Biochemical aspects of the cell wall of Fusarium oxysporum
Schoffelmeer, E.A.M.

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CHAPTER 1

GENERAL INTRODUCTION

Introduction
Fusarium oxysporum f.sp. lycopersici
Fungal cell walls
Chitin
Chitin synthesis
Classification of chitin synthases
Chitin synthases of Aspergillus species
Catalytic sites in chitin synthases
Targeting of chitin synthases
Glucans
Cell wall proteins (CWPs)
Posttranslational transformations of proteins
N- and O-glycosylation of excreted proteins
Glycosylphosphatidylinositol (GPI) -anchor attachment
Cross-linking of proteins into the cell wall
GPI anchor mediated linkage of CWPs
Linkage of CWPs without a GPI anchor
Putative cell wall proteins
Proteins with amino acid repeats
Effect of loss of cell wall proteins
Cell wall proteins that mediate adhesion
Fimbriae
Hydrophobins of plant pathogenic fungi
Enzymes involved in cell wall construction
Outline of this thesis
Fungi are eukaryotic, heterotrophic organisms, for which approximately 80,000 species have been described. They are widespread, being most abundant in areas of high humidity. Within this group of organisms symbiotic, saprophytic and parasitic species are found, but also species with both saprophytic and parasitic life-styles. Fungi with a symbiotic life style are found in association with roots of plants, and form a mycorrhiza. This mutually beneficial association guarantees an ample flow of dissolved salts to the plant and the fungus is supplied with carbohydrates. Saprophytic fungi play an important role in recycling organic debris. Especially their role in the decay of dead plants is very significant, as fungi have the ability to use cellulose as carbon source. Fungi may have deleterious effects on humans e.g. species that cause lung diseases; others have an indirect effect on human life by causing loss of live stock or crops. The latter is vividly illustrated by the story of the Irish potato famine in 1845, which was caused by the potato blight fungus *Phytophthora infestans*. Fungi are also used in manufacturing certain types of cheese, beer, wine and bread, and in the production of useful antibiotics, notably penicillin.

Fungi are classified into either the Pseudomycota or the Eumycota. The latter group contains three divisions, notably the Dikaryomycota, the Zygomycota and the Chytridiomycota. The Ascomycotina and Basidiomycotina represent the two subdivisions of the Dikaryomycota. Fungi of the former group are characterized by asci in which the sexually produced spores are hold. They also can produce spores (conidia) asexually on erect hyphae, the conidiophores. Within the Ascomycotina unicellular (yeasts), mycelial and dimorphic fungi can be found. Dimorphic fungi not only produce hyphae but also propagate by production of yeast-like cells. Spore germination is triggered by favorable conditions, which results in the case of mycelial fungi in development of a germ tube. This hyphal tube will eventually branch, thus creating a network of hyphae, which is called a mycelium. In the mycelium of the Ascomycotina septal walls develop some distance behind the extending hyphal tip. These septa have pores of various types linking the adjacent hyphal compartments. In most cases these pores are large enough to permit passage of protoplasm and even the movement of nuclei between compartments (Carlile 1995). In case hyphae are damaged these septal pores became blocked in a short period of time. This type of damage control is thought to be a function of small cytoplasmatic particles known as Woronin bodies (Fig.1), which are only found in filamentous fungi. Hyphal growth has some advantages over unicellular growth.
as areas can be crossed with low nutritional value e.g. nutrients can be passed on to neighboring cells. Furthermore, extension of the hyphal tip allows hyphae to penetrate solid substrates such as wood and living tissues of plants and animals. In relation to this invasive growth, turgor plays an important role, as does the excretion of large amounts of hydrolytic proteins (Sietsma and Wessels 1994).

Fusarium oxysporum f. sp. lycopersici

*Fusarium oxysporum* is a soil-borne fungus that is found throughout the world. The species belongs to the Deuteromycetes (Fungi Imperfecti), a group of fungi for which the sexual (perfect) form has not (yet) been identified. Characteristic for this species are the banana-shaped macro-conidia, asexually produced spores, consisting of three to five cells. Within the genus *Fusarium*, *F. oxysporum* is classified within the section Elegans. This section is closely related to taxa in the section Liseola with perfect forms in the Gibberella fujikuroi complex (Burns et al. 1991). Because these perfect forms belong to the Ascomycotina, *F. oxysporum* is considered to be a member of this subdivision.

*F. oxysporum* is one of the fungi with both saprophytic and parasitic life-styles. As a plant-pathogen it causes essentially two types of plant diseases, notably bulb or root rot and vascular wilts. Vascular wilt is caused in a broad range of host plants e.g. vegetables, flowers and plantation crops. However a high degree of host-specialization is observed, as individual strains are able to induce disease in particular hosts only. Based on host range, the species is subdivided into more than 80 formae speciales (Armstrong and Armstrong 1981). *Fusarium oxysporum* f. sp. lycopersici is the causal agent of *Fusarium* wilt of tomato and its host range is restricted to *Lycopersicon* species. Tomato plants growing in *Fusarium*-contaminated soil become infected through the root system. Germ tubes of spores or the mycelium enter tomato plants mainly via wounds or at the point of formation of lateral roots (Agrios 1997). The mycelium traverses the root cortex and finally colonizes the xylem vessels. Wilting of the plant is probably the result of vessel clogging by mycelium, spores, gels, and tyloses.
(protrusions of adjacent xylem parenchyma cells into the xylem vessels). Finally the fungus invades all tissues of the plant, thereby reaching the surface of the dead plant, and starts to sporulate profusely.

**Fungal cell walls**

The cell wall is of prime importance to the fungus. They not only provide strength to withstand turgor pressure that is needed for invasive growth, but they are also involved in cell-cell interaction and adhesion and they function as a molecular sieve for larger molecules. On average, cell walls of species belonging to the Ascomycotina consist of 60-70% glucose and 15-20% amino sugars, predominantly N-acetyl-glucosamine (Sietsma and Wessels 1994). Glycoproteins, which account for ~3.5% of the cell wall contain the minor sugars, notably galactose, mannose and sometimes xylose (Sietsma 1994). The glucose residues are linked via β-1,3-, β-1,6- and α-1,3-linkages, thus forming polysaccharides that are collectively known as glucans. Modification of β-1,3-glucans occurs by addition of one or more β-1,6-linked glucose residues. N-acetyl-glucosamine residues are linked through β-1,4-linkages, thus forming a linear polysaccharide known as chitin.

**Chitin**

Chitin (glucosaminoglycan) is an essential structural component of the fungal cell wall. Mutants of *Aspergillus nidulans* that are impaired in chitin synthesis display cell wall defects that result in lysis of the cell, indicating that chitin contributes to the structural rigidity of the cell wall (Borgia and Dodge 1992). After synthesis of chitin it can be subjected to modifications such as de-acetylation and cross-linking to β-1,3-glucan. De-acetylation results in stretches of glucosamine that is also observed for chitin in the wall of *F. oxysporum* (Fukamizo *et al.* 1996). Linkage of chitin to β-1,3-glucan has been observed in cell walls of *S. cerevisiae* (Mol and Wessels 1987). Chemical analysis of this bond in *S. cerevisiae* has shown that the reducing end of chitin is linked to the non-reducing end of β-1,3-glucan via a β-1,4-linkage (Kollar *et al.* 1997). Chitin is not merely restricted to the fungal kingdom, but is also a major component of the exoskeleton of arthropods, and is present as a minor compound in cell walls of Oomycota, a division of the Pseudomycota (Mort-Bontemps *et al.* 1997). Evolutionarily the Oomycota are related to algae rather than to the Ascomycotina and Basidiomycotina (Förster *et al.* 1990). Just as in plants, in Oomycota cellulose (β-1,4-linked glucose) is a major structural component.
Chitin synthesis

Chitin synthesis has been most thoroughly investigated for the yeast *S. cerevisiae* (Bulawa 1993; Cabib *et al.* 1996). In this organism three chitin synthetases, Chs1, Chs2 and Chs3 have been found. Chs1 is the major activity found in yeast cells and represents about 90% of the measurable chitin synthetase activity in a wild-type strain. The *in vivo* activity of both Chs1 and Chs2 rely on processing by proteases and are therefore zymogenic enzymes. The main difference between Chs3 and the other two synthetases is that Chs3 does not seem to need activation by proteases.

Chitin is synthesized by plasma membrane-bound synthases that catalyze the transfer of GlcNac from UDP-GlcNac to a growing chain of β-1,4-linked GlcNac residues. After synthesis, the chitin chains crystallize and form microfibrils in a non-enzymatic process. Incorporation of radio-active N-acetyl-glucosamine shows that chitin synthesis occurs in the hyphal tip of the extending hyphal tube (Wösten *et al.* 1991; Galun *et al.* 1992). Furthermore, its synthesis sharply decreases towards the base of the hyphal extension zone.

Classification of chitin synthases

Based on amino acid sequence similarity between zymogen type chitin synthases Chs1p and Chs2p of *S. cerevisiae* and Chs1p of *C. albicans* Bowen *et al.* (1992) designed degenerated primers to amplify a 600 bp region from the DNA of 14 taxonomically diverse fungal species. Alignment of the deduced amino acid sequences by the program CLUSTAL revealed three classes (Class I, Class II and Class III) into which the sequences could be divided. This analysis clustered Chs2p of *S. cerevisiae* within the Class II synthases, whereas Chs1p was not related to any of the proposed classes. Class III synthases are not found in *S. cerevisiae*, but are only present in filamentous fungi. DNA sequences homologous to the CHS3 gene of *S. cerevisiae* were not detected in this PCR, as only one of the primers had sufficient homology to the CHS3 sequence. As this classification did not cover all synthases a Class IV synthase was introduced, for those synthases homologous to Chs3 (CHS3) of *S. cerevisiae* (Din *et al.* 1996). This classification was expanded to Class V for those synthases that share no homology with Chs3 (CHS3) (Specht *et al.* 1996). In summary, *S. cerevisiae* contain chitin synthases that belong to Class II (CHS2), IV (CHS3) and the unrelated Chs1, whereas filamentous fungi possess chitin synthases that belong to Class I to V.

Chitin synthases of *Aspergillus* species

Chitin synthases of *Aspergillus fumigatus* are encoded by at least eight different genes, designated chsA to G and csmA (Table 1) (Mellado *et al.* 1995, Mellado *et al.* 1996 a and 1996 b, Borgia *et al.* 1996, Aufauvre-Brown *et al.* 1997 and Fujiwara *et al.* 1997). From the
Table 1: Chitin synthases of *Aspergillus fumigatus* and *nidulans*.

<table>
<thead>
<tr>
<th>species</th>
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<th>Class</th>
<th>% chitin reduction</th>
<th>function</th>
<th>reference</th>
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<td><em>A. fumigatus</em></td>
<td>chsA</td>
<td>I</td>
<td>not determined</td>
<td>unknown</td>
<td>Mellado et al. 1995</td>
</tr>
<tr>
<td></td>
<td>chsB</td>
<td>II</td>
<td>not determined</td>
<td>unknown</td>
<td>Mellado et al. 1995</td>
</tr>
<tr>
<td></td>
<td>chsC</td>
<td>III</td>
<td>0</td>
<td>redundant</td>
<td>Mellado et al. 1995; 1996b</td>
</tr>
<tr>
<td></td>
<td>chsD</td>
<td>*</td>
<td>20</td>
<td>unknown</td>
<td>Mellado et al. 1995; 1996a</td>
</tr>
<tr>
<td></td>
<td>chsF</td>
<td>IV</td>
<td>not determined</td>
<td>unknown</td>
<td>Mellado et al. 1995</td>
</tr>
<tr>
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<td>chsG</td>
<td>III</td>
<td>0</td>
<td>growth, conidiation</td>
<td>Mellado et al. 1996b; Borgia et al. 1996</td>
</tr>
<tr>
<td></td>
<td>csmA</td>
<td>V</td>
<td>not determined</td>
<td>unknown</td>
<td>Fujiwara et al. 1997</td>
</tr>
<tr>
<td><em>A. nidulans</em></td>
<td>chsA</td>
<td>II</td>
<td>not determined</td>
<td>redundant; A+D</td>
<td>Yanai et al. 1994; Motoyama et al. 1997</td>
</tr>
<tr>
<td></td>
<td>chsB</td>
<td>III</td>
<td>not detected</td>
<td>essential for growth</td>
<td>Yanai et al. 1994; Borgia et al. 1996</td>
</tr>
<tr>
<td></td>
<td>chsC</td>
<td>I</td>
<td>not determined</td>
<td>redundant</td>
<td>Motoyama et al. 1994</td>
</tr>
<tr>
<td></td>
<td>chsD1</td>
<td>IV</td>
<td>33</td>
<td>A+D conidiation</td>
<td>Motoyama et al. 1997</td>
</tr>
<tr>
<td></td>
<td>chsE</td>
<td>IV</td>
<td>30 to 40</td>
<td>redundant</td>
<td>Specht et al. 1996</td>
</tr>
<tr>
<td></td>
<td>chsD2</td>
<td>V</td>
<td>30 to 40</td>
<td>growth, conidiation</td>
<td>Specht et al. 1996</td>
</tr>
</tbody>
</table>

* = chsD of *A. fumigatus* does not fit within the known classes. However, it reveals a low but significant similarity to chitin synthases and to other N-acetylglucosaminytransferases, notably NodC from *Rhizopus* spp. and HasA from *Streptococcus* spp.

related species *A. nidulans* so far six chitin synthase genes have been cloned, which are also numbered chsA to E (Table 1). To distinguish the *A. fumigatus* synthases from those of *A. nidulans* the suffix Af or An will be used to mark these synthases. The large number of genes suggests a specific function for each synthase in fungal growth and development. At this
moment the function of AfchsA (Class I), AfchsB (Class II), AfchsD, AfchsF (Class IV) and AfcsmA (Class V) have not been investigated yet. Disruption of AfchsC (Class III), AnchsA (Class II), AnchsC (Class I) or AnchsE (Class IV) does not show an apparent phenotype and therefore these genes are considered to be redundant. Disruption of other genes resulted in an aberrant phenotype as shown by i) altered hyphal morphology, ii) changes in both hyphal morphology and reproductive structures or iii) impaired conidiophore and conidium development.

To date AnchsB is the sole example of chitin synthase that only influences hyphal morphology. Disruption of AnchsB causes growth arrest of the hyphal tube immediately after germination of the conidia, which was accompanied by inflated hyphal tips (Yanai et al. 1994). Analysis of a similar AnchsB disruptant by Borgia et al. (1996) showed that these disruptants were capable of forming slow-growing colonies. In addition these disruptants form highly branched and disorganized hyphae, indicating that AnchsB is important for normal hyphal morphology. Surprisingly, no difference in chitin content between the AnchsB disruptant and wild type strain was detected, indicating that this isoenzyme synthesizes only a minor, but essential part of the chitin content. AnchsB is 89% identical to AfchsG, suggesting that these two Class III enzymes may be functional homologs (Mellado et al. 1996 b). Despite this similarity AfchsG determines not only hyphal morphology but is also involved in construction of reproductive structures. Disruption of AfchsG results in highly branched hyphae, production of fewer conidiophores and impaired separation of conidia.

Effects on both hyphal morphology and reproductive structures are observed for the Class V chitin synthases AfchsE and AnchsD2. Disruption of AfchsE causes cessation of sporulation and growth rates were indistinguishable from the wild-type strain (Aufauvre-Brown et al. 1997). Severe morphological defects are observed at the microscopical level showing balloon-shaped swellings along the length of the hyphae and occasionally at tips. Reproductive structures (conidiophores) that are also affected consist of a central stalk (stipe) that ends in a spherical structure (vesicle). This vesicle is decorated with numerous conidia carrying structures (phialides). Conidiophores of this mutant have severe abnormalities, including swollen phialides, collapsed stipes and swollen vesicles. Over the entire length of the protein, AfchsE is 80% identical to AnchsD2 (Specht et al. 1996), suggesting that they may be functionally equivalent. Analysis of the AnchsD2 disruptant indicates that this might be the case. Disruption of AnchsD2 caused a severe reduction in radial growth, reduced conidiation and morphological defects such as swollen conidia that lyse, subapical swelling of hyphae and appearance of multiple balloon-shaped regions along the length of hyphae (Specht et al. 1996). A substantial fraction of the hyphal tips (> 50%) shows lysis at sub-apical locations. Lysis of hyphal tips, swelling of conidia and production of conidia was remedied in
the presence of an osmotic stabilizer indicating that chitin synthesized by AnchsD2 contributes to the structural integrity of the cell wall of germinating conidia, hyphae and conidiophore vesicles. AfchsE as well as AnchsD2 contribute substantially to the total amount of chitin in the cell wall, 25% and 30%-40% respectively (Table 1). However, the growth and morphological defects cannot be merely explained by a reduction in chitin synthesis. Both AnchsD1 (33%) and AnchsE (30%-40%) also contribute highly to the total amount of chitin but display no defect or a mild defect in morphology in the deletion strain (Table 1). It appears, therefore, that Class V chitin synthases synthesize a specific portion of chitin that is crucial to morphogenesis in this filamentous fungus.

Chitin synthase genes that encode AnchsA and AnchsD1 appear to be solely involved in conidiation. A single disruption of either AnchsA or AnchsD1 has no effect. Disruption of both genes, however, caused a severe defect in conidiation (Motoyama et al. 1997).

Catalytic sites in chitin synthases

In search for putative catalytic sites of zymogen type synthases the synthases of the Oomycete Saprolegnia monoica and Ascomycete fungi Neurospora crassa, S. cerevisiae, Candida albicans, Aspergillus nidulans, Rhizopus oligosporus and the Basidiomycete Ustilago maydis were aligned (Mort-Bontemps et al. 1997). A total of six homologous domains were found, suggesting that these domains are involved in synthesis of chitin. Chitin synthases are characterized as processive glycosyl transferases that transfer multiple sugar residues to the acceptor. Saxena et al. (1995) analyzed several glycosyl transferases by hydrophobic cluster analysis (HCA). This method detects similarities in the three-dimensional structures of proteins that show very limited amino acid sequence similarity. In this way one conserved domain was found in the N-terminal half (domain A) and one in the C-terminal half (domain B) (Fig. 2). Two conserved aspartic acid (Asp) residues are present in the A domain which are thought to be part of the catalytic site. By mutational analysis of CHS2 of S. cerevisiae it was demonstrated that the second aspartic acid (Asp441) plays an essential role in the catalytic activity of the enzyme (Nagahashi et al.
Domain B also contains a conserved aspartic acid together with a motif consisting of Q-R-R-R-W (Saxena et al. 1995) (Fig. 2). Mutational analysis confirmed that the aspartic acid and Q-R-R-R-W motif are essential for catalytic activity of Chs2 (Nagahashi et al. 1995) and Cal1 (CsIII activity) (Cos et al. 1998). The QRRRW motif is also conserved in the C-terminal domain of class I, II, III, IV and V synthases from A. fumigatus and A. nidulans. Recently another region of 67 amino acids (amino acids 748 to 815 in Fig. 2) was found in the C-terminal half of Chs2 that was part of the catalytic site. This region is conserved in Class I and II synthases of other filamentous fungi (Yabe et al. 1998).

Targeting of chitin synthases

Chitin synthase activity is found in hyphal tips of the growing hyphal tube and during septum formation (Galun et al. 1981, Wösten et al. 1991 and Sietsma and Wessels 1994). Site specific synthesis of chitin might require a vectorial transport of these. A vectorial transport of chitin synthases in a vesicle-based transport system has been suggested for Neurospora crassa hyphae (Sietsma et al. 1996). In these hyphae vesicle enclosed chitin synthases (chitosomes) were more abundant in the vicinity of the hyphal tip (Sietsma et al. 1996), suggesting that they are specifically targeted towards this site. Transport of chitosomes might be driven by a myosin-actin-based system. In N. crassa a myosin I has been implicated to participate in transport of vesicles to the growing tip (McGoldrick et al. 1995). Myosin I contain an amino-terminal domain that binds to actin and a carboxyl-terminal lipid-binding domain that enables it to associate with membranes. In this way myosin I associated vesicles can travel along the polarized actin cytoskeleton towards the hyphal tip. Other research points out the possibility that a myosin domain is part of the N-terminal domain of the chitin synthase itself. Recently, genes of A. nidulans and Pyricularia oryza have been cloned that encode Class V chitin synthases (csmA - Table 1) that contain a putative myosin motor-like domain (Fujiwara et al. 1997 and Park et al. 1999). This suggests that a subclass of Class V chitin synthases (Class VI) does not depend on vesicle directed transport but might be guided to specific sites in the plasma membrane by a direct association with the actin cytoskeleton.

Glucans

Glucans are polymers of glucose in which glucose residues are either α or β-linked. α-Glucan only consists of 1,3 linked glucose residues and has been found throughout the hyphal wall of Neurospora crassa (Marshall et al. 1997). Recently a gene encoding an α-1,3-glucan synthase has been cloned from the fission yeast S. pombe (Hochstenbach et al. 1998; Katayama et al. 1999). The predicted amino acid sequence reveals several trans membrane helices, suggesting that this protein is membrane associated. Indeed, this protein was found to
be associated with the plasma membrane (Katayama et al. 1999). Such a location would enable the synthase to deliver the α-1,3-glucan directly into the expanding cell wall.

β-Glucans can be subdivided into two distinct polymers namely β-1,3-glucan and β-1,6-glucan. β-1,3-Glucans can be divided in an alkali-soluble and alkali-insoluble form. The latter results from a covalent linkage of β-1,3-glucan to chitin, making it insoluble in alkali (Mol and Wessels 1987 and Kollár et al. 1995). Synthesis of β-1,3-glucan is most likely performed by a membrane bound synthase; a synthase for β-1,6-glucan has not been identified yet.

β-1,3-Glucan synthase (UDP-glucose; 1,3-D-glucan 3-β-D-glucosyltransferase) catalyses the formation of β-1,3-glucan polymers, an indispensable component of the fungal cell wall (Borgia and Dodge 1992). β-1,3-glucan is synthesized at the cytoplasmic face of the plasma membrane by the transfer of glucose residues from UDP-glucose to a growing chain of β-1,3-glucan extending across the plasma membrane. This synthase consists of at least one catalytic and one regulatory subunit (Beauvais et al. 1993). In S. cerevisiae two genes, FKS1 and FKS2, are essential for β-glucan synthesis and might encode the catalytic subunit of β-1,3-glucan synthases. Fks1p is the major protein responsible for glucan synthase activity during vegetative growth, while Fks2p appears to be important for sporulation (Mazur et al. 1995) and repair (Ram et al. 1998 a). In order to synthesize glucan the catalytic subunit requires a soluble GTP-binding protein. The regulatory subunit that performs this function in S. cerevisiae has been identified as the GTP binding protein Rho1 (Quadota et al. 1996). FKS1 homologs in filamentous fungi have been cloned from A. fumigates (Beauvais et al. unpublished, Gene bank entry g2149092), N. crassa (Bezerra and Azevedo unpublished, Gene bank entry g3025867), A. nidulans (Kelly et al. 1996) and the dimorphic fungus C. albicans (Mio et al. 1997). Comparison of the deduced amino acid sequence reveals a significant sequence similarity indicating that glucan synthases are conserved in both yeasts and mycelial fungi. As the S. cerevisiae, A. fumigatus (Beauvais et al. 1993) and A. nidulans (Kelly et al. 1996) synthases need GTP for their activity, it suggests the presence of Rho-like subunits in these fungi as well.

Cell wall proteins (CWPs)

The fungal cell wall is not only composed of the polymeric carbohydrates chitin and glucan but it also contains cell wall proteins (CWPs) that often are extensively glycosylated. CWPs can perform various functions in the cell wall. In S. cerevisiae they are involved in wall permeability, cell wall construction, mating and cell-cell aggregation. In addition, CWPs of C. albicans have been described that play a role in establishing an infection court, such as fimbrial proteins that mediate adhesion to receptors on human epithelial cells.
<table>
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<tr>
<th>species</th>
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<th>AA no.</th>
<th>N-sites</th>
<th>S/T (%)</th>
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<td>26</td>
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<td>mainly hyphal tip</td>
<td>Ramon et al. (1999)</td>
</tr>
<tr>
<td>Coccioides immitis</td>
<td>Ag2</td>
<td>194</td>
<td>0</td>
<td>17.5</td>
<td>yes</td>
<td>hyphae</td>
<td>Zhu et al. (1996a-l)</td>
</tr>
<tr>
<td>Blastomyces dermatitidis</td>
<td>WI-1</td>
<td>1146</td>
<td>0</td>
<td>8.5</td>
<td>no</td>
<td>yeast</td>
<td>Hogan et al. (1995)</td>
</tr>
</tbody>
</table>

* - personal communication, 1) number of amino acids, 2) number of N-glycosylation sites, 3) percentage of serine (Ser) and threonine (Thr) residues, 4) presence of a putative glycosylphosphatidylinositol (GPI)-anchor attachment/cleavage site and 5) location of the cell wall protein (CWP)
General introduction

The amount of protein present in the cell walls of Ascomycotina is generally a few percent. In several *Fusarium* species comparable amounts are present, ranging from 3 to 7% (Barran *et al.* 1975, Sivan and Chet 1989, Barbosa 1993, Schoffelmeer *et al.* Chapter 2 of this thesis). Two groups of cell wall glycoproteins can be distinguished. One group is not extractable by SDS and thought to be covalently linked to β-glucan. The second group can be solubilized by SDS under reducing conditions and is non-covalently bound. Among the members of the second group it is difficult to discriminate between genuine cell wall proteins and contaminating cellular proteins (Klis, 1994). Features that are shared by most CWPs of *S. cerevisiae* are the presence of a N-terminal signal peptide, a GPI-anchor addition signal at their C-terminus and serine- and threonine-rich regions (Caro *et al.* 1997). Based on these features the complete genome of *S. cerevisiae* was screened. In this way 38 potential CWPs were identified (Caro *et al.* 1997). The number of cell wall proteins (CWP) that originate from filamentous fungi and that have been characterized is limited (Table 2). Therefore, most of our knowledge about these proteins stems from *S. cerevisiae* and *C. albicans* cell wall research. CWPs of the latter have recently been reviewed by Chaffin *et al.* (1998).

Posttranslational modifications of proteins

Proteins that contain an N-terminal signal sequence are directed into the secretory pathway and may undergo several post-translational modifications. In this pathway three types of post-translational modification of proteins can be performed; i) attachment of N-linked glycan chains to asparagine; ii) attachment of O-linked glycan chains to serine or threonine residues, and iii) attachment of a glycosylphosphatidylinositol (GPI) anchor at the COOH terminus of membrane and cell wall proteins.

Depending on the proteins, glycan side chains may contribute to their conformation, stability and appropriate targeting. Furthermore, specific carbohydrate structures are known to participate in biological recognition processes (Herscovics and Orlean 1993). GPI-anchored proteins are destined for the plasma membrane where they are displayed on the outer surface of the membrane.

N- and O-glycosylation of excreted proteins

Glycosylation of proteins is a stepwise process involving the consecutive action of many gene products. This process has been studied in great detail for *S. cerevisiae* (Herscovics and Orlean 1993). Sugar addition is performed on selected asparagine residues (N-linked glycosylation), and on serine and threonine residues (O-linked glycosylation). N-glycan chains are linked to the amide group of asparagine within the tripeptide, Asn-X-Thr/Ser, where X may be any amino acid except proline. Initiation of N-glycosylation occurs in the
rough endoplasmic reticulum (ER) by addition of the preformed oligosaccharide \( \text{Glc}_2\text{Man}_9\text{GlcNac}_2 \) to these asparagine residues. Maturation of this chain by addition of additional monosaccharides occurs in the Golgi apparatus. \( O \)-linked glycosylation of proteins is performed by the sequential addition of monosaccharides to OH groups of selected serine or threonine residues. Whereas for \( N \)-linked glycosylation a defined consensus sequence for sugar addition has been established, no such clear-cut motif has been resolved for \( O \)-glycosylation.

The initial reactions of protein \( N \)-glycosylation proceed identically in all eukaryotic cells. Protein \( O \)-glycosylation proceeds differently in fungal and higher eukaryotic cells. In contrast to fungi, most \( O \)-glycosylation reactions in higher eukaryotic cells proceed in the Golgi and commence with the attachment of \( N \)-acetylglactosamine (Roth et al. 1994). In glycoproteins of yeast the length of the \( O \)-glycosidical linked glycan chain varies between one and five hexoses and is solely composed of mannose. \( O \)-glycan chains derived from cell wall glycoproteins of \textit{Fusarium} spp. on the other hand consist of heteropolymers of Man, Gal, GlcA (glucuronic acid), Glc, Rha, amino sugars and phosphate (Jikibara et al. 1992 a). Some \( O \)-chains of this organism are of low molecular weight consisting of one to six residues (Jikibara et al. 1992 b). One of these small chains was identified as a \( \text{Man}\alpha-1,2\text{Man} \)-ol containing an ethanolamine residue, which is linked via a phosphodiester bond to Man. A similar structure is present in GPI-anchors. However, in this case mannose is linked to the protein moiety instead of the ethanolamine residue excluding the possibility that this structure is part of a GPI-anchor. Two larger \( O \)-linked heteropolymers with an estimated molecular weight of \( 8.2 \times 10^4 \) (± 456 sugar residues) and \( 3.1 \times 10^4 \) (± 172 residues) were found to be mainly composed of repeating glycan units (Jikibara et al. 1992 c). These units consist of a \( \beta \)-1,6-Gal backbone of five residues, which is branched by four glycan chains of variable length.

\textit{Glycosylphosphatidylinositol (GPI) anchor attachment}

Addition of a glycosylphosphatidylinositol (GPI) anchor to selected proteins is known to occur in animal cells, yeast and protozoa (Takeda and Kinoshita 1995). This type of protein modification was not yet known for plants. Recently, also GPI-anchored plasma membrane proteins have been detected in \textit{Nicotiana tabacum} protoplasts (Takos et al. 1997). Biochemical evidence for GPI-anchor linked arabinogalactan-proteins has been presented for the plant species \textit{Nicotiana alata} and \textit{Pyrus communis} (Yeul et al. 1998). Furthermore, for other plant arabinogalactan-proteins GPI-anchor attachment sites have been predicted (Schultz et al. 1998). GPI-anchoring of proteins has the following advantages: it increases mobility of these proteins in the membrane as compared to proteins linked by a transmembrane domain (Udenfriend and Kodukula 1995) and it allows excretion of the
General introduction

protein, often in response to a specific signal (Müller and Bandlow 1993). In *S. cerevisiae*, GPI-anchors are also essential to link cell wall proteins to β-glucan (Klis 1994; Kollár et al 1997; Kapteyn et al. 1999).

![Diagram of GPI anchor-mediated linkage](image)

**Fig. 3** Glycosylphosphatidylinositol (GPI) anchor mediated linkage of an extracellular protein to the plasma membrane (A) and the subsequent linkage of cell wall proteins (CWPs) via a remnant of this GPI anchor to β-glucan (B). The exact events that result in membrane dissociation of the protein and subsequent linkage to the mannan core of the GPI anchor are not known. Etn = ethanolamine, GlcN = glucosamine, GPI-anchor = glycosylphosphatidylinositol anchor, HF = hydrofluoric acid, I = inostol, P = phosphate, and PDE = phosphodiesterase.

GPI anchors (Fig. 3A) consist of a core glycan (Manα1-2Manα1-6Manα1-4GlcNH2α1-) linked via ethanolamine phosphate to the C terminus of the protein. The glucosamine is linked via inositol to a glycerolipid moiety (or occasionally a ceramide) that is embedded in the plasma membrane (McConville and Ferguson 1993). Biosynthesis of the GPI moiety is a multi-step process that consists of the sequential transfer of sugars and phosphoethanolamine (EtnP) residues from donor molecules to phosphatidylinositol (PtdIns) (reviewed by Takeda and Kinoshita 1995; Udenfriend and Kodukula 1995). Biosynthesis of the GPI-anchor is initiated on the cytoplasmic face of the ER, and is finished on the luminal side of the ER. It is then transferred en bloc to eligible glycoproteins.

Proteins destined to be GPI-anchored contain in addition to an N-terminal signal sequence also a signal at their C-terminus. The N-terminal signal sequence directs the protein into the
ER and is subsequently removed. In the ER the C-terminal signal peptide is removed during the process of GPI addition. The new C-terminal amino acid residue that results from this proteolytic event is linked to the GPI moiety. In the nascent protein the amino acid which is destined to become the new C-terminus and accept the GPI-anchor has been designated the $\omega$ site, residues trailing this site are called $\omega+1$ and $\omega+2$. By comparison of proteins that have had their $\omega$ sites determined and from site mutational studies, an accurate prediction can be made for the cleavage sites at the COOH-terminus (Udenfriend and Kodukula 1995). Such a site-directed mutagenesis of the human placental alkaline phosphatase (PLAP) showed the following amino acid requirements for the $\omega$ and $\omega+2$ site (Gerber et al. 1992). Only Gly, Ala, Ser, Cys, Asp and Asn are allowed as $\omega$ site whereas the $\omega+2$ site only permits Gly, Ala and Ser. Mainly small amino acids constitute the $\omega+1$ site. Similar experiments for the Gas1 protein of \textit{S. cerevisiae} show the same subset of amino acids with small side chains at the $\omega$ site (Nuoffer et al. 1993). A hydrophilic hinge region of five to seven amino acids that is needed for optimal GPI anchor addition follows the tri-peptide cleavage/attachment site. The exact composition of this hinge region is not important. The hydrophobic part of the COOH-terminal signal peptide varies from about 10 to 30 mainly hydrophobic amino acids.

\textbf{Cross-linking of proteins into the cell wall}

Ovalently bound SDS-resistant CWPs can be released from the cell wall by cell wall hydrolytic enzymes such as $\beta$-1,3-glucanases (Zymolyase, laminarinase and Quantzyme) and $\beta$-1,6-glucanase. Release of CWPs from isolated cell walls by $\beta$-glucanases indicates that these proteins are physically linked to $\beta$-glucans. Evidence for such a linkage was obtained from the analyses of glucanase-released CWPs using antibodies directed against $\beta$-1,6- and $\beta$-1,3-glucan. This showed that Quantzyme-released CWPs from \textit{S. cerevisiae} contained $\beta$-1,6- as well as $\beta$-1,3-glucan (Montijn et al. 1994; Kapteyn et al. 1995; Kapteyn et al. 1996). Similar observations have been reported for other Ascomycotina, notably \textit{C. albicans} (Kapteyn et al. 1994), \textit{F. oxysporum} (Schoffelmeer et al. 1996; Chapter 3 of this thesis) and \textit{Aspergillus niger} (Brul et al. 1997), suggesting that linkage of CWPs to $\beta$-glucans is widespread within this class of fungi. The order in which both glucan types were connected to the CWPs of \textit{S. cerevisiae} was resolved by treating Quantzyme-released CWPs with endo-$\beta$-1,6-glucanase. This treatment resulted in loss of both $\beta$-1,6- and $\beta$-1,3-glucan, indicating that $\beta$-1,3-glucan is linked through a $\beta$-1,6-glucan moiety to the CWPs (Kapteyn et al. 1996) (Fig. 3B).
General introduction

GPI anchor mediated linkage of CWPs

Apart from the N- and O-linked glycan chains several glucanase extractable CWPs from *S. cerevisiae* contain a carbohydrate chain consisting of β-linked glucose, mannose and (N-acetyl)glucosamine (van Rinsum *et al.* 1991; Montijn *et al.* 1994). The latter two monosaccharides are also present in the core glycan of GPI-anchors that consist of three mannose residues and one glucosamine residue. Attachment of such a GPI-anchor has been demonstrated for the α-agglutinin of *S. cerevisiae* (Lu *et al.* 1994). For other CWPs like α-agglutinin anchor protein, flocculin and Cwp2, the primary translation product of the corresponding gene shows a putative GPI-anchor attachment signal (Klis, 1994 and Caro *et al.* 1997). Involvement of O-glycan chains in cross-linking Cwp2 to β-glucan was investigated by introduction of an IgA protease recognition site immediately N-terminal from the ω site. Cleavage of this site in glucanase extracted Cwp2 resulted in loss of the β-1,6-glucan chain from the protein (van der Vaart *et al.* 1996). As no serine or threonine residues were lost from the protein, O-glycosylation cannot be an attachment site for the β-1,6-glucan, indicating that a GPI anchor mediates the linkage between protein and β-1,6-glucan (Fig. 3B).

Biochemical evidence also supports GPI-anchor mediated linkage. Treatment of β-1,3-glucanase-released CWPs of *S. cerevisiae* and *C. albicans* with aqueous hydrofluoric (HF) acid or phosphodiesterase released β-1,6-glucan epitopes from the CWPs as judged from Western analysis (Kapteyn *et al.* 1995, 1996). Both treatments break phosphodiester bonds that can be found in GPI-anchors (Fig. 3) (Sipos 1995), indicating that this structure could mediate the linkage between CWPs and β-1,6-glucan.

Additional evidence showing that a GPI anchor mediates linkage of CWPs into the cell wall of *S. cerevisiae* is extensive. It has been shown that addition of the C-terminal thirty amino acids of α-agglutinin, constituting the GPI-attachment signal, to α-galactosidase allows localization of the hybrid protein in the cell wall (van Berkel *et al.* 1994). Mutant proteins deficient in the C-terminal hydrophobic sequence were secreted into the culture medium, as described for the CWPs α-agglutinin (Wojciechowicz *et al.* 1993; Lu *et al.* 1995) Cwp1 (Shimoi *et al.* 1995) and Flo1 (Bony *et al.* 1997). Furthermore, tagging of two *S. cerevisiae* cell wall mannoproteins, Cwp1 and Cwp2, with the green fluorescent protein (GFP) resulted in incorporation of fluorescent fusion proteins into the cell wall (Ram *et al.* 1998 b). If the GPI-anchor signal sequence is absent from the GFP-CWP fusion protein it results in secretion of the proteins into the culture medium.

Genes encoding putative GPI-anchored CWPs have been cloned from several species belonging to the Ascomycotina (Table 2), indicating that GPI-anchoring, as a means to cross link CWPs to the cell wall could be a more general property. This is illustrated by *Ag2* of *Coccidioides immitis* (Zhu *et al.* 1996a and 1996b), *HYR1* (Bailey *et al.* 1996) and *HWP1*
Chapter 1

(Staab et al. 1996 and 1998) of C. albicans, MPI of Penicillium marneffei (Cao et al. 1998) and FEM1 of F. oxysporum (Schoffelmeer et al. chapter 4 of this thesis). Although potential GPI-attachment sites have been found in the predicted proteins of these Ascomycete fungi, addition of a GPI anchor and its role in cell wall attachment still needs to be established.

Linkage of CWPs without a GPI anchor

Although it might seem that addition of a GPI anchor is a common and perhaps preferred mechanism to link CWPs to cell wall polysaccharides, it is probably not the only method, as is illustrated by the following examples. In S. cerevisiae four PIR proteins (PIR = proteins with internal repeats) were identified in the cell wall that could be released by mild alkaline treatment (Mrsa et al. 1997). Subsequent cloning of the genes showed that the primary translation product did not contain a C-terminal sequence required for GPI anchoring. This ruled out the possibility that a GPI anchor links these proteins into the cell wall. The alkaline conditions used release O-glycan chains from the glycoprotein in a process called β-elimination. Recently, it has been shown that O-chains mediate the linkage of PIR proteins to β-1,3-glucans (Kapteyn et al. 1999a). From the dimorphic fungus Yarrowia lipolytica the gene YWP1 was cloned that encodes a hyphal CWP (Ramon et al. 1996). Analysis of the primary translation product reveals at the N-terminus a secretion signal sequence whereas a GPI-anchor attachment site is lacking. Since this CWP can only be released from the hyphal cell wall by glucanase digestion (Zymolyase), it suggests a covalent linkage to β-1,3-glucan via an as yet unknown bond. In view of the PIR data it is tempting to speculate that YWP1 is linked to β-1,3-glucan via O-chains as has been suggested for PIR proteins (Kapteyn et al. 1999).

Other CWPs that lack a GPI addition site are WI-1 and CIH1 of Blastomyces dermatitidis and Colletotrichum lindemuthianum, respectively (Hogan et al. 1995; Perfect et al. 1998). WI-1 is a major target of cellular and humoral immunity in infected humans. Binding studies demonstrated that WI-1 is an adhesin, which mediates attachment to mammalian cells. Targets for the WI-1 adhesins are the β2 chain integrins in the plasma membrane. The bean pathogen C. lindemuthianum produces intracellular hyphae (IH, the functional equivalent of the haustorium) that develop inside plant cells and functions as a specialized feeding structure. These hyphae persist up to four days after infection marking the biotrophic phase. After this the fungus switches to the necrotrophic phase. CIH1 has been cloned from this fungus and appeared to encode a fungal proline-rich glycoprotein that is specifically secreted to the walls of IH. Northern analysis showed that the CIH1 gene is only expressed in planta, suggesting that it is necessary to establish or maintain a biotrophic interaction. As yet nothing is known about the link that retains WI-1 and CIH1 into the cell wall.
General introduction

Putative cell wall proteins

In filamentous fungi the ability to distinguish self and non-self is essential because of their capacity to undergo hyphal fusion that lead to the formation of a so-called heterokaryon. Heterokaryon formation between different isolates is controlled by specific loci termed *het* loci. Heterokaryotic cells formed between strains of different *het* genotype are rapidly destroyed or strongly inhibited in their growth. In *N. crassa*, at least 11 loci, including the mating type locus, affect the capacity to form a heterokaryon between different isolates (Perkins 1988). The gene present at the *het-C* locus has been cloned and appeared to encode a protein that possesses an N-terminal signal peptide sequence but not a C-terminal GPI anchor addition signal (Saupe et al. 1996). In its N-terminal part this protein contains a leucine-zipper domain which is implicated in protein-protein interactions. The hydrophilic glycine-rich C-terminal domain of Het-C is similar to glycine-rich domains found in plant cell wall proteins. This homology together with the expected extracellular location suggests that the protein is associated with the fungal cell wall (Saupe et al. 1996). The presence of the leucine-zipper motif fits in a simple model describing how these proteins might function. In this model *het* gene products trigger incompatibility via protein-protein interaction between alternate *het* gene products.

Other putative cell wall proteins were found in *N. crassa* by screening an EST (expressed sequence tag) library (http://www.kumc.edu/research/fgsc/craslib.html). One of the ESTs designated *NEM1* (*Neurospora* extracellular matrix protein 1) was found during a screen of this library with the *F. oxysporum* CWP gene *FEM1* (*Fusarium* extracellular matrix protein - Chapter 3 of this thesis). Another EST of this fungus was found to be homologous to the *PIR1* gene of *S. cerevisiae*. *FEM1* is thought to be located within the cell wall via a remnant of the GPI anchor (Chapter 4). O-glycan chains might be involved in cross-linking of PIR proteins to β-1,3-glucan (Kapteyn et al. 1999). Hence, both systems to cross-link CWPs to β-glucans might also be present in *N. crassa*.

Proteins with amino acid repeats

The *PIR1*, *PIR2* and *PIR3* proteins of *S. cerevisiae* contain sequence of 18 or 19 amino acid residues that are tandemly repeated seven to ten times (ToH-E et al. 1993). These PIR proteins are thought to be necessary to make the yeast cell more tolerant to a sudden increase in temperature. Also the product of the *FLO1* gene which is involved in flocculation (aggregation of yeast cells) contains three different repeat units (Teunissen et al. 1993). These proteins which are composed of several amino acid repeats can also be found in CWPs of other fungi. Qid74 of *Trichoderma harzianum* consists of five incomplete and nine complete...
copies of a 59 amino acid repeat unit (Rey et al. 1998). At the N-terminal part of CIH1 (C. lindemuthianum) a small series of short repetitive motifs of three types is found, notably LPEP, YKPK and VEGPYKPK (Perfect et al. 1998). Within the N-terminal domain of HWP1 (C. albicans) 13 tandem repeats which are 10 amino acids in length are present. Repeats of variable length are found throughout the mature YWP1 (Y. lipolytica). A total number of 30 highly conserved repeats, 24-amino acids in length, are present in WI-1 (B. dermatitidis) comprising a large part of the mature protein. Except for the repetitive sequences in WI-1, that is homologus over a stretch of 17 residues to the Yersiniae invasin's adhesive domain, no function can be ascribed to the repeats of the other aforementioned proteins.

**Effect of loss of cell wall proteins**

Whether CWPs are essential for survival of the fungus has been studied for the S. cerevisiae proteins Cwp1, Cwp2, Tip1, Srp1 (van de Vaart 1995), and four CWPs belonging to the PIR family (Mrsa et al. 1997). Disruption of these genes resulted in loss of the protein from the cell wall. However, only in a CWP2 deletion strain the thickness of the cell wall protein layer appeared to be reduced (van der Vaart et al. 1995). There was, however, no effect on growth rate indicating that Cwp1, Cwp2, Tip1, Srp1 and PIR proteins are not essential for cell growth (Shimoi et al. 1995, van de Vaart 1995 and Mrsa et al. 1997). Deletion of YWP1 also showed that loss of Ywp1 from the cell wall of Y. lipolytica is not essential for growth, osmotic stability and formation of mycelium. It is conceivable that other CWPs compensate for the loss of Ywp1. Growth of the CWP2 deletion strain is impaired only in the presence of Calcofluor White or Congo Red (van der Vaart et al. 1995), chemicals that cause a change in the cell wall structure and aggravate cell wall defects (Ram et al. 1994).

**Cell wall proteins that mediate adhesion**

*Fimbriae*

Fimbriae are flexible, long (0.5 to 20 µm), narrow (7 nm), and erect appendages that are present at the outside of many fungi (Celerin and Day 1998). These structures appear similar to pili or fimbriae found on the surface of prokaryotic cells. In the anther smut fungus Microbotryum violaceum fimbriae are thought to be involved in mating. Detailed studies of these fimbriae showed that they are composed of a 74 kDa glycoprotein subunit and a nucleic acid compound, fimbrial-RNA (reviewed by Celerin and Day 1998). Whether this fRNA has a specific function remains to be resolved. The glycan moiety of the protein consists partially of α-linked mannose (Castle et al. 1996). Amino acid sequences of a small number of peptides (12.5 % of the entire protein) showed amino acid motifs known to be present in
General introduction

collagens, components of the extracellular matrix in mammalian systems (Celerin et al. 1996). Furthermore, the fimbrial protein could be partially digested by collagenases. These observations indicate that this protein may be a type of collagen. Subunits of fimbriae have been immunolocalized throughout the cell wall but are most abundant at the inside of the cell wall and/or at the plasma membrane. To explain this, a model has been proposed in which fimbrial subunits migrate from the surface of the plasma to the outside of the cell wall. At the surface of the cell wall these subunits polymerize into fimbrial structures (Celerin et al. 1997).

Hydrophobins of plant pathogenic fungi

Originally hydrophobins have been described for the Basidiomycete Schizophyllum commune. Their genes were essentially cloned as mRNAs that were highly expressed during formation of aerial hyphae and fruit bodies (Wessels 1993). Later it was shown that hydrophobins are secreted into the culture medium and are present on hyphae. Analysis of the purified Sc3 hydrophobin of S. commune revealed that this protein is able to self-assemble into a protein membrane, a rodlet layer similar to the one observed on hyphal walls (Wösten et al. 1993). Such a rodlet layer is formed at water/air interfaces and facilitates the emergence of aerial hyphae. In addition hydrophobins have been detected as well on surfaces of fungal spores and infection structures of plant pathogenic fungi or fruiting bodies (Kershaw and Talbot 1998). Hydrophobins are now widely found among the fungal taxa and have been reported in 20 species, including members of the Ascomycotina, Basidiomycotina and Zygomycota.

Their small size (± 100 amino acids), high hydrophobicity and the presence of eight cysteine residues that are spaced in a particular manner characterize hydrophobins. Apart from these characteristics there is little amino acid sequence homology (Wessels 1994). The cysteine residues are thought to form disulfide bridges. Experimental evidence for this has been provided for the cerato-ulmin (CU) toxin, a hydrophobin from Ophiostoma ulmi. In this hydrophobin there are two disulphide bonds (Cys7-S-S-Cys17 and Cys18-S-S-Cys30) in the N-terminal domain and two disulphide bonds (Cys46-S-S-Cys57 and Cys58-S-S-Cys69) in the C-terminal domain (Fig. 4) (Yaguchi et al. 1993). Comparison of the hydropathy pattern of several hydrophobins revealed two classes, designated class I and class II (Wessels 1994). These classes are exemplified by MPG1 from Magnaporthe grisea that represents a class I hydrophobin and CU from Ophiostoma ulmi that represents a class II hydrophobin (Fig. 4). Putative basic structures for each class of hydrophobins can be given assuming that a similar pairing of Cys residues like that observed for the CU toxin occurs in other hydrophobins (Kershaw and Talbot 1998) (Fig. 4). Between members of class I hydrophobins the first, second and fourth loop vary greatly in length. The greatest divergence is in the length of loop
two, which varies from 22 to 39 amino acids. Also the distance between each domain varies considerably. In contrast the class II hydrophobins contain loops which are more invariant in length and the spacing between each domain is also invariant. In both class I and II hydrophobins the second and fourth loop is predominantly hydrophobic.

Fig. 4 Schematic representation of putative hydrophobin structures based on intramolecular disulfide linkages determined for cerato-ulmin (Yaguchi et al. 1993). A Class I hydrophobin is exemplified by MPG1 from Magnaporthe grisea and a Class II hydrophobin is represented by cerato-ulmin (CU) from Ophiostoma ulmi. The arrow indicates the site where the N-terminal signal peptide is cleaved to generated the mature protein. Adapted from Kershaw and Talbot 1998.

In fungi hydrophobins are needed to form aerial hyphae, to protect spores from desiccation and they are involved in adhesion. For the Sc3 hydrophobin it was demonstrated that it plays a role in adhesion of fungal walls to hydrophobic surfaces (Wösten et al 1994). Leaf surfaces are coated with a layer of wax, which gives the leaf its water repellent (hydrophobic) property. Therefore, hyphae that are coated with hydrophobins might facilitate infection of the host-plant. Several genes encoding hydrophobins have been cloned from plant pathogenic fungi, notable cerato-ulmin (CU), (Bowden et al. 1994), CRP (Zang et al 1994), HCF-1 (Spanu 1997) and MPG1 (Talbot et al. 1993) which were obtained from Ophiostoma ulmi, Cryphonectria parasitica, Cladosporium fulvum and Magnaporthe grisea, respectively.

The dimorphic pathogen O. ulmi is the causal agent of wilt of elms known as the Dutch elm disease. This vascular pathogen secretes the CU toxin, which is able to cause wilting of the host, indicating that this protein could be a key pathogenicity factor. This idea is opposed by several recent observations. Naturally occurring CU deficient strains have been found that retained their normal pathogenicity, suggesting that CU may not be required for pathogenicity
(Brasier et al. 1995). This view was corroborated by disruption of CU in the aggressive O. novo-ulmi that did not result in a reduced virulence on elm (Bowden et al. 1996). Likewise, overproduction of CU in the less aggressive O. ulmi did not alter its virulence towards elm (Temple et al. 1997). Overproduction of CU, however, revealed other functions for this hydrophobin. In this tree-pathogen relation the fungus depends for its dispersal on the bark beetle. It was therefore interesting to find that yeast-like cells of the CU overproducing strain adhere in larger numbers to the bark beetle, suggesting a relationship between hydrophobicity and the adhesive properties of the fungal surface (Temple et al. 1997). The presence of hydrophobins could be advantageous to the fungus, as the load of infectious propagules carried by the beetle is a major factor in the initiation of infection. Furthermore, yeast-like cells of the overproducing strain are more resistant to desiccation (Temple et al. 1997). This as well could be advantageous to the fungus as desiccation most likely occurs during flight of the beetle. This idea is supported by the observation that the proportion of bark beetles carrying viable spores declines dramatically after a period of flight (Webber and Gibbs 1989).

The hydrophobin HCF-1 has been obtained from the tomato pathogen Cladosporium fulvum. Spores of this pathogen germinate on leafs and enter the plant via stomata in the leaf. In this pathogenic relation hydrophobin HCF-1 might be involved in adherence of the hyphal tube to the leaf surface. A HCF-1 knockout strain was tested on plants and showed no difference in pathogenicity compared to the wild-type strain (Spanu 1998). This indicates that HCF-1 is not important for infection of tomato.

Brutal force to gain access to its host is used by the rice blast fungus Magnaporthe grisea. After germination of conidia on the leave surface, a short germ tube is formed which differentiates at the tip to produce the infective cellular structure, the appressorium. Infection proceeds by tight adhesion of the appressorium to the leaf surface, followed by the build-up of a high internal turgor pressure which allows a penetration peg to break through the cuticle and reach the underlying epidermal cell layer. The hydrophobin MPG1 could be involved in adhesion of the appressorium to the leaf surface, as MPG1 is highly expressed 12 h after infection. This timing coincides with development of the appressoria. Experimental proof for the involvement of MPG1 in infection of rice was obtained by disruption of MPG1 (Talbot 1993). Loss of MPG1 caused a marked reduction of symptoms and lesions. Most significant was the reduced ability of the disruptant strain to form appressoria. In addition carbohydrates, proteins or glycoproteins present in the extracellular matrix support adhesion and enable hyphae to differentiate into infection structures (Mendgen et al. 1996 and Xiao et al. 1994). There is probably a sequence of events in which hydrophobins are important for the first contact after which gel-like material bind the hyphae firmly to the substrate.
Chapter 1

Enzymes involved in cell wall construction

To assemble and expand a cell wall, not only chitin and glucan-synthases, but also hydrolases to break existing bonds and transglycosidases to form new bonds are necessary. Furthermore, glycoproteins that are destined for the cell wall need to be properly processed in order to link them to β-glucans. The polysaccharides, notable glucan and chitin, are subjected to several modifications, such as extension and branching of β-1,3-glucan, deacetylation of chitin and coupling of chitin to β-1,3-glucan.

In *S. cerevisiae* the protein Gas1 is linked to the outside of the plasma membrane via a GPI-anchor, and is thought to be involved in cell wall assembly. Two functions are proposed for Gas1, it might play a role in the retention of β-1,3-glucan and/or β-glucosylated proteins and it might function as a transglycosidase (Ram *et al.* 1998; Popolo and Vai 1999). Elongation and branching of β-1,3-glucan is performed by β-1,3-glucanosyltransferase. The enzymes that perform these modifications have been isolated from *A. fumigatus* (Hartland *et al.* 1996; Mouyna *et al.* 1998). One transferase is responsible for the elongation of β-1,3-glucan chains (Hartland *et al.* 1996). To give an example: a glucan of 11 glucose (G) residues is cleaved by the enzyme releasing G₆ from the reducing end of the substrate molecule. The remaining G₅ is then transferred to another G₁₁ molecule acting as an acceptor, to form the G₁₆ transferase product. In this way products can be produced that increase in size as the initial transferase product can be reused either as donor or acceptor. The second transferase, a Bgl2 homolog from *S. cerevisiae* and *C. albicans*, introduces branches in β-1,3-glucan via β-1,3-/β-1,6- branch points (Mouyna *et al.* 1998). Disruption of this gene revealed no distinct phenotype in *A. fumigatus* or *S. cerevisiae*, suggesting that it is not essential for wall construction. Transferases that are involved in linkage of chitin to β-1,3-glucan are not discovered yet.

Outline of this thesis

The cell wall of *Fusarium oxysporum* f. sp. *lycopersici* is thought to play a role in the interaction with its host plant tomato. Components that make up the outside of the cell wall are interesting in this respect, as they are the first to come into contact with host tissue. It is known from *in vitro* experiments that fungal cell wall components such as oligosaccharides derived from chitin and β-glucan and also glycoproteins interact with the plant. Glycoproteins are the most versatile as they induce or suppress plant defense responses or can be involved in adhesion of the hyphae to plant tissues. The long-term aim of the research described in this thesis was to obtain information on cell wall components from *F. oxysporum* f.sp. *lycopersici* that most likely interact *in vivo* with its host tomato. It was, therefore, necessary to obtain a
basic understanding of the chemical composition and also of the structure of the cell wall of this fungus. As limited information was available about the *Fusarium oxysporum* cell wall experiments were initiated to provide such information (chapter 2). These analyses demonstrated that the outside of this cell wall consists predominantly of glycoprotein, which are exposed to the environment. From this study it became clear that cell wall glycoproteins (CWPs) are tightly associated with the cell wall. In order to investigate this linkage CWPs were released from the cell wall by various enzymatic and chemical treatments. Chapter 3 describes the analysis of CWPs and their linkage to polysaccharides within this wall. This work suggests that *Fusarium* CWPs contain a C-terminal modification that is needed for GPI-anchoring. To provide further evidence for such a view experiments were started to clone a gene that encodes a CWP (chapter 4). Cloning of such a gene would also provide a tool to study the role of this cell wall protein in the interaction of *Fusarium* with its host the tomato plant. The results described in chapter 4 show that the primary translation product of the cloned CWP (FEM1) contains a N-terminal signal sequence, is rich in Ser and Thr residues (potential O-glycosylation sites) and possess a C-terminal signal sequence that has the hallmark of a GPI-anchor cleavage/attachment site. In order to verify that we cloned the right gene a DNA construct was designed to tag the FEM1 protein with histidin residues. The tagged FEM1 protein had an identical molecular weight as the native FEM1 indicating that we cloned the right gene. To obtain evidence for GPI-anchoring of FEM1 a DNA construct was designed to create a C-terminal truncated FEM1. Analysis of cell walls obtained from transformants of *Fusarium* expressing the modified gene showed that C-terminal truncated FEM1 is not incorporated in the cell wall. Hence, FEM1 contains a functional GPI-anchor cleavage/attachment site that is essential for incorporation of this protein into the cell wall. The results described in chapter two to four are discussed in chapter five.

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