i>Penicillium brevis-compactum</i>	Eurotiomycetes	16-32	(1→3)		Leal <i>et al.</i> (1992)
<i>Penicillium decumbens</i>	Eurotiomycetes	16-32	(1→3)		Leal <i>et al.</i> (1992)
<i>Penicillium expansum</i>	Eurotiomycetes	26-43	(1→3)		Parra <i>et al.</i> (1994)

<i>Ramalina celastri</i>	Lecanoromycetes	28	(1→3), (1→4) (3:1)	1800	Stuelp <i>et al.</i> (1999)
<i>Sphaerophorus globosus</i>	Lecanoromycetes		(1→3), (1→4) (2:3), 6% (1→6)		Takeda <i>et al.</i> (1972)
<i>Stereocaulon japonicum</i>	Lecanoromycetes		(1→3), (1→4) (3:1)	64	Yokota & Shibata (1978) Yokota <i>et al.</i> (1979)
<i>Stereocaulon pauschale</i>	Lecanoromycetes		(1→3), (1→4) (4:1)	135	Hauan & Kjølborg (1971)
Subphylum: Archaeascomycetes					
<i>Schizosaccharomyces pombe</i>	Schizosaccharomycetales	28	(1→3), (1→4) (7%)	207	Bacon <i>et al.</i> (1968) Bush <i>et al.</i> (1974) Manners & Meyer (1977)

¹Note that of the three major taxonomic groups, Euascomycetes, Archaeascomycetes, and Hemiascomycetes, so far α -glucans have not been identified in the Hemiascomycetes. ²DP: degree of polymerization. ³Anamorph of *Ajellomyces dermatitidis*. ⁴Anamorph of *Ajellomyces capsulatus*. ⁵Anamorph of *Ajellomyces capsulatus* var. *farcinosus*. ⁶Nigeran is an α -glucan with alternating (1→3)-linked and (1→4)-linked α -GlcP residues.

Table I-B. Basidiomycetous fungi whose cell walls contain (1→3)- α -glucan¹

Species	Taxonomic class	Wall dry weight (%)	Type of linkages	DP	References
Subphylum: Hymenomycetes					
<i>Agrocybe cylindracea</i>	Homobasidiomycetes		(1→3)	3500	Kiho <i>et al.</i> (1989) Yoshida <i>et al.</i> (1996)
<i>Ananita muscaria</i>	Homobasidiomycetes		(1→3)	260	Kiho <i>et al.</i> (1994) Yoshida <i>et al.</i> (1996)
<i>Armillaria mellea</i>	Homobasidiomycetes		(1→3), (1→4) (1→3)		Sanchez Hernandez <i>et al.</i> (1993)
<i>Coriolus versicolor</i>	Homobasidiomycetes		(1→3), (1→4) (1:2)		Hirase <i>et al.</i> (1970)
<i>Cryptococcus albidus</i>	Heterobasidiomycetes		(1→3)		Bacon <i>et al.</i> (1968)
<i>Cryptococcus neoformans</i>	Heterobasidiomycetes	33	(1→3), (1→4) (3%)	85	James <i>et al.</i> (1990)
<i>Cryptococcus terreus</i>	Heterobasidiomycetes		(1→3)		Bacon <i>et al.</i> (1968)
<i>Ganoderma lucidum</i>	Homobasidiomycetes		(1→3)	1200	Chen <i>et al.</i> (1998a) Chen <i>et al.</i> (1998b)
<i>Laetiporus sulphureus</i>	Homobasidiomycetes	75-88	(1→3)		Jelsma & Kreger (1978) Jelsma & Kreger (1979) Takeo & Matsuzaki (1983)
<i>Lentinus edodes</i>	Homobasidiomycetes		(1→3), (1→4) (5.3:1); branched	600	Shida <i>et al.</i> (1978) Zhang <i>et al.</i> (1999) Zhang <i>et al.</i> (2000) Zhang <i>et al.</i> (2002)
<i>Piptoporus betulinus</i> ²	Homobasidiomycetes	44-53	(1→3)	55-90	Jelsma & Kreger (1978) James & Cherniak (1990) Bacon (1968) Duff (1952)
<i>Polyporus tumulosus</i>	Homobasidiomycetes	15	(1→3)		Angyal <i>et al.</i> (1974) Ralph & Bender (1965)
<i>Schizophyllum commune</i>	Homobasidiomycetes	28	(1→3)		Siehr (1976) Sietsma & Wessels (1977) De Vries & Wessels (1975) Wessels <i>et al.</i> (1972)
<i>Tremella mesenterica</i>	Heterobasidiomycetes	25-38	(1→3)		Jelsma & Kreger (1978) Reid & Bartnicki-García (1976)

¹Note that of the three major taxonomic groups, Urediniomycetes, Ustilaginomycetes, and Hymenomycetes, so far α -glucans have been identified only in the Hymenomycetes. ²Formerly known as *Polyporus betulinus*.

shape of the cell was retained. Furthermore, for the dimorphic fungi *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *P. brasiliensis*, correlations between α -glucan levels and virulence were observed. These three fungi have α -glucan levels of approximately 35-45% of total cell-wall carbohydrates in their virulent yeast form, whereas in their non-virulent mycelial form, α -glucan was almost absent (Kanetsuna *et al.*, 1974; Hogan & Klein, 1994; San-Blas *et al.*, 1977). Additionally, a *H. capsulatum* yeast strain was isolated that showed no virulence towards mice. Analysis of its cell wall demonstrated that α -glucan was absent (Klimpel & Goldman, 1988), suggesting that cell-wall α -glucan might affect virulence (Kügler *et al.*, 2000).

Recently, a gene encoding an α -glucan synthase, *ags1⁺/mok1⁺*, was identified in fission yeast (Hochstenbach *et al.*, 1998; Katayama *et al.*, 1999). A hydropathy plot of the amino-acid sequence indicated an integral membrane protein consisting of three domains: a C-terminal multipass transmembrane domain, an intracellular domain, and an N-terminal extracellular domain (**Fig. 3**). A BLAST search revealed that the intracellular domain may act as a synthase, whereas the extracellular domain has sequence homology with amylases, and therefore might function as a transglycosylase (Hochstenbach *et al.*, 1998) (**Fig. 2B**). Importantly, a temperature-sensitive mutant with a point-mutation in the extracellular domain of Ags1p caused a three-fold decrease in α -glucan levels together with a rounded cell morphology when grown at a semi-permissive temperature. At a restrictive temperature cells lysed, indicating that α -glucan has an essential role in maintaining fission-yeast morphology (Hochstenbach *et al.*, 1998). With the completion of the genome project of fission yeast (Wood *et al.*, 2002), genes homologous to *ags1⁺* were identified, designated *mok11⁺*, *mok12⁺*, *mok13⁺*, and *mok14⁺* (**Fig. 3**) (Hochstenbach *et al.*, 1998, Katayama *et al.*, 1999). Their functions remain unknown, but disrupting each or all *mok⁺* genes did not lead to a noticeable phenotype in vegetatively-grown cells (Katayama *et al.*, 1999). By applying DNA-microarray analyses, Mata *et al.* (2002) showed that *ags1⁺* is downregulated during sporulation, whereas its homologs are upregulated. Together, these results indicate that *mok⁺* genes may be involved in the synthesis of the ascospore wall. α -Glucan synthases homologous to Ags1p are also found in other fungi such as the human pathogenic fungus *A. fumigatus*, which has two homologs (with Genbank accession numbers AAL18964 and AAL28129) that both possess the multidomain structure characteristic for the fission-yeast Ags1p (**Fig. 3**). Also in *C. neoformans*, *Neurospora crassa*, *S. commune*, and *Phanerochaete chrysosporium* genes homologous to fission-yeast *ags1⁺* were identified (**Fig. 3**).

Questions remain, however, regarding the molecular mechanism of α -glucan biosynthesis. It is unknown how the biosynthesis is initiated, what type of nucleotide is involved in chain elongation and how the synthesis is regulated. Latest studies indicate that a GTPase, Rho2p, may be involved in regulating α -glucan biosynthesis, since overexpression of Rho2p leads to increased levels of α -glucan (Hirata *et al.*, 1998; Calonge *et al.*, 2000).

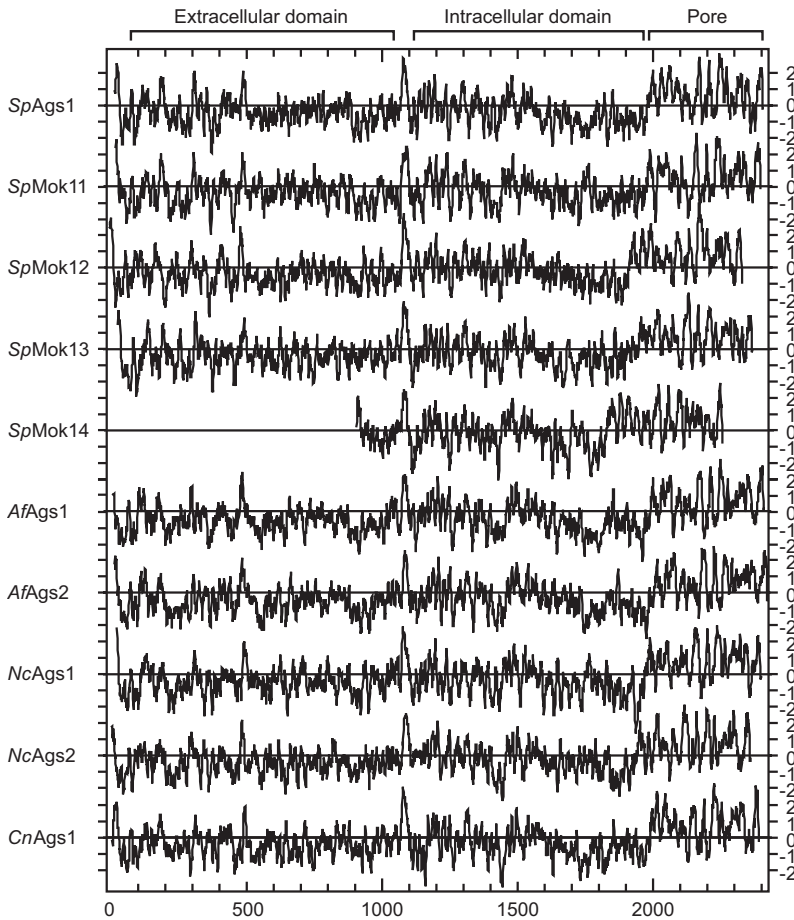


Fig. 3. Fungal α -glucan synthases display a multiple domain structure. Hydropathy plots were generated according to Kyte and Doolittle (1982). Note that the plot of *CnAgs1* is based on preliminary sequence data of strain B3501 (serotype D) obtained from The Institute for Genomic Research through the website at <http://www.tigr.org>. *Sp*: *Schizosaccharomyces pombe*; *Af*: *Aspergillus fumigatus*; *Nc*: *Neurospora crassa*; *Cn*: *Cryptococcus neoformans*.

Chitin

Chitin, a linear chain of (1 \rightarrow 4)- β -*N*-acetyl-D-glucosamine residues, is present in many fungi as a cell-wall component at levels of up to 60% (Bartnicki-García, 1968). In *S. cerevisiae*, chitin is present at approximately 2% (w/w) of the total cell wall and is localized predominantly at the bud scars as a polysaccharide consisting of on average 190 *N*-acetylglucosamine residues, but is also dispersed in the lateral walls where it is covalently linked to (1 \rightarrow 3)- β -glucan and (1 \rightarrow 6)- β -glucan (Hartland *et al.*, 1994; Klis *et al.*, 2002). From X-ray crystallography studies, three crystalline forms are known, denoted α , β , and γ , of which the α -form is found in fungi (Wessels & Sietsma, 1982). α -Chitin forms a two-fold single helix that is stabilized by 3-OH \cdots O-5 hydrogen bonds. Its conformation is very similar to

cellulose, which is consistent with its role as a structural polysaccharide (Chandrasekaran, 1997).

Chitin synthases are integral membrane proteins (**Fig. 2C**) that require UDP-*N*-acetylglucosamine as a donor. Three chitin synthases have been identified in *S. cerevisiae*. The significance of *CHS1* and *CHS2* for cell integrity has long been controversial. Based on gene-disruption experiments, the group of Cabib concluded that *CHS2* is essential for vegetative growth, whereas *CHS1* is not (Silverman *et al.*, 1988, Bulawa *et al.*, 1986). Studies by Bulawa and Osmond (1990), however, revealed that *chs2* null mutants are viable when grown in synthetic minimal medium and do not display a detectable deficiency in chitin. Therefore, a third chitin synthase, Chs3p, must be responsible for the observed chitin synthase activity. Cloning and disruption of the respective genes showed that Chs3p is responsible for chitin dispersed in the bud ring and the lateral wall, whereas Chs2p catalyzes the synthesis of chitin that forms the primary septum dividing the mother cell from the daughter cell (Shaw *et al.*, 1991; Cabib *et al.*, 1993). Importantly, a deletion of both Chs2p and Chs3p led to inviable cells (Shaw *et al.*, 1991). Chs1p was shown to act as a repair enzyme utilized at the end of cytokinesis (Valdivieso *et al.*, 1999).

Genes homologous to *CHS1* and *CHS2* have been identified in many other fungi, such as *C. albicans*, *A. nidulans*, *A. fumigatus*, and *S. pombe* (Munro & Gow, 2001; Arellano *et al.*, 2000; see Bowen *et al.*, 1992 for classification of a large number of fungal chitin synthases into three major groups). In fission yeast, two homologous genes were identified. Depletion or overexpression of *chs1*⁺ did not lead to noticeable defects during vegetative growth, whereas ascospore formation was strongly affected, indicating that the gene is solely required for sporulation (Arellano *et al.*, 2000). The function of *chs2*⁺ is unknown, but DNA-microarray analyses demonstrated that this gene, like *chs1*⁺, is upregulated during meiosis (Mata *et al.*, 2002), and therefore may also display a function during sporulation. On the contrary, Sietsma and Wessels (1990) suggested that chitin may be synthesized also during vegetative growth. They showed that approximately 10% of the β -glucan isolated from vegetatively-grown fission yeast (strain CBS356) is insoluble under alkaline conditions, whereas chitinase or nitrous acid treatment led to fully alkali-soluble β -glucan. Furthermore, the authors were able to detect chitin-synthase activity during vegetative growth. When a fission-yeast strain with a disrupted chitin synthase was investigated, they found that the β -glucan was completely alkali-soluble (J.H. Sietsma, personal communication). These results suggested that chitin may be synthesized during vegetative growth. However, because the authors used a homothallic yeast strain (CBS356), which contains both h⁺ and h⁻ cells, and because they harvested the yeast cells in the late exponential phase, it cannot be excluded that due to nutrient-depletion, mating and subsequent sporulation occurred, leading to activation of chitin synthases.

Glycogen and amylose

Amylose ((1→4)- α -glucan) and glycogen ((1→4)- α -glucan with (1→6)-linked branches) are polysaccharides found in many organisms, both bacteria and eukaryotes. Glycogen is

generally considered to be a storage compound, providing carbon and energy. Also fungi such as *S. cerevisiae*, *S. pombe*, and *H. capsulatum* accumulate glycogen, which is degraded under nutrient-deprived conditions (Kane & Roth, 1974; Inoue & Shimoda, 1981; Garrison & Boyd, 1978). Glycogen synthesis has been studied extensively in mammals where it is initiated by a protein designated 'glycogenin'. This protein synthesizes short (1→4)-linked α -glucoooligomers of four to eight residues from UDP-Glc that serve to prime glycogen synthesis by glycogen synthase (Alonso *et al.*, 1995; Whelan, 1998). In *S. cerevisiae* two genes encoding glycogen synthases, *GSY1* and *GSY2*, were identified (Farkas *et al.*, 1990; Farkas *et al.*, 1991). The synthases catalyze the addition of (1→4)-linked glucose residues from UDP-Glc to the non-reducing end of linear (1→4)- α -glucoooligosaccharides (François & Parrou, 2001). Like in the mammalian counterpart, (1→4)- α -glucoooligosaccharides are synthesized from UDP-Glc by yeast glycogenin, Ggl1p and Ggl2p, and act as a primer.

Genes homologous to *S. cerevisiae* *GSY* were found in the fungus *N. crassa*, but are absent from fission yeast *S. pombe* (Cid *et al.*, 2002; Hochstenbach *et al.*, 1998). In fission yeast, a glycogen-like material is accumulated during sporulation (Beltran *et al.*, 2000; Inoue & Shimoda, 1981). Genes containing sequence similarity to bacterial glycogen synthases were identified in fission yeast, namely *ags1⁺*, *mok11⁺*, *mok12⁺*, *mok13⁺*, and *mok14⁺* (Hochstenbach *et al.*, 1998; Katayama *et al.*, 1999). Surprisingly, studies indicate that Ags1p functions to synthesize (1→3)- α -glucan rather than (1→4)- α -glucan (Hochstenbach *et al.*, 1998; Katayama *et al.*, 1999). The functions of the *mok⁺* homologs still need to be determined.

Cellulose

Although cellulose, a (1→4)- β -glucan, is not found in fungi, recent discoveries concerning its biosynthesis led to new insights into polysaccharide synthesis. Cellulose biosynthesis has been studied thoroughly in bacteria and plants. *In vitro* studies indicated that cellulose synthesis is Mg^{2+} dependent and uses UDP-Glc as a donor (Gibeaut & Carpita, 1994). It was further reported that a primer consisting of (1→4)-linked β -glucose residues may be required for chain initiation (Blaschek *et al.*, 1983), but the actual need for a primer has long been controversial (Delmer & Amor, 1995). Recently, a breakthrough was achieved in the understanding of cellulose biosynthesis with the identification of sitosterol- β -glucoside as the primer for cellulose biosynthesis. In their paper, Peng *et al.* (2002) demonstrated that first a glucose residue is transferred onto a sitosterol molecule, forming sitosterol- β -glucoside. This molecule is then transferred to a glucosyltransferase, Cesa-1, that upon addition of UDP-Glc, elongates the glucoside via (1→4)-linkages, forming a sitosterol-celloooligosaccharide. A membrane-bound cellulase subsequently liberates the celloooligosaccharide from the sitosterol, for use as a primer to initiate cellulose biosynthesis catalyzed by a second glucosyltransferase, Cesa-2. No doubt, these findings will lead to new insights into the biosynthetic mechanisms of other polysaccharides (Read & Basic, 2002).

The fungal cell wall as a target for the development of fungicides

Infections caused by human pathogenic fungi threaten increasing numbers of patients with suppressed immune systems, such as patients undergoing organ transplants or chemotherapy, and patients infected with the human immunodeficiency virus. Some important pathogenic fungi include the dimorphic fungi *H. capsulatum*, *B. dermatitidis*, and *P. brasiliensis* that all are non-virulent in their mycelial form, but become pathogenic when in their yeast form (Kanetsuna *et al.*, 1974; Hogan & Klein, 1994; San-Blas *et al.*, 2002). *A. fumigatus* causes life-threatening mycosis, and due to the absence of adequate medication, death rates can reach up to 80% of infected patients (Beauvais & Latgé, 2001). *C. albicans*, on the contrary, is a dimorphic fungus that requires the ability to switch from a yeast form to a mycelial form for virulence (Lo *et al.*, 1997; Mitchell, 1998). Infections with *C. albicans* are common to immunosuppressed patients, but occasionally also healthy individuals are infected.

Current medication against fungal infections includes polyenes (fungicidal) and azoles (fungistatic) that both act on the plasma membrane of the fungal cell. The polyenes, such as amphotericin B, form complexes with membrane sterol, namely ergosterol, resulting in the formation of transmembrane pores that leak vital cellular ions, such as K^+ and Mg^{2+} . Since the polyenes also bind to mammalian cholesterol, toxicity is common (Georgopapadakou, 1998). A second drawback is the increasing fungal resistance to polyenes, that is based on alterations in fungal membrane lipids, and is found especially in *Fusarium* and *Trichosporon* species (Georgopapadakou, 1998). The second group of antifungals, the azoles, also act on fungal ergosterol, but rather than binding to it like the polyenes, it affects ergosterol biosynthesis by inhibiting a demethylation step catalyzed by cytochrome P-450_{DM} leading to perturbation of the plasma membrane function (Georgopapadakou, 1998). The toxicity of azoles to humans is low, but since azoles are fungistatic rather than fungicidal, life-time medication is necessary. In addition, resistance is emerging in *Candida* species and in *C. neoformans* (Kelly *et al.*, 1996; Perea *et al.*, 2001; Cowen *et al.*, 2002; Georgopapadakou, 1998).

The fungal cell wall is unique among eukaryotes and therefore enzymes that are involved in its biosynthesis form ideal targets for the development of highly-specific antifungal drugs (reviewed in Kurtz & Rex, 2001). (1→3)-β-Glucan and chitin are cell-wall polymers that are both essential for cell viability and occur in most fungi. Accordingly, their synthases have been chosen for the development of novel cell-wall targeted fungicides (Georgopapadakou & Tkacz, 1995; Radding *et al.*, 1998; Onishi *et al.*, 2000; Ohyama *et al.*, 2000; Feldmesser *et al.*, 2000; De Pauw, 2000; Georgopapadakou, 2001; Kurtz & Rex, 2001). For example, polyoxins and nikkomycins inhibit chitin synthesis, whereas echinocandins, the related pneumocandins, and papulacandins inhibit (1→3)-β-glucan synthesis. Although the development of antifungals targeted against chitin synthesis stalled in the first phase of clinical development (Georgopapadakou, 2001), the echinocandin caspofungin inhibiting (1→3)-β-glucan synthesis has recently been approved for clinical use (Hussar, 2001; Ruhnke &

Maschmeyer, 2002).

Unfortunately, *C. neoformans* is not vulnerable towards inhibitors of (1→3)-β-glucan and chitin synthesis (Georgopapadakou, 2001). In addition, fungal strains resistant towards candins have already been identified in *C. albicans* and the model-yeast *S. cerevisiae* (Douglas *et al.*, 1994b; el-Sherbeini & Clemas, 1995; Kurtz *et al.*, 1996), indicating that the search for new targets has to continue. Other cell wall components that are potentially interesting targets for antifungals are (1→6)-β-glucan and (1→3)-α-glucan. The former is present in the cell wall only in minor amounts, but is an essential component for cell-wall rigidity since structural studies made clear that it interconnects mannoproteins with (1→3)-β-glucan and chitin. Also α-glucan has recently been shown to be essential for cell morphogenesis (Hochstenbach *et al.*, 1998) and therefore enzymes that are involved in the synthesis and assembly of (1→6)-β-glucan and (1→3)-α-glucan form interesting chemotherapeutic targets.

Aim and outline of the thesis

The fungal cell wall is a highly complex network of glycoproteins and polysaccharides. Enzymes that are involved in the synthesis and assembly of cell-wall components have gained increasing interest since they form ideal targets for the development of antifungal drugs. For this purpose, understanding the structure and biosynthesis of the individual cell-wall components is essential. The mechanism of how α-glucan is synthesized is barely understood and it is unknown whether this polysaccharide is covalently linked to other cell-wall components. The aim of this thesis is to obtain information on the biosynthesis of fungal cell-wall α-glucan by elucidating its chemical structure. As a first step, we make use of fission yeast *S. pombe* as a model organism, because mutants are available or engineerable and its genome sequence is known (Wood *et al.*, 2002). It was shown that fission-yeast α-glucan synthase, Ags1p, is a transmembrane protein, that may exert several discrete functions, such as the synthesis of α-glucan, the transport of this polysaccharide through the plasma membrane and the assembly or cross-linking to the cell-wall matrix (Hochstenbach *et al.*, 1998). To investigate the different functions of Ags1p, we study the chemical structures of α-glucan isolated from wild-type fission yeast as well as from a mutant that is defective in α-glucan biosynthesis.

In **Chapter 2**, the chemical structure of fission-yeast α-glucan is investigated. We show that fission-yeast α-glucan forms an individual fibrillar network that is not covalently linked to other cell-wall components such as β-glucan. By using a mutant that is defective in α-glucan biosynthesis, we propose that a single enzyme is involved in the synthesis and coupling of two α-glucan polymers and we speculate that a primer might be necessary for the initiation of α-glucan biosynthesis.

To identify the chemical structure of this speculative primer, we degrade the α-glucan by an (1→3)-α-glucanase and elucidate the chemical structure of the non-hydrolyzed material (**Chapter 3**). For this purpose, we purify and characterize an (1→3)-α-glucanase

from *Trichoderma harzianum*, which is described in the **Supplementary data** at the end of Chapter 3. We demonstrate that the speculative primer is composed of (1→4)- α -glucooligomers with varying chain length.

In **Chapter 4**, the structure elucidation of three α -glucan fractions isolated from fission yeast ascospores is described. We show that the ascospore wall contains two mainly (1→3)-linked α -glucans that only differ in their degree of polymerization. Both α -glucans are composed fundamentally different from that of vegetatively-grown cells, indicating that a different biosynthetic mechanism may be involved. The third α -glucan fraction is composed solely of (1→4)-linked glucose residues. This fraction is ascribed to amylose, which may be a storage carbohydrate accumulated during sporulation.

In **Chapter 5**, we investigate whether the chemical structure of α -glucan is conserved among fungi. We determine the chemical structure of α -glucans from seven Ascomycetes and Basidiomycetes and find that in two species, the α -glucans are composed similar to that of fission-yeast cell walls, whereas the α -glucans from four fungi are composed similar to that of fission-yeast spore walls. The seventh fungus may contain both structural types. These results indicate that two biosynthetic mechanisms of α -glucan biosynthesis may be conserved in evolution.

The data obtained in this thesis are the result of a collaboration between a group involved in carbohydrate chemistry and molecular biologists. The combination of chemical analyses and molecular biology proved an extremely strong tool in unraveling the complex mechanisms that are involved in fungal α -glucan biosynthesis.

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