Biochemical aspects of the cell wall of Fusarium oxysporum
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CHAPTER 5

GENERAL DISCUSSION

The plant pathogenic fungus *Fusarium oxysporum* f.sp. *lycopersici* is the causal agent of wilt disease in tomato. Infection by this soil-borne pathogen occurs by direct penetration of root epidermal cells or via natural wounds in the root system caused by emerging lateral roots (Beekman 1987; Agrios 1997). After penetration of the root the fungus traverses the root cortex until it reaches the xylem vessels. At this stage the fungus is able to colonize the entire plant. If the host is ineffective in mounting defense responses, the plant succumbs to the fungus.

Inside the plant there is an intimate contact between the fungal cell wall and host tissue. Therefore, it is assumed that the fungal cell wall plays an important role in the interaction with its host. On the one hand, several fungal cell wall components such as glycoproteins or oligosaccharides derived from chitin and glucan, can be sensed by the plant and trigger a defense response (Yoshikawa et al. 1993; Hahn 1996). On the other hand, fungal cell walls possess properties that might be important for a successful colonization of the plant, mediating adhesion to roots, confer resistance to cell wall hydrolytic enzymes (e.g. chitinases and β-1,3-glucanases) and forming a barrier to anti-fungal plant compounds.

Chemical composition of the *Fusarium* cell wall

The composition of the cell wall is not only important to maintain strength and shape of the hyphal tube, but in plant pathogenic species it might also determine the outcome of the interaction with the host. If this is the case, it is conceivable that especially cell wall components exposed at the outside of the cell wall contribute to the interaction with their host plant. Therefore, we set out to determine the structural organization of the *Fusarium* cell wall (Chapter 2). Chemical analysis of this wall revealed several macromolecular structures notably, polysaccharides composed of β-1,3-, β-1,6- or α-1,3-linked glucose (glucan), β-1,4-linked (N-acetyl)glucosamine (chitin) and glycoproteins (Chapter 2 and 3). These polysaccharides are commonly found in cell walls of Ascomycotina (Gooday 1994). Two β-1,3-glucan fractions can be discerned in fungal cell walls, notably an alkali-soluble and an alkali-insoluble fraction (Klis 1994). In the *Fusarium* cell wall the alkali-soluble β-1,3-glucan is branched by monomeric β-1,6-linked glucose residues (Fig. 1) (Bruneteau et al. 1992). Branching of β-1,3-glucan is carried out among others by an extracellular glucosyltransferase...
(Bgl2) that is a major protein in the cell wall of *S. cerevisiae* (Mrsa *et al.* 1993). The branches introduced by Bgl2 appear to be variable in length as is shown for *C. albicans* and *A. fumigatus*. In *C. albicans* Bgl2 introduces a branch in β-1,3-glucan by addition of laminaritrioses via a β-1,6-linkage to a laminarioligosaccharide (Goldman *et al.* 1995). In the filamentous fungus *A. fumigatus* a Bgl2 homolog introduces a branch into the β-1,3-glucan by transfer of laminaribiose units that are linked via a β-1,6- bond (Mouyna *et al.* 1998). Also the β-1,6-linked single-glucose branches in the *Fusarium* β-1,3-glucan may be attached by a Bgl2 homolog, as has been postulated by Wessels (1994). X-ray diffraction studies showed the presence of α-1,3-glucan in the alkali-soluble cell wall fraction. This was a novel compound that had not been demonstrated before in *Fusarium* cell walls. The alkali-insoluble fraction of this cell wall was represented by β-1,3- glucan and chitin (Chapter 2). In yeast, alkali-insolubility of β-1,3- glucan has been shown to result from a covalent link between this polymer and chitin (Mol and Wessels 1987; Kollár *et al.* 1995).

In filamentous fungi, chitin and β-1,3- glucan are important for the structural integrity of the cell wall (Borgia and Dodge 1992) and also determine the shape of the hyphal tube (Gooday 1994). So far, no specific function has been assigned to α-1,3-glucan in filamentous fungi. In the fission yeast *Schizosaccharomyces pombe* α-glucan appears to be essential for cell wall integrity (Hochstenbach *et al.* 1998; Katayama *et al.* 1999). Loss of α-glucan synthase activity in a temperature-sensitive mutant results in cell lysis, whereas mutant cells grown at the semipermissive temperature contain less α-glucan and display aberrant cell morphology (Hochstenbach *et al.* 1998). This indicates that α-glucan is essential for fission yeast morphogenesis. Whether α-1,3-glucan performs such a critical role in filamentous fungi needs to be evaluated experimentally.

**Structural organization of the *Fusarium* cell wall**

Electron microscopic observations revealed a cell wall consisting of an outer electron-dense layer and an inner electron-translucent layer (Chapter 2). The outer layer is thought to be mainly composed of glycoproteins whereas the inner layer is mainly composed of polysaccharides (Fig. 1). Indeed, the proteinaceous nature of the outer layer was confirmed by its sensitivity to proteases. This layer was further analyzed by fluorescence microscopy using the mannose-binding lectin concanavalin A (ConA). The fluorescein (FITC) coupled ConA was bound to the entire hyphal wall showing that mannose is evenly distributed over the outside of the cell wall. Analysis of CWPs from a *Fusarium* spp. revealed that mannose is a constituent of glycan chains attached to these proteins (Jikibara *et al.* 1992 a). We were able to confirm this for *F. oxysporum* as protease treatment of the hyphae reduced the labeling of
the hyphal wall by ConA-FITC. In line with this observation is the affinity of ConA for β-1,3-glucanase extracted cell wall glycoproteins (CWPs) (Chapter 3).

![Diagram](image)

**Fig. 1** Schematic representation of the cell wall of *Fusarium oxysporum*. Etn = ethanolamine, GPI-anchor = glycosylphosphatidylinositol anchor and P = phosphate

Based on the electron translucent appearance of the inner layer we assumed that this layer mainly consisted of polysaccharides. This was investigated by fluorescence microscopy using the lectin wheat germ agglutinin (WGA) that binds specifically to chitin. In untreated hyphae WGA-FITC did not bind to the cell wall, indicating that chitin is not exposed at the outside of the cell wall. Only after protease treatment WGA-FITC was able to bind to the cell wall, suggesting that chitin is covered by a layer of glycoproteins and is therefore a part of the inner...
layer of the cell wall (Fig. 1). Presence of β-1,3-glucan in the inner layer of the cell wall could be inferred from the of X-ray diffraction analysis. Chitin and β-1,3-glucan are present in the alkali-insoluble part of the *Fusarium* cell wall (Chapter 2). As chitin and β-1,3-glucan are thought to be covalently linked it is likely that β-1,3-glucan is also part of the electron-transparent inner layer. The location of α-1,3-glucan within the *Fusarium* cell wall remains to be resolved. For fungi like *Schizopyllum commune* (Wessels et al. 1983), *Neurospora crassa* and *Aspergillus nidulans* α-1,3-glucan is thought to be present in the outer matrix of the wall (Marshall et al. 1997). Electron microscopic studies of the *N. crassa* cell wall with antibodies raised against α-1,3-glucan revealed that this glucan was not only present at the outside of the hyphal wall but also can be found throughout the hyphal wall (Marshall et al. 1997). *Fusarium, Aspergillus nidulans* and *N. crassa* are members of the Ascomycotina. Therefore, it is possible that a similar distribution of α-1,3-glucan exists in the *Fusarium* cell wall.

In conclusion, the *F. oxysporum* cell wall is a bi-layered structure in which the inner layer predominantly consists of polysaccharides and the outer layer is essentially composed of glycoproteins.

**Characterization of linkages between cell wall components**

To understand both the structure and morphogenesis of the cell wall, it is necessary to know how its constituents are organized and cross-linked to each other to yield a fabric strong enough to resist turgor pressure. Cross-linking of various cell wall constituents is best studied for the cell wall of *S. cerevisiae*. In this cell wall covalent linkages exist between chitin and β-1,3-glucan (Mol and Wessels 1987; Kollár et al. 1995). Furthermore, CWPs are either linked via a β-1,6-glucan to β-1,3-glucan or linked via β-1,6-glucan to chitin (Kollár et al. 1997; Kapteyn et al. 1999; Smits et al. 1999). Direct and indirect evidence also indicates that these linkages exist in the cell wall of *Fusarium oxysporum* (this thesis).

**Linkage between chitin and glucan**

A physical link between chitin and β-1,3-glucan in the cell wall of *F. oxysporum* was suggested by the presence of both compounds in the alkali-insoluble cell wall fraction (Chapter 2) (Fig. 1). Evidence for such a link has been obtained from *S. commune* (Wessels et al. 1983) and *S. cerevisiae* (Mol and Wessels 1987; Kollár et al. 1995) cell wall research. In *S. commune* β-1,3-glucan is initially alkali-soluble and during its migration to the subapical part of the hyphal tube it is slowly converted into an alkali-insoluble form (Wessels et al. 1983). Alkali-insolubility of β-glucan was thought to be caused by a covalent linkage with chitin. Evidence for this assumption was obtained by treating yeast cell walls with chitinase that converted the alkali-insoluble β-1,3-glucan into the alkali-soluble form (Mol and Wessels
1987). Analysis of the linkage region between chitin and \( \beta-1,3 \)-glucan in \textit{S. cerevisiae} cell walls showed that the reducing end of a chitin chain is linked via a \( \beta-1,4 \)-bond to the non-reducing end of a \( \beta-1,3 \)-glucan (Kollár et al. 1995) (Fig. 1). An enzyme involved in cross-linking of these polysaccharides has not been found yet.

**Linkage between cell wall glycoproteins and \( \beta \)-glucans**

As observed by electron microscopy, it was not possible to remove the external glycoprotein layer in the cell wall of \textit{F. oxysporum} by a treatment with hot SDS/\( \beta \)-mercaptoethanol (Chapter 2). Therefore, it was assumed that SDS/\( \beta \)-mercaptoethanol resistant CWPs in this cell wall are covalently bound to structural cell wall components, notable \( \beta-1,3 \)-glucan and chitin. In the \textit{S. cerevisiae} cell wall most of these proteins are covalently bound to \( \beta \)-glucan (Rinsum et al. 1991) and more specifically to \( \beta-1,6 \)-glucan (Montijn et al. 1994; Kollár et al. 1997 and Kapteyn et al. 1999). Therefore we investigated whether in \textit{Fusarium} a similar association exists between CWPs and \( \beta \)-glucan (Chapter 3). Release of CWPs by \( \beta-1,3 \)-glucanases provides indirect evidence that these proteins are covalently linked to \( \beta-1,3 \)-glucan (Chapter 3) (Fig. 1). Analyzing these CWPs with antisera that specifically recognize \( \beta-1,3 \)- or \( \beta-1,6 \)-glucan further tested this idea. This Western analyses showed that \( \beta-1,3 \)- as well as \( \beta-1,6 \)-glucan is attached to these proteins. Furthermore, it was demonstrated that the bond between \textit{Fusarium} CWPs and \( \beta-1,3 \)-/\( \beta-1,6 \)-glucan was sensitive to a treatment with aqueous hydrofluoric acid (HF). Release of the \( \beta-1,3 \)-/\( \beta-1,6 \)-glucan by aqueous HF has also been reported for CWPs from \textit{S. cerevisiae} (Kapteyn et al. 1996) and \textit{C. albicans} (Kapteyn et al. 1995). This treatment is known to cleave phosphodiester bonds that are present in GPI-anchors of plasma membrane-linked proteins (Sipos et al. 1995). HF-mediated release of \( \beta-1,3 \)-/\( \beta-1,6 \)-glucan indicates the presence of GPI-anchored CWPs in the cell wall of \textit{Fusarium}. These observations suggest that mycelial cell walls of \textit{Fusarium} may contain a similar structural unit as found in the budding yeast \textit{Saccharomyces cerevisiae}, namely CWP-\( (-P-\rightarrow \beta 1,6\text{-glucan} \rightarrow \beta 1,3\text{-glucan} \)) (Fig. 1).

To obtain evidence for GPI-anchored CWPs, we set out to clone a gene that encodes such a protein (Chapter 4). For this purpose a partial amino acid sequence was obtained from a CWP that was released from the cell wall by aqueous HF. The primary translation product of this gene, designated \textit{FEM1} (\textit{Fusarium} Extra cellular Matrix protein 1), revealed several characteristics that are known for \textit{S. cerevisiae} CWPs (Caro et al. 1997). Interestingly, the C-terminal part of \textit{FEM1} contains a hydrophobic amino acid sequence that resembles a GPI attachment signal (Gerber et al. 1992; Nuoffer et al. 1993). To obtain experimental evidence for the assumption that the hydrophobic C-terminus functions as a GPI-anchor attachment site a C-terminal truncated \textit{FEM1} was created. As a result of this modification \textit{FEM1} could not be
detected in cell walls of *F. oxysporum* expressing the altered *FEM1* (Chapter 4). This strongly suggests that the hydrophobic C-terminus is needed for cell wall incorporation of *FEM1* most likely via a GPI-anchor. In conclusion, the release of *FEM1* by aqueous HF in conjunction with the presence of a GPI attachment signal, supports the concept of CWPs being linked through a GPI-anchor to the β-1,3-/β-1,6-glucan (Fig. 1). Gradually, more data have become available that indicate that GPI-anchoring of CWPs is not only a trait of *S. cerevisiae* CWPs but might be widespread among the Ascomycotina. Besides *FEM1* of *Fusarium* also MPI of *Penicillium marneffei* (Cao et al. 1998) and HYR1 of *Candida albicans* (Bailey et al. 1996) contain putative GPI-anchoring signal peptides. Based on the homology to *FEM1* we also identified a gene of *Neurospora crassa* that encodes a putative CWP containing a GPI-anchor attachment site (Chapter 4). Whether these proteins are linked to a β-1,3-/β-1,6-glucan remains to be resolved.

**Processing of GPI-anchored CWPs**

**Release of GPI anchored CWPs from the plasma membrane**

The final destination of GPI-anchored CWPs is the outside of the fungal cell wall. Therefore, these proteins are most likely transiently incorporated into the plasma membrane to be subsequently converted into a cell wall bound-protein. Secretion of proteins in *Aspergillus niger* (Wösten et al. 1991) and other filamentous fungi (Peberdy 1994) occurs at the tip of growing hyphae only. The cell wall at this part of the hyphal tube evidently allows passage of larger molecules. Due to subapical rigidification of the wall a small time window is available for large
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CWPs to migrate to the outside of the cell wall. As we have shown for *Fusarium*, the major CWPs have a molecular weight of ± 200 kDa (Chapter 3). The estimated permeability threshold of mature (subapical) fungal cell walls is around 20 kDa (Money 1990; Peberdy 1994). Therefore, it is unlikely that these proteins could migrate through the mature cell wall. Transition of the plasma membrane-bound CWP to a cell wall-bound form requires several steps, including release from the plasma membrane and coupling to β-glucans. As not all GPI-anchored proteins are destined for the cell wall, the cell needs to discriminate between GPI-anchored proteins that are retained at the plasma membrane and those GPI-anchored proteins that need to be incorporated into the cell wall. So, how does the cell distinguish between these proteins?

In *S. cerevisiae* a signal that might determine whether a GPI-anchored protein is retained within the plasma membrane has been hypothesized to be a di-basic motif, N-terminal from the GPI attachment site (Caro *et al.* 1997; Vossen *et al.* 1997). GPI-anchored proteins that lack this motif would become incorporated into the cell wall. This concept was tested by introduction of this di-basic motif in the yeast CWP Cwp2. The modified protein was, however, not permanently located into the plasma membrane (van der Vaart 1997).

Cross-linking of GPI-anchored CWPs to β-glucan

Incorporation of GPI-anchored CWPs into the cell wall requires the release of these proteins from the plasma membrane. Enzymes that could release these proteins are phosphoinositid phospholipases. Indeed, for an GPI-anchored cAMP-binding protein of *S. cerevisiae* its release from the plasma membrane has been ascribed to phospholipase activity (GPI-PLC - Fig. 2) (Müller and Bandlow 1993; Müller *et al.* 1996). However, phospholipases are not necessarily involved in the maturation of GPI-anchored CWPs, since a remnant of the GPI anchor is present in the cell wall that lacks inositol. Loss of inositol was demonstrated for the α-agglutinin of *S. cerevisiae* (Lu *et al.* 1994). The GPI structure of this protein was metabolically labeled with [3H] tagged inositol and fatty acids. Cell wall bound α-agglutinin was found to have lost the radioactive labeled compounds. Upon transition of GPI-anchored CWP from the plasma membrane to the cell wall, the GPI-anchor of α-agglutinin must have been processed between the mannan chain and inositol (Fig. 2). Furthermore, a biochemical study of a 100 kDa mannoprotein shows that the phosphoethanolamine and mannose chain of the GPI-anchor are still present in the glucan-bound CWP (Kollár *et al.* 1997). More specifically this has also been demonstrated for the CWP Tip1 (Fujii *et al.* 1999). These observations indicate that the glucosamine-inositol-phosphate-lipid structure is lost from the GPI-anchor attached to the CWP (Fig. 2). Therefore, a glycosidic cleavage rather than a phospholipidic cleavage occur.
Involvement of glycoproteins in the interaction with the plant

As has been demonstrated in Chapter 2 the outer layer of the Fusarium oxysporum cell wall is composed of glycoproteins. These glycoproteins are exposed to the environment and are thought to play a role in the interaction of F. oxysporum with its host the tomato plant. This notion is supported by the fact that fungal glycoproteins are implicated in adhesion of spores and hyphae to plant surfaces (Epstein and Nicholson 1997) and suppression (Basse et al. 1992; Kato et al. 1993) or elicitation (Yoshikawa 1993; Hahn 1996) of plant defense responses.

Suppression of defense responses is best known for the interaction between pea and the fungus Mycosphaerella pinodes (Kato et al. 1993). In this plant-pathogen interaction a protein has been identified as the suppressor molecule. Importantly, this protein has been found in the intercellular space of plant tissue. Although this protein is not cell wall derived it points out that proteins can be a source of molecules that suppress a plant defense response. Not only proteins function as a suppressor. As has been shown in a tomato cell suspension, oligosaccharides derived from yeast invertase also suppress plant defense responses (Basse et al. 1992). Besides the suppressor molecules also elicitor active fragments were obtained from the yeast invertase. A combination of the suppressor active oligosaccharide connected to a peptide backbone result in induction of a defense response in tomato cells, and is therefore an elicitor molecule.

Not only soluble glycoproteins but also cell wall derived glycoproteins possess elicitor activity, which is illustrated by Phytophthora megasperma f.sp. glycinea soybean interaction and Puccinia graminis wheat interaction. In the cell wall of P. megasperma f.sp. glycinea a 42-kDa cell wall glycoprotein is identified as an elicitor. More specific, elicitor activity of this protein resides in a 13-meric oligopeptide (Hahlbrock et al. 1995). Also, in the interaction between wheat and Puccinia graminis an elicitor has been identified. The elicitor activity was isolated from germ tube cell walls and resides in the glycan portion of a 67 kDa glycoprotein (Kogel et al. 1988). In vivo studies show that the glycoprotein elicitor from Puccinia graminis is present in the fungal cell wall during infection of its host plant wheat (Marticke et al. 1998).

Whether FEM1 has a role in the interaction between F. oxysporum and the tomato plant can now be tested. On the one hand FEM1 can be purified due to the His tag attached to this protein (Chapter 4). Subsequently the reaction (phytoalexin production, pH shift in the cell suspension) of a tomato cell suspension can be monitored after being exposed to the purified FEM1. The same type of experiment can also be performed with tomato plants by monitoring the phytoalexin production. On the other hand F. oxysporum transformants that lack the CWP
FEM1 (FEM1 knock out) in their cell wall can be tested for their ability to colonize the tomato plant.

REFERENCES


Peberdy, J.F. (1994) Protein secretion in filamentous fungi - trying to understand a highly productive black box. TIBTECH 12: 50-57


van der Vaart (1997) Identification and characterization of cell wall proteins of *Saccharomyces cerevisiae*. Thesis University of Utrecht, pp 112


Yoshikawa, M., Yamaoka, N. and Takeuchi, Y. (1993) Elicitors: Their significance and primary modes of
action in the induction of plant defence reactions. Plant Cell Physiol. 34: 1163-1173