The role *of* **repellents** *and* **hydrophobins** *in* ***Ustilago maydis***

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The role *of* **repellents** *and* **hydrophobins** *in* ***Ustilago maydis***

De rol van repellents en hydrofobines in *Ustilago maydis*

(met een samenvatting in het Nederlands)

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door
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geboren op 8 april 1954 te Groningen

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CHAPTER 1

General introduction

Ustilago maydis belongs to the heterobasidiomycetes. This fungus is the causal agent of smut in *Zea mays* (maize) and *Euchleana mexicana* (Mexican teosinte) (Figure 1A; 2B). The ability to infect maize makes *U. maydis* both feared and valued. Although yield losses are generally below 2%, the disease has a severe economical impact worldwide. Losses represent billions of dollars a year, which is partly explained by the fact that *U. maydis* mainly infests sugar maize, which has a higher sales prize than dent corn. Moreover, fouling of the harvest by spores of *U. maydis* leads to export loss due to quarantine restrictions (Martínez-Espinoza *et al*., 2002).

Infested corncobs display large kernels consisting of plant tumor tissue, which are filled with fungal mycelium and black spores (Figure 2B). *U. maydis* does not produce toxins during infection. Consumption of infected plants or cobs thus does not pose a health risk for humans and animals. In fact, sweet corn invaded by *U. maydis*, called huitlacoche or the Mexican truffle, has been consumed since ancient times in Mexico and is nowadays also considered a delicacy in the USA (Figure 1B). Huitlacoche is even registered as an official crop in some states of the USA. The galls can be used in various dishes and have a very rich mushroom-like taste. In research, *U. maydis* is considered an important model organism to study fungal pathogenicity and fungal dimorphism.

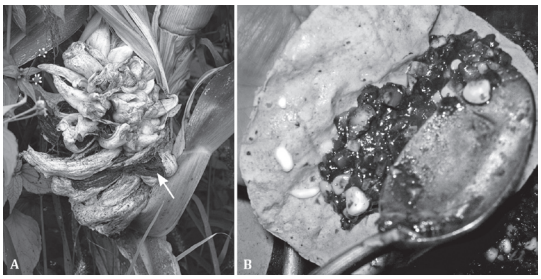

Figure 1: (A) Tumors in a corncob infected by *U. maydis*. The galls break open (indicated by an arrow) resulting in the release of large numbers of black pigmented spores. (B) Dish prepared with huitlacoche.

The life cycle of *U. maydis*

In its yeast-like form, the dimorphic fungus *U. maydis* grows saprophytic and is haploid. Upon mating of two compatible sporidia, a dikaryon is formed that grows filamentous and which can infect the plant. Fusion of haploid cells is regulated by the *a* mating type locus, which is bi-allelic. Both the *a1* and *a2* variants encode precursors of lipopeptide pheromones and receptors for the pheromones of the opposite mating type. The genes encoding the pheromone precursors are called *mfa1* and *mfa2*, whereas the receptors are encoded by *pra1* and *pra2*, respectively. The receptors enable *U. maydis* yeast-like sporidia to sense a partner of the opposite mating type (Bölker *et al*., 1992, Spellig *et al*., 1994). Upon pheromone recognition, cells form conjugation tubes. These tubes grow towards the compatible partner, eventually resulting in fusion of the tubes (Snetselaar *et al*., 1996). The fusion cell harbors the nuclei of both partners. These nuclei do not fuse and the resulting

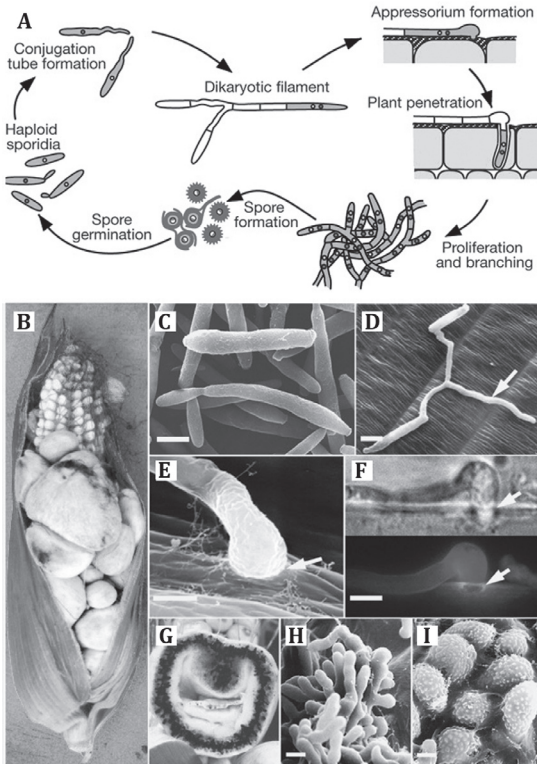

Figure 2: (A) Developmental stages in the *U. maydis* life cycle. (B) Tumor formation in maize. (C) Scanning electron microscopy (SEM) image of haploid sporidia. (D) SEM image of mated sporidia on plant epidermis; arrow denotes dikaryotic filament. (E) SEM image of appressorium; arrow marks entry point. (F) Top, differential interference contrast image of appressorium; bottom, epifluorescence image of fungal cell wall stained with calcofluor white and endocytotic vesicles stained with FM4-64. The bright ring indicates active secretion and endocytosis at the fungus–plant interface; arrows indicate the penetration point. (G) Black teliospores visible in tumor section. (H) SEM image of sporogenous hyphae and early stages of spore development. (I) SEM image of ornamented teliospores. Scale bars represent 5 µm. Figure copied from Nature: Kämper *et al.* (2006), with permission from the publisher.

filament is therefore dikaryotic.

Post-fusion development of the dikaryotic cell depends on the multi-allelic *b* locus. Only when cells harbor different alleles of the *b* mating type locus a stable dikaryon is formed. At least 30 different *b* alleles exist, each encoding a bE and bW homeodomain protein. The bE and bW proteins can form heterodimers when they are derived from different alleles (Kämper *et al*., 1995). Such heterodimers function as transcriptional activators and repressors. As a result of their activity, a long filament is formed, which is capable to initiate invasion of the host. It should be noted that filament formation not only results from mating. Haploid (monokaryotic) strains can display filamentous growth under conditions that cause low cAMP levels (Gold *et al*., 1994, Ruiz-Herrera *et al*., 1995). Such conditions probably include environmental cues and stress, like low pH, nitrogen source, nitrogen concentration, cold-shock (Ruiz-Herrera & Martinez-Espinoza, 1998, Ruiz-Herrera *et al*., 1995) and the presence of lipids (Klose *et al*., 2004). However, monokaryotic filaments are not able to invade the host plant. In contrast, the dikaryotic hyphae enter the plant by means of an appressorium that manifests itself as a swelling of the hyphal tip (Figure 2E). In nature, invasion occurs through stomata and silks, the male floral organ of maize, or directly, by penetrating the epidermis (Snetselaar & Mims, 1992). Unlike the pathogenic fungus *Magnaporthe grisea* (de Jong *et al*., 1997), these appressoria do not build up a turgor pressure that enables the fungus to enter the plant by mechanical force. Instead, the appressorium seems to be instrumental to redirect growth by 90° to be able to invade the plant.

Upon invasion through the epidermis, the cell walls of the host are broken, but the plasma membrane is left intact (Snetselaar & Mims, 1992, Snetselaar, 1993). Inside the plant, *U. maydis* mainly grows in the intracellular space. Initially, hyphae do not branch and cytoplasm is only maintained in the tip region. As a result, subapical parts are left empty. Eventually, however, hyphae start to branch. At this stage, filaments become completely filled with cytoplasm. The fungus induces formation of the characteristic large tumors in the meristemic tissue of the host, which are most conspicuous in kernels of the cob. Inside these tumors or galls, the fungus proliferates, giving rise to formation of black pigmented teliospores. These diploid spores are dispersed in the environment. After germination they undergo meiosis, resulting in formation of haploid sporidia (For a summary of the stages in the life cycle of *U. maydis* see Figure 2A). As mentioned, sporidia have to fuse with a compatible partner to become pathogenic. However, haploid strains have been constructed that are pathogenic independent from mating. Such strains (e.g. SG200) carry a chimeric *b* locus consisting of *b*E and *b*W alleles of different mating types (Bölker *et al*., 1992).

Only part of the life cycle can be accomplished when *U. maydis* grows on artificial media. In liquid media mating does not occur. In contrast, mating and subsequent formation of filaments, including aerial hyphae, do occur on medium containing activated charcoal. This can also be accomplished by growing *U. maydis* in a water droplet placed on a hydrophobic substrate such as Teflon. Such substrates mimic the hydrophobic surface of the plant leaf (Chapter 3). However, a mycelial network and teliospores are not formed on artificial media and in a water droplet. For this, yet undefined plant signals are needed.

Genetic control of filament formation and pathogenicity

The shift from a non-pathogenic yeast-like sporidium to a pathogenic filamentous dikaryon is mediated by complex signaling pathways. Both self and non-self cues lead to activation of signaling cascades, of which many components have been described and analyzed (Kahmann & Kämper, 2004). Two main cascades exist (Figure 3) that show high levels of interplay (Lee *et al*., 2003, Feldbrügge *et al*., 2004). The cAMP dependent PKA (protein kinase A) pathway is activated by environmental signals such as pH, nitrogen source, nitrogen limitation, and exposure to air. Activation triggers release of the α-subunit of the G-protein, Gpa3 which, through action of Uac1, results in synthesis of cAMP. Binding of cAMP to the PKA complex, consisting of regulatory subunits of Ubc1 and catalytic subunits of Adr1 (Gold *et al*., 1994, Gold *et al*., 1997) affects downstream enzymes and transcription factors. The other pathway is a MAP kinase cascade (Müller *et al*., 2003). Through this cascade, involving MAPKKK Kpp4 and MAPKK Fuz7, two MAP kinases, Kpp2 and Crk1, can be triggered. Kpp2 is activated upon pheromone induction (Müller *et al*., 1999),

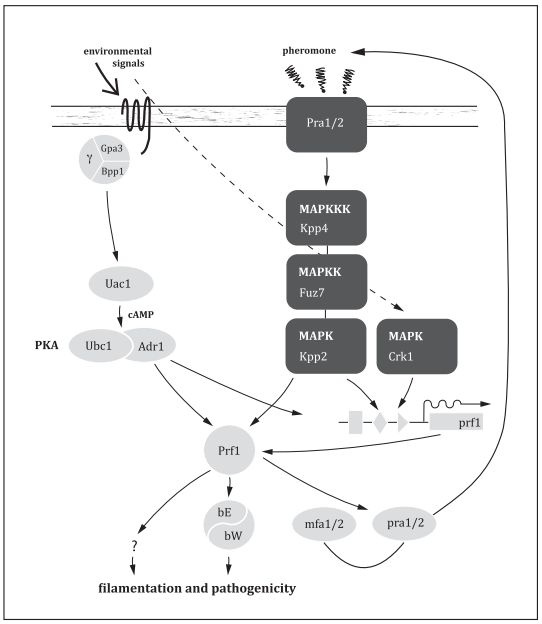

Figure 3: Signaling pathways involved in *U. maydis* development. Pheromone response and environmental signals trigger the PKA pathway and the MAP-kinase cascade, resulting in filament formation and pathogenic development.

whereas Crk1 is likely activated in reaction to nutritional cues (Garrido & Pérez-Martín, 2003, Garrido *et al*., 2004). Both PKA and MAPK pathways activate Prf1 (Hartmann *et al*., 1999). This pheromone response factor is post-translationally activated, while its gene is transcriptionally regulated by autoregulation, pheromone induction and nutrition (Brefort *et al*., 2005, Hartmann *et al*., 1996, Hartmann *et al*., 1999). The transcription factor Prf1 regulates expression of the genes in the *a* and *b* mating type loci (Hartmann *et al*., 1999) and is thus a main regulator of the fusion and post-fusion events in *U. maydis.*

Regulation of the cell cycle is important in development of *U. maydis.* Prior to cell fusion, cells arrest in G2. Probably, this is to synchronize the cell cycles of the partners. After fusion, but prior to invasion, cells also arrest in G2. At this point plant signals are needed to initiate further propagation (García-Muse *et al*., 2003). The G2-arrested dikaryotic hyphae grow at their tip and leave empty, septated compartments behind. The initial growth mode in planta is similar. Two complexes of a cyclin-dependent protein kinase and a cyclin regulate transition from G2 to the M phase. The cyclin-dependent protein kinase Cdk1 and the mitotic cyclin Clb2 form one complex, whereas the second complex consists of Cdk1 and Clb1 (Flor-Parra *et al*., 2006). G2 cell cycle arrest results from down-regulation of *clb1*. The transcriptional regulator Biz1 is responsible for this down-regulation. Biz1, the *b*-dependent zinc finger protein, is expressed post-fusion and its expression depends on the presence of an active bE/bW heterodimer. Haploid strains that over-express *biz1* show elongated growth, and resemble dikaryotic filaments in G2 arrest 24 h after induction (Flor-Parra *et al*, 2006). On the other hand, deletion of *biz1* results in a-virulence. Appressorium formation and hyphal proliferation in planta are affected in such a mutant strain (Flor-Parra *et al*., 2006).

The bE/bW heterodimer acts by binding to *b* binding sequences (*bbs*) in the promoter region of genes (Brachmann *et al*., 2001, Romeis *et al*., 2000). So far only

few genes (Brachmann *et al*., 2001, Kahmann & Kämper, 2004, Romeis *et al*., 2000, Scherer *et al*., 2006) were shown to be direct targets of bE/bW. However, many other genes are regulated indirectly by *b*. Among these are the first genes described as *b*-dependent; *eg1*, (Schauwecker *et al*., 1995), that encodes an endo-glucanase, and *rep1* (Wösten *et al*., 1996) and *hum*2 (Bohlmann *et al*., 1994) that encode cell wall proteins. Recently, over 300 so-called *b*-dependent genes were indentified by microarray analysis, many of these genes are involved in the release of cell-cycle arrest following plant invasion (Scherer *et al*., 2006).

The role of hydrophobins in filamentous fungi

As mentioned above, *hum2* and *rep1* were among the first genes that were identified as being (indirectly) regulated by the bW/bE heterodimers. These genes encode a hydrophobin and a hydrophobin-like protein, respectively. Hydrophobins have not been found in yeasts but generally occur in filamentous fungi (Wösten & Wessels, 1997). In these fungi they fulfill a wide variety of functions (Wösten, 2001). Hydrophobins enable hyphae to grow out of a moist environment into the air. Moreover, they make fungal structures hydrophobic. This is essential to provide stability to aerial structures such as aerial hyphae, to allow spore dispersal by wind or insects, to enable gas exchange via air channels in mushrooms, and to mediate attachment of fungi to hydrophobic surfaces such as that of a host. The latter is important in pathogenic and mutual beneficial symbiotic interactions. Hydrophobins were discovered in the mushroom forming fungus *Schizophyllum commune* in a screen for genes that are highly expressed during aerial growth. *SC3* was found to be highly up-regulated during formation of aerial hyphae, whereas *SC1* and *SC4* became highly active during formation of fruiting bodies. During these stages, their mRNA-levels reach 1-4 % of the total mRNA (Dons *et al*., 1984, Mulder & Wessels, 1986). Because of their high expression, it was decided to sequence *SC1*, *SC3* and *SC4*. Remarkably, these genes belonged to a new gene family encoding small secreted proteins (Schuren & Wessels, 1990). These proteins contain eight conserved cysteine residues and a relative high number of hydrophobic residues. The latter explains why these proteins were dubbed hydrophobins (Wessels *et al*., 1991ab). Wessels distinguished class I and class II hydrophobins based on the spacing of the cysteine residues, the hydropathy patterns and solubility characteristics (Wessels, 1994). Class I hydrophobins are present in both ascomycetes and basidiomycetes, whereas class II hydrophobins only occur in ascomycetes (Wösten, 2001). The Class II hydrophobins are beyond the scope of this Chapter and for these proteins I refer to Linder *et al*. (2005).

The class I hydrophobin SC3 of *S. commune* is the best studied class I hydrophobin. Experimental evidence suggests that other class I hydrophobins behave similarly (Wösten & de Vocht, 2000). SC3 self-assembles at hydrophilic-hydrophobic

interfaces such as those between water and air, water and oil and water and a hydrophobic solid such as Teflon (Wösten *et al*., 1993; 1994ab; 1995). During the self-assembly process, the water-soluble form of SC3 (Wang *et al*., 2004) organizes itself into amyloid fibrils (Wösten & de Vocht, 2000, Butko *et al*., 2001). Self-assembly proceeds via intermediate states called the α-helical form and the β-sheet 1 state (de Vocht *et al*., 1998; 2002). The amyloid fibrils of SC3 form an amphipathic protein film that has a characteristic ultrastructure consisting of a mosaic of 10 nm wide parallel rods. This mosaic is also known as the rodlet layer. The side of the protein film that is exposed to the water is highly hydrophilic (water contact angle of about 40 degrees), whereas the side exposed to the air, an oil, or a hydrophobic solid is extremely hydrophobic (water contact angle of about 110 degrees; which is similar to that of Teflon) (Wösten *et al*., 1993; 1994a; 1995). This explains why self-assembled SC3 is so highly surface-active. With a lowering of the surface tension to as low as 24 mJ m^{-2}, it is the most surface-active molecule that has been identified in nature (Wösten *et al*., 1999). The four disulfide bridges in SC3 prevent self-assembly in an aqueous environment such as within the secretory pathway (de Vocht *et al*., 2000). This explains why cysteine residues are so highly conserved in hydrophobins.

Inactivation of *SC3* in *S. commune* affected formation of aerial hyphae and attachment to hydrophobic surfaces (van Wetter *et al*., 1996, Wösten *et al*., 1994a). These observations combined with immuno-localization and the properties of SC3 led to a model of formation of aerial hyphae (Wösten *et al*., 1994b; 1999) as well as to a model for attachment of fungi to hydrophobic surfaces (Wösten *et al*., 1994a). These models will now be described, starting with the formation of aerial hyphae (Figure 4A). Germination of a spore results in a vegetative mycelium. This mycelium initially does not express *SC3*. At this stage, hyphae cannot grow into the air simply because the water surface tension acts as a barrier. The *SC3* gene is induced after a certain mass of mycelium is formed that can support growth of aerial structures. The water-soluble form of the hydrophobin is secreted into the aqueous substrate. It forms an amphipathic protein film at the medium-air interface, which dramatically lowers the water surface tension. As a result, hyphae can grow into the air. These hyphae continue secreting SC3. These molecules can not diffuse into the medium. Instead, they accumulate at the interface between the air and the aqueous cell wall. As a result, SC3 self-assembles providing the hypha with a hydrophobic coating. This coating prevents the hypha to fall back into the substrate, for instance during rain. The model of hyphal attachment is essentially the same (Figure 4B). Hyphae that grow over a hydrophobic solid secrete SC3. These molecules assemble at the interface between the solid and the cell surface. In this way, the incompatible surfaces that make up the interface are bridged, thus gluing the hypha to the solid.

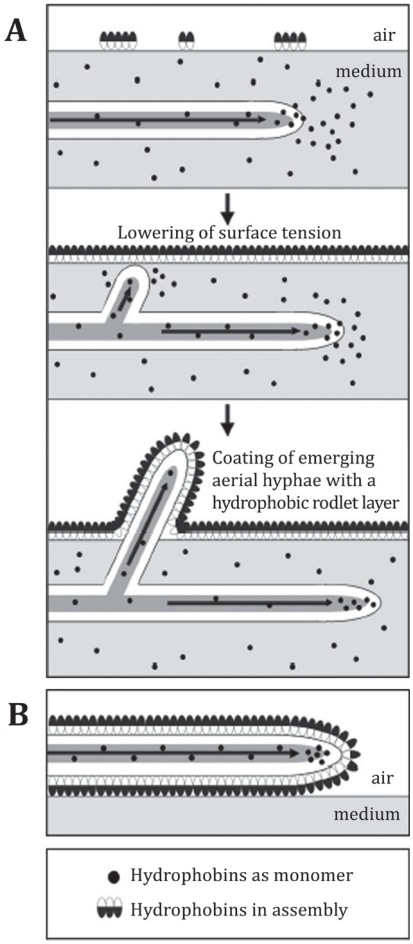

Figure 4: (A) The *SC3* gene is activated when *S. commune* has formed a feeding substrate mycelium. As a result, hyphae secrete the water-soluble form of the SC3 hydrophobin into the environment. At the medium-air interface, SC3 assembles into an amphipathic membrane. This dramatically lowers the water surface tension, enabling hyphae to breach the interface to grow into the air. SC3 secreted by aerial hyphae cannot diffuse into the medium. Instead, it assembles at the interface between the hydrophilic cell wall and the air. This renders the aerial hyphae hydrophobic, preventing them to fall back under wet conditions. (B) Attachment of hyphae of *S. commune* to a hydrophobic substrate (e.g. Teflon) is mediated by SC3. The interface between the hydrophilic cell wall of the fungus and the hydrophobic substrate induces the formation of an amphipathic membrane. This membrane bridges these two incompatible surfaces, thus gluing them together (Taken from de Jong, 2006).

Inactivation of the *MPG1* hydrophobin gene of *Magnaporthe grisea* resulted in spores that lacked a rodlet layer (Talbot *et al*., 1993; 1996). In addition, the mutant strain did not attach to a hydrophobic solid and appresoria were not formed. The latter could be complemented by adding cAMP (Soanes *et al*., 2002). From this it was concluded that attachment of the fungus to a hydrophobic solid (e.g. that of the host) renders a signal that induces the formation of the appressorium. The MPG1 hydrophobin would thus have three functions in *M. grisea.* It coats spores, it attaches the fungus to the hydrophobic surface of the host to enable mechanical penetration; and it provides signals that the fungus grows over the host surface, resulting in the activation of appressorium formation. This result suggests that hydrophobins are multifunctional proteins that are not tailored to fulfill specific functions. Indeed, various hydrophobin genes could complement rodlet formation at the surface of spores of *M. grisae* in which the *MPG1* hydrophobin gene was inactivated (Kershaw *et al*., 1998). However, biophysical characterization and genetic studies have revealed that class I hydrophobins probably did evolve to fulfill specific functions. Like SC3 (see Text box 1), SC4 of *S. commune* and ABH1 and ABH3 of the white button mushroom *Agaricus bisporus* were purified (Lugones *et al*., 1996; 1998, van Wetter *et al*., 2000). Upon self-assembly, they form a protein film with an air-exposed side similar in hydrophobicity as SC3. However, the hydrophilicity at their water-exposed side varies (water contact angles between 45 and 71 degrees). Moreover, it was shown that SC3 and SC4 have different lectin-like activities (van Wetter *et al*., 2000). The different properties of SC3 and SC4 explain why introduction of *SC4*

behind the *SC3* promoter in a *SC3* mutant strain could only partially complement for the absence of SC3. Although formation of aerial hyphae was restored, only a partial complementation was observed for hyphal attachment (van Wetter *et al*., 2000). The latter was explained by proposing that the sugar binding activity of SC3 is adapted to the cell surface of aerial hyphae of *S. commune,* whereas SC4 would have more affinity to the cell surface of hyphae within the fruiting bodies. Here, it provides air channels with a hydrophobic lining preventing water to penetrate. In this way, the mushroom ensures gas exchange (Lugones *et al*., 1999, van Wetter *et al*., 2000).

The role of repellents and hydrophobins in the life cycle of *U. maydis*

The purification procedure to isolate hydrophobins from cell walls of *S. commune* (see Text-box 1) was adopted to isolate hydrophobins from *U. maydis* (Wösten *et al*., 1996). To this end, proteins were compared that were extracted from cell walls of the diploid strain FBD11 (*a1a2b1b2*) displaying filamentous growth on charcoal plates and the nearly isogenic strain FBD11-21 (*a1a2b2b2*) growing yeast-like on the same medium. An abundant peptide was found in the extract from FBD11, which was absent in the extract of FBD11-21. This peptide, called Rep1-2, ran at an apparent molecular weight of about 8 kDa. The N-terminal sequence of the peptide was determined and used to isolate the *rep1* gene. Remarkably, *rep1* did not encode a hydrophobin. Instead, it encodes a 652 aa pre-pro-protein, which consists of a signal sequence for secretion and 12 repeated sequences (Figure 5A). A Kyte-Doolittle hydropathy plot revealed a repeating pattern of alternating hydrophilic and hydrophobic regions (Figure 5B). Each repeat, with the exception of the 11th and 12th repeat, ends with Kex2-like protease recognition sequence. Processing in the endoplasmic reticulum leads to 10 small amphipathic peptides with a size ranging between 34-55 amino acids and one larger peptide of 229 aa (Figure 5B). These processing products were collectively called repellents. Rep1-2 is one of these repellents. Inspection of the SDS-PAA gels, however, did not reveal candidate bands that could represent the other peptides. Therefore, the purification protocol was adapted. Proteins in the TFA extract of SDS-

Text-box 1:
Purification of class I hydrophobins

Self-assembled class I hydrophobins are highly insoluble. Their protein films do not dissociate in most aqueous and organic solvents. It can only be dissolved in highly concentrated formic acid or TFA (de Vries *et al*., 1993, Wessels *et al*., 1991ab). These properties were used to develop a protocol to purify SC3 and other class I hydrophobins from a fungal mycelium (Wessels, 1997, Wösten *et al*., 1993). A homogenate of mycelium is first extracted with 2% SDS at 100 °C. After washing with water and drying, the cell wall fraction is extracted with TFA. The dissociated hydrophobin molecules are taken up in 60% ethanol after removing the TFA with a stream of nitrogen gas. After centrifugation, the ethanol is removed from the supernatant by dialysis, resulting in an aqueous hydrophobin solution (Wösten *et al*., 1993). These water-soluble molecules are capable again of self-assembly at a hydrophilic-hydrophobic interface. Similarly, hydrophobins can be purified from the culture medium of static or liquid shaken cultures. In this case, electro-bubbling or rapid shaking is used to assemble all hydrophobin in solution. Assembled hydrophobin is then pelleted by centrifugation. After drying, the pelleted material is treated with TFA, and taken up in water after a 60% ethanol step.

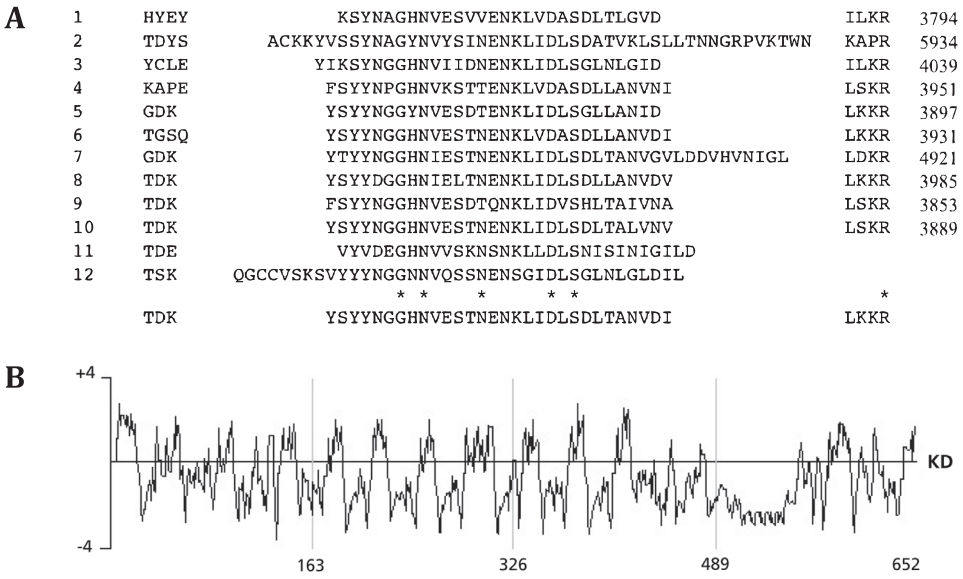

Figure 5: The *rep1* gene encodes a pre-pro-protein that is processed in the secretory pathway into 10 peptides with a length of 34-55 amino acids and one larger peptide of 229 amino acids. **(A)** Alignment of the repeated sequences contained in the Rep1 pre-pro-protein and the deduced consensus sequence of these peptides (Wösten *et al*., 1996). The KR sequences at the C-terminus represents a typical Kex2 recognition site (Fuller *et al*., 1988). The Kex2 protease in *U. maydis* may also recognize PR residues (Park *et al*., 1994) as present in Rep1-2. Residues indicated with an asterisk are conserved. (B) Kyte-Doolittle hydropathy plot of Rep1 (Kyte & Doolittle, 1982).

treated cell walls were taken up in 50% formic acid, and immediately analyzed by mass spectrometry after separation by HPLC. In this way, all repellents were detected in the cell walls of FBD11 except for Rep1-3, Rep1-6 and the 229 aa cleavage product. From the fact that the repellents were found in cell walls of the FBD11 strain but not in those of FBD11-21 it was concluded that at least the *b* mating type locus was involved in the expression of *rep1* (Wösten *et al*., 1996). Using diploid strains that were either heterozygous for the *a* or *b* mating type loci it was concluded that both mating type loci affect expression of *rep1*. As a result, *rep1* is 50-fold up-regulated in the dikaryon compared to the haploid sporidia. Brachmann *et al*. (2001) confirmed that *rep1* is under (indirect) control of the bW/bE heterodimers by using a haploid strain in which compatible *b*-genes were placed under control of an inducible promoter. This study showed that *rep1* was highly expressed 5 h after induction.

Haploid strains in which *rep1* was inactivated did not display an apparent phenotype. In contrast, a cross between such strains resulted in colonies with only few aerial hyphae. Moreover, surface hydrophobicity of the colony was severely reduced (Wösten *et al*., 1996). This phenotype is similar to that of the *SC*3 deletion strain of *S. commune* (van Wetter *et al*., 1996, Wösten *et al*., 1994). In contrast to deletion of the *MPG1* hydrophobin gene of *M. grisea*, *rep1* deletion had no effect

on pathogenicity. These data suggest that repellents have partially replaced hydrophobins in *U. maydis.* Alternatively, repellents might function indirectly for instance by anchoring hydrophobins to the cell wall (Wösten *et al*., 1996). The latter could not be excluded since two hydrophobins were identified in the genome sequence of *U. maydis* (see **Chapter 3**).

Scope of this thesis

The expression and function of repellents and hydrophobins of *U. maydis* were studied in this Thesis. **Chapter 2** describes an in situ hybridization protocol that makes use of peptide nucleic acid (PNA) probes. The system was set up in *S. commune* using probes for 18S RNA and *SC3* mRNA. It was shown that all hyphae at the periphery of a colony of *S. commune* express *SC3*, indicating that they all contribute to secretion of SC3 in the medium. The fact that 18S rRNA was detected at higher levels in the apical compartment of hyphae indicates that this compartment is more active in protein synthesis than subapical compartments. In contrast to *SC3* mRNA, 18S rRNA was not detected at the extreme apex. These data show that RNA is not evenly distributed in hyphae of *S. commune*. In **Chapter 4** the in situ hybridization protocol was successfully adopted to detect 18S rRNA and *rep1* mRNA in *U. maydis*. mRNA of *rep1* was detected in filaments but not in yeast cells, which was confirmed using GFP as a reporter. Moreover, it was shown by in situ hybridization that *rep1* mRNA, but not 18S rRNA, was more abundant in the tip of filaments when compared to sub-apical regions. This suggests that *rep1* mRNA is actively transported to the region where its encoded protein is expected to be released into the environment.

In **Chapter 3** the hydrophobin genes *hum2* and *hum3* are described. Gene *hum2* encodes a typical class I hydrophobin, while *hum3* encodes a hydrophobin domain which is preceded by 17 repeat sequences with predicted KEX-2 processing sites. Gene *hum*2 was expressed during aerial growth but expression of *hum*3 could not be detected. Deletion of *hum*2 but not *hum*3 affected aerial growth and surface hydrophobicity in a cross between compatible haploid strains. However, inactivation of *rep1* had a much more dramatic effect on aerial growth and wettability of the colony surface. It was also shown that *rep1* but not the hydrophobin genes are involved in attachment of hyphae to a hydrophobic solid. This led to the conclusion that repellents have replaced hydrophobins in *U. maydis* in attachment and formation of aerial hyphae.

In **Chapter 4 it** is shown that a synthetic Rep1-1 peptide assembles into amyloid fibrils. These fibrils reduced the water surface tension to as low as 36 mJ m^{-2}. ThT staining showed that amyloid fibrils are present at the surface of filaments and yeasts cells that had been exposed to air. No staining was observed when yeast

cells were grown in liquid medium. In contrast, yeast cells in liquid medium did stain when *rep1* was expressed from the constitutive *O2tef* promoter. This shows that repellents form amyloids in vivo in the absence of a water-air interface. Interestingly, hyphae from a *rep1* deletion strain also stained with ThT. This indicates that other proteins also have the capacity to form these fibrils in the cell wall.

In **Chapter 5** the *rep1* deletion strain was used to study changes in gene expression during aerial growth. Microarray analysis revealed that only 31 genes had a fold change in expression ≥ 2. Twenty-two of these genes were up-regulated and half of them encode small secreted proteins (SSP's) with unknown functions. Seven of the SSP genes and two other genes that are over-expressed in the *Δrep1* strain encode secreted cysteine-rich proteins (SCRP's). Such proteins may represent apoplastic effectors that interact with the environment. The SCRP gene um00792 showed the highest up-regulation in the *rep1* mutant strain. Using GFP as a reporter, it was shown that this gene is over-expressed in the layer of hyphae at the medium-air interface. The results in this chapter show that only minor changes occur in the expression profile when *U. maydis* forms aerial structures. This contrasts the situation in *Schizophyllum commune* and *Streptomyces coelicolor*.

In **Chapter 6** the results are summarized and discussed.

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CHAPTER 2

In situ hybridization in filamentous fungi using peptide nucleic acid probes

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ABSTRACT
Fluorescent DNA and peptide nucleic acid (PNA) probes were used for in situ hybridizations in colonies of *Schizophyllum commune* and *Aspergillus niger*. DNA probes for 18S rRNA did not diffuse through the cell wall after mild chemical fixation. After permeabilizing the cell wall with lysing enzymes or slow freezing and embedding, hybridization was still poor and not reproducible. In contrast, PNA probes did diffuse through the cell wall after mild chemical fixation and reproducible fluorescent signals were obtained. The rRNA signal was most intense in the apical compartment of hyphae of *S. commune*. Within this compartment, the signal was lower at the extreme apex. Apparently, ribosomes are unevenly distributed in hyphae. In *S. commune*, the mRNA of the *SC3* gene was also detected with a PNA probe. The ratio between 18S rRNA and *SC3* mRNA signals were variable between hyphae and their compartments. This is the first report of using PNA probes for in situ hybridization of mRNA in fungi. The method provides a powerful tool to study gene expression.

INTRODUCTION
Filamentous fungi colonize moist substrates by forming a mycelium consisting of hyphae that extend at their apices, while branching sub-apically. At a certain moment, hyphae escape the substrate to form aerial hyphae and aerial reproductive structures, the most conspicuous being the fruiting bodies (e.g. mushrooms and brackets). Aerial structures are fed by the vegetative colonizing mycelium. This is facilitated by a continuous cytoplasm resulting from perforated septa that separate the hyphal compartments.

Hardly anything is known about gene expression in the vegetative colonizing mycelium at the hyphal and compartment level. Gene expression has been studied in mushrooms by in situ hybridizations using DNA probes (Peñas *et al*., 1998; de Groot *et al*., 1999). The cell wall prevents diffusion of the DNA probe into the hypha. Therefore, in situ hybridizations were performed on sections of a few micrometers thickness. However, only part of the hyphae will be accessible for the probe after sectioning. Moreover, due to the length/width ratio, longitudinal sections of whole hyphae are almost impossible to obtain (Ruiters & Wessels, 1989). As an alternative to sectioning, the cell wall can be removed with lytic enzymes as is done in yeasts (Chartrand *et al*., 2000). However, hyphal integrity is lost upon protoplasting. Therefore, cell walls were permeabilized by brief treatments with cell wall-lytic enzymes (Ruiters & Wessels, 1989).

Peptide nucleic acid (PNA) probes have been used to detect rRNA in situ in bacteria and yeasts (Worden *et al*., 2000; Stender *et al*., 2001; Perry-O'Keefe *et al*., 2001; Rigby *et al*., 2002) and mRNA in mammalian cells (Yokomori *et al*. 2001, Larsen *et*

al., 2003). PNA probes are synthetic DNA mimics with a peptide backbone consisting of repetitive units of *N*-(2-aminoethyl)glycine (Egholm *et al*., 1993; Nielsen *et al*., 1994). The synthetic backbone of PNA results in a more rapid and stronger binding during hybridization with complementary strands of DNA (Egholm *et al*., 1993). Moreover, PNA is not degraded by nucleases or proteases (Demidov, 1994) and due to its relative hydrophobic character it penetrates the yeast cell wall following mild fixation (Stender *et al*., 2001; Rigby *et al*., 2002).

In this study fluorescent PNA probes were used for in situ hybridizations in filamentous fungi. We show for the first time that in this way mRNA can be detected without permeabilization of the cell wall and enhancement of the signal. This enables easy and reproducible monitoring of gene expression.

MATERIALS AND METHODS

Strains and growth condition

Aspergillus niger N402 (Bos *et al*., 1988) and the co-isogenic *Schizophyllum commune* strains 4-40 (*MAT*A43*MAT*B43, CBS 340.81) and 72-3 were used in this study. The latter strain is a derivative of 4-39 (*MAT*A41*MAT*B41, CBS 341.81) that contains a disrupted *SC3* gene (Wösten *et al*., 1994a; van Wetter *et al*., 1996). *A. niger* and *S. commune* were grown in a thin agarose layer or in sandwiched cultures to maintain the hyphal orientation during in situ hybridizations. Cultures were placed in water-saturated containers using minimal medium with glucose as carbon source. Minimal media for *A. niger* and *S. commune* were described by de Vries *et al*. (2002) and Dons *et al*. (1979), respectively. *A. niger* was grown at 30 °C, while *S. commune* was grown at 25 °C . To obtain the thin agarose layer, 0.2 ml minimal medium (1.5 % agarose; 80 °C) was pipetted onto a microscope slide and immediately covered with a cover slip (24 x 50 mm). After solidifying, the cover slip was removed. The resulting layer of agarose (0.17 mm) was inoculated with a piece of mycelium and covered with a cellophane membrane (unlaquered, Versteden Papier, Tilburg, the Netherlands) wetted in minimal medium. For sandwiched cultures (Wösten *et al*., 1991) a perforated polycarbonate (PC) membrane (diameter 76 mm, pore size 0.1 µm; Osmonics, GE Water Technologies) was placed on the surface of 20 ml minimal medium (1.5 % agar) solidified in a 9 cm Petri dish. 2 ml of 1.25 % agarose (80 °C) was spread over the surface of the PC membrane resulting in an agarose layer of 0.45 mm thickness. After solidifying, a small piece of mycelium was inoculated at the center of the agarose layer, after which it was topped with a second PC membrane.

In situ hybridization with PNA probes

Cultures of *A. niger* and *S. commune* were fixed for 2 h at 20 °C with freshly prepared 4 % paraformaldehyde in PBS, pH 7.4. After washing with buffer, cultures were placed overnight at –20 °C in excess 50 % ethanol (v/v). Fixed colonies stored for

up to 3 months at –20 °C were found to be suitable for hybridization experiments. Pieces (22 x 22 mm) of the agarose slabs were cut and rehydrated in PBS for 20 min. After washing once with the same buffer, they were placed on a cover slip (22 x 22 mm). The remaining fluid was removed with a piece of filter paper and the cover slip with the agarose slab was placed in a hybridization-chamber (Corning). 50 µl of hybridization buffer (100 mM NaCl, 0.5 % SDS in 25 mM Tris-HCl pH 9.0) was added, after which a Hybri-slip (22 x 22 mm; Molecular Probes, Invitrogen) was placed on top of the buffer. The hybridization buffer contained 120 nM EuUni PNA probe and/or 300 nM *SC3* PNA probe. The eukaryotic 18S rRNA EuUni probe (ACCAGACTTGCCCTC) (Perry-O'Keefe *et al*., 2001) and the *SC3* (accession number M32329) mRNA probe (TCCGACACCGATGA) were (5') N-terminally labeled with fluorescein and Cy3, respectively. These probes had a Tm between 65 and 70 °C at the concentrations used (Applied Biosystems). Hybridization was performed at 54 °C in a water bath for 1 h. The agarose slabs were transferred to 6-well plates and washed 3 times 20 min with TE (10 mM Tris-HCl pH 9.0, 1 mM EDTA) at 54 °C. Slabs were mounted on microscope slides with Vectashield mounting medium (Vector Laboratories). Specificity of the rRNA hybridization was checked by incubating rehydrated fixed cultures for 1 h at 37 °C in 0.5 x PBS, pH 7.4 in the absence or presence of 0.5 mg ml^{-1} RNase A and 83 U ml^{-1} RNase I.

In situ hybridization with DNA probes

Thin agarose cultures were fixed, treated with 50 % ethanol and rehydrated as described above. The agarose slabs (22 x 22 mm) were washed in 40 % formamide, 2 x SSC for 5 min. This was followed by overnight hybridization at 37 °C in the same buffer supplemented with 5 mM sodium phosphate, pH 7.0, 10 mM vanadylribonucleoside complex, 1 mg ml^{-1} RNase-free BSA, 40 U RNase inhibitor (Rnase OUT, Invitrogen), 0.4 mg ml^{-1} sheared herring sperm DNA, 0.4 mg ml^{-1} tRNA and 25 nM DNA probe (Chartrand *et al*., 2000). A 5' end Alexa Red 594 labeled 18S rRNA probe (TATACGCTATTGGAGCTGGAATTACCGCGGCTGCTGGCACCAGACTTGC) was used (Eurogentec), the complementary strand serving as a control. Agarose slabs were washed two times for 15 min with preheated 40 % formamide, 2 x SSC at 37 °C. This was followed by washing for 15 min at 20 °C with 2 x SSC, 0.1 % Triton X-100 (once), and 1 x SSC (twice). Colonies were mounted on microscope slides as described above.

Cell walls were permeabilized by slow freezing and embedding methods (Bourett *et al*., 1998). Alternatively, they were permeabilized by treating with lytic enzymes from several sources (Sigma; Boom, Meppel, The Netherlands; Applied Plant Research, Horst, The Netherlands). These lytic enzymes had been treated with bentonite to remove RNases (Ruiters & Wessels, 1989).

Fluorescence microscopy

Fluorescein fluorescence was monitored using a FITC filter set, while a Cy3 filter was used for detection of Cy3 and Alexa Red 594. Fluorescence light microscopy was done using a Zeiss Axioscope 2PLUS equipped with a HBO 100 W mercury lamp and a Photometrics Cool SNAP camera (1392 x 1024 pixels) connected to a computer. Confocal Laser Scanning Microscopy was performed at a resolution of 1024x1024 pixels with a Zeiss LSM 5 Pascal equipped with an argon 488 nm and a helium/neon 543 laser line. Maximal intensity in the Z-axis was projected. Sequential scans were made in the case of co-hybridization experiments.

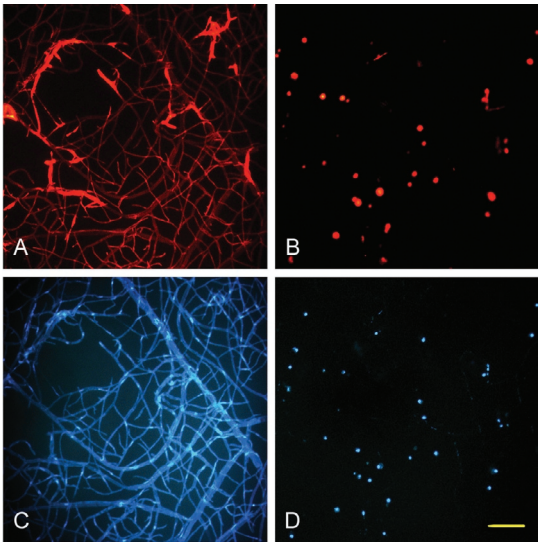

Figure 1: In situ hybridization of 18S rRNA in a colony of *S. commune* grown between a PC membrane and a cover slip (A) and derived protoplasts (B) using an Alexa Red 594 labeled DNA probe. Staining with DAPI served as a control (C, D). Fluorescence was monitored using a fluorescence light microscope. Bar represents 20 µm.

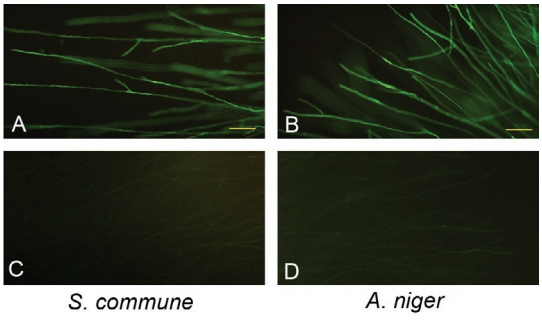

Figure 2: In situ hybridization of 18S rRNA in colonies of *S. commune* (A, C) and *A. niger* (B, D) using a fluorescein labeled PNA probe with (C, D) and without (A, B) prior RNase treatment. Fluorescence was monitored using a fluorescence light microscope. Bar represents 40 µm.

RESULTS AND DISCUSSION

The aim of this study was to establish a rapid and reproducible in situ hybridization procedure for filamentous fungi. DNA and PNA probes for 18S rRNA were hybridized to colonies of *S. commune and A. niger* grown in thin agarose layers. After mild chemical fixation, no signals were detected after hybridization with the Alexa Red 594-labeled 18S rRNA DNA probe (not shown). In contrast, intense signals were obtained with protoplasts (Figure 1B). Protoplasting, however, disrupted the hyphal integrity. Therefore, we attempted to permeabilize the cell wall to allow the DNA probe to penetrate the hyphae without interfering with the morphology. To this end, colonies of *S. commune* and *A. niger* were briefly treated with lysing enzymes (Ruiters & Wessels, 1989) or with slow freezing and embedding procedures (Bourett *et al*., 1998). In both cases low signals were observed except for hyphae that had been broken (Figure 1A) or

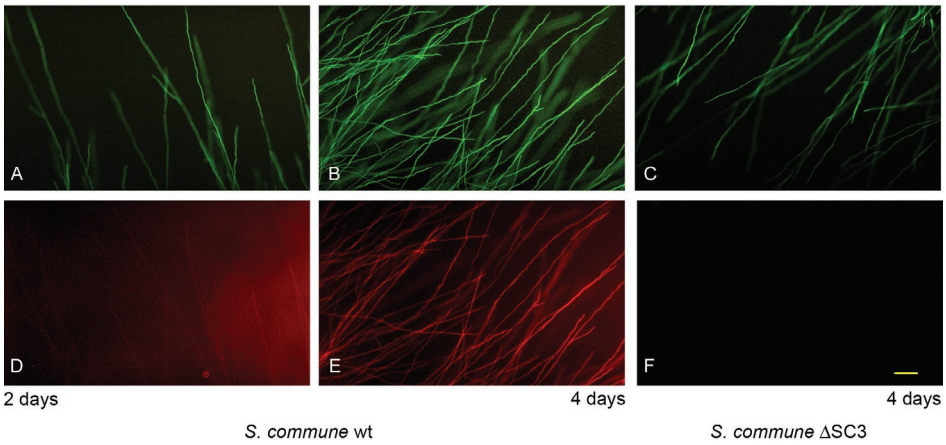

Figure 3: In situ co-hybridization of 18S rRNA (A, B, C) and *SC3* mRNA (D, E, F) in sandwiched colonies of *S. commune* strains 4-40 (A, B, D, E) and 72-3 (C, F) using a fluorescein and a Cy3 labeled PNA probe, respectively. Cultures were grown for 2 days (A, D) and 4 days (B, C, E, F). Fluorescence was monitored using a fluorescence light microscope. Bar represents 40 μm.

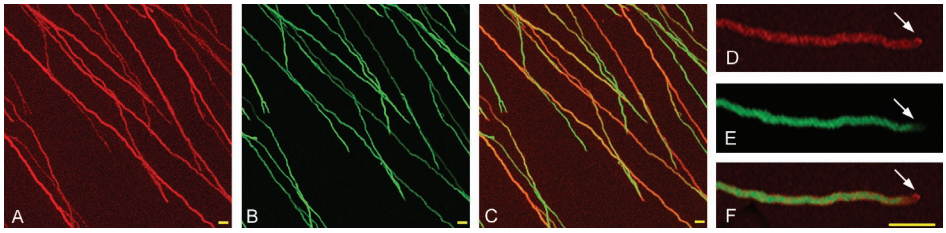

Figure 4: In situ co-hybridization at the periphery of a colony of *S. commune* strain 4-40 using the Cy3 labeled *SC3* PNA probe (A, D) and the fluorescein-labeled 18S rRNA PNA probe (B, E). In (C) and (F) an overlay is shown. Fluorescence was monitored using a confocal laser scanning microscope. Bar represents 20 μm. Arrow indicates the tip of the hypha.

when the hyphal tip had ballooned. From these experiments it was concluded that in situ hybridization with DNA probes was not reproducible in *S. commune* and *A. niger*. Moreover, the procedures proved to be elaborate.

In contrast to the DNA probe, the fluorescein 18S rRNA PNA probe penetrated the hyphae of colonies of *S. commune* and *A. niger* after mild chemical fixation (Figure 2). When the colonies were pre-treated with RNase, no signal was obtained. From this it can be concluded that the probe specifically bound RNA.

To establish whether PNA probes can also localize mRNA in fungal colonies, a Cy3 labeled PNA probe was used to detect mRNA of the *SC3* gene of *S. commune*. The *SC3* gene encodes a hydrophobin, which has multiple functions. It allows hyphae to escape from the aqueous environment into the air (Wösten *et al*., 1999; van Wetter *et al*., 1996), makes aerial hyphae hydrophobic (Wösten *et al*., 1993; 1994b), attaches hyphae to hydrophobic surfaces (Wösten *et al*., 1994a), and influences the cell wall architecture (van Wetter *et al*., 2000). The *SC3* gene is not expressed in

young cultures (Mulder & Wessels, 1986). It is activated after a feeding submerged mycelium has been formed. In agreement, no hybridization signal was obtained with the *SC3* PNA probe in 2-day-old colonies grown in thin agarose layers or in a sandwiched culture (Figure 3D). In contrast, strong fluorescence was observed in 4-day-old colonies (Figure 3E). The *SC3* PNA probe did not hybridize with 2- and 4-day old colonies of a strain in which the *SC3* gene had been disrupted (Figure 3F). From this it is concluded that the probe specifically bound to *SC3* mRNA. Interestingly, all hyphae in the growing zone of the colony were fluorescent after hybridization with the *SC3* PNA probe, although the intensity of the signal varied (Figure 4A). This suggests that all these hyphae contribute to secretion of SC3 into the medium. Signals of the ribosomal probe were also variable (Figure 4B) and the intensity did not correlate with that of the *SC3* probe (Figure 4C). This raises the intriguing question whether *SC3* mRNA is translated with the same efficiency in the different hyphae. Our findings may imply that high accumulation of mRNA, as assessed by Northern analysis, not necessarily correlates with a similar increase in protein.

Fluorescence was more intense in the apical compartment compared to subsequent compartments when hyphae of *S. commune* were hybridized with the 18S rRNA probe (Figure 4B). In those cases that a subapical compartment had the same intensity as the apical compartment, a branch had formed at this site. Such a subapical compartment can be considered to be apical also. In co-hybridization experiments, *SC3* mRNA was shown to be more evenly distributed than the 18S probe (Figure 4A). From these data we conclude that the differences in intensity obtained with the 18S probe are not due to local differences in penetration of the probe. Therefore, it may very well be that the apical compartment is most active in protein synthesis.

Within the apical compartment, homogenous hybridization was observed with the *SC3* probe (Figure 4D). In contrast, fluorescence of hyphae was weaker at the apex after hybridizing with the 18S rRNA PNA probe (Figure 4E). In 15 out of 45 hyphae a lower signal was observed at the extreme apex, corresponding to the zone where the Spitzenkörper resides. In the other hyphae the zone with the lower signal was up to 2 hyphal diameters. Transmission electron microscopy has indicated that ribosomes are less abundant at the apex compared to subapical parts (Heath, 1994). Our findings thus support the observation that ribosomes are not evenly distributed throughout the hypha.

The results obtained with the *SC3* probe imply that PNA probes can be used in in situ hybridizations to detect mRNAs in filamentous fungi. *SC3* is highly expressed reaching levels up to 1% of the mRNA (Mulder & Wessels, 1986). In this case the

mRNA was detected by using a single fluorescent probe only. Less abundant mRNAs may need the use of multiple probes hybridizing to the same mRNA. The use of catalyzed signal enhancement of PNA probes in fungi, as was done in mammalian systems (Yokomori *et al*. 2001, Larsen *et al*., 2003) will be hampered by the presence of the cell wall, as is the case with DNA probes.

ACKNOWLEDGEMENTS
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CHAPTER 3

Repellents have functionally replaced hydrophobins in mediating attachment to a hydrophobic surface and in formation of hydrophobic aerial hyphae

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ABSTRACT
Ustilago maydis contains one repellent and two class I hydrophobin genes in its genome. The repellent gene *rep1* has been described previously. It encodes 11 secreted repellent peptides that result from the cleavage of a precursor protein at KEX2 recognition sites. The hydrophobin gene *hum2* encodes a typical class I hydrophobin of 117 aa, while *hum3* encodes a hydrophobin that is preceded by 17 repeat sequences. Most of these repeats are separated, like the repellent peptides, by KEX2 recognition sites. Gene *hum2*, but not *hum3*, was shown to be expressed in a cross of two compatible wild-type strains, suggesting a role of the former hydrophobin gene in aerial hyphae formation. Indeed, aerial hyphae formation was reduced in a Δ*hum2* cross. However, the reduction in aerial hyphae formation was much more dramatic in the Δ*rep1* cross. Moreover, colonies of the Δ*rep1* cross were completely wettable, while surface hydrophobicity was unaffected and only slightly reduced in the Δ*hum2* and the Δ*hum2*Δ*hum3* cross, respectively. It was also shown that the repellents and not the hydrophobins are involved in attachment of hyphae to hydrophobic Teflon. Deleting either or both hydrophobin genes in the Δ*rep1* strains did not further affect aerial hyphae formation, surface hydrophobicity and attachment. From these data it is concluded that hydrophobins of *U. maydis* have been functionally replaced, at least partially, by repellents.

INTRODUCTION
The life cycle of *Ustilago maydis*, the causal agent of smut in *Zea mays* (maize) and *Euchlaena mexicana* (Mexican teosinte), is characterized by distinct morphological and nuclear states. Fusion of compatible yeast-like sporidia results in a filamentous pathogenic dikaryon. The dikaryon needs the plant to differentiate and to complete its life cycle with karyogamy and the production of diploid teliospores (Banuett, 1992; Banuett & Herskowitz, 1988; Christensen, 1963). These teliospores undergo meiosis ultimately producing haploid sporidia.

Fusion of haploid cells and development of an infectious dikaryon only occur when the two partners carry different alleles in the *a* and *b* mating type loci. The *a* locus controls cell-fusion through a pheromone-based recognition system (Bölker *et al.*, 1992), while the multiallelic *b* locus regulates post-fusion steps of pathogenic development including hyphal growth. The *b* locus encodes two unrelated homeodomain proteins, bE and bW (Gillissen *et al.*, 1992). These proteins form heterodimers when they are derived from different alleles (Kämper *et al.*, 1995) and as such regulate a number of genes (Bohlmann, 1996; Brachmann *et al.*, 2001; Romeis *et al.*, 2000; Wösten *et al.*, 1996). One of the genes that is strongly up-regulated by the bE/bW heterodimer is the *rep1* gene (Wösten *et al.*, 1996). It encodes a pre-pro-protein, consisting of a signal sequence for secretion and twelve repeats. Each repeat, with the exception of the last two, ends with a Kex2-

like protease recognition sequence. The pro-protein is cleaved in the endoplasmic reticulum resulting in ten repeats of 34-55 amino acids and a larger peptide of 229 aa. The latter not only consists of the last two repeats of Rep1 but also contains a proline-rich region (Figure 1). The 10 small repeats and the 229 aa polypeptide are collectively known as repellents. Eight of the repellents were identified in cell walls of aerial hyphae where they reside as SDS-insoluble but TFA extractable proteins (Wösten *et al.*, 1996).

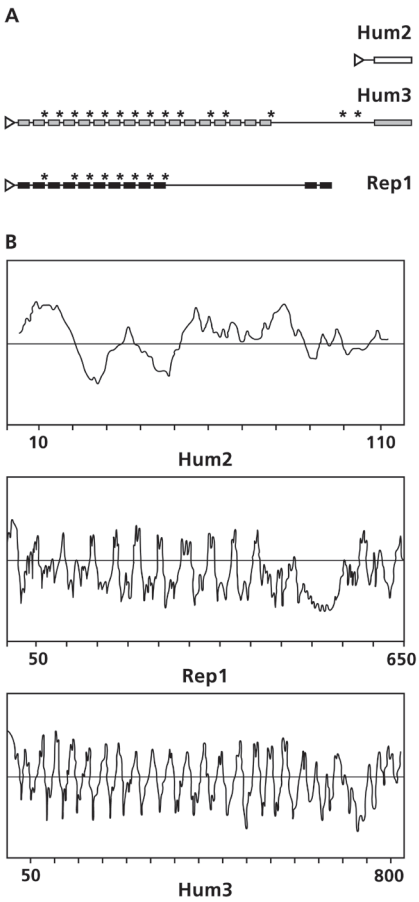

Figure 1: Schematic representation (A) and hydropathy patterns (B) of proteins encoded by *rep1, hum2* and *hum3* of *U. maydis*. Small boxes represent repeat sequences, large boxes hydrophobin domains. ▷ Indicates a signal sequence, while * indicates the presence of a Kex2 recognition sequence.

Disruption of *rep1* results in a dramatic reduction of aerial hyphae formation and the loss of hyphal hydrophobicity (Wösten *et al.*, 1996). Moreover, outgrowth of hyphae from a water droplet onto a hydrophobic surface is affected. The phenotype of the Δ*rep1* strain is very similar to that of a *Schizophyllum commune* strain in which the *SC3* hydrophobin gene is deleted (van Wetter *et al.*, 1996; Wösten *et al.*, 1994b). Moreover, solubility of the repellents in the cell wall is very similar to that of the class I hydrophobin SC3 (de Vries *et al.*, 1993; Wessels *et al.*, 1991; Wösten *et al.*, 1996). Here, we studied the role of two hydrophobin genes of *U. maydis*. These genes, called *hum2* and *hum3*, do not function in attachment of hyphae to hydrophobic Teflon. Moreover, they are not involved in surface hydrophobicity and pathogenicity and are only partly responsible for aerial growth. Repellents have thus functionally replaced the hydrophobins in formation of hydrophobic aerial hyphae and in attachment of hyphae to a hydrophobic surface.

MATERIAL AND METHODS

Strains

Cloning was done in *Escherichia coli* DH5α. The repellent gene *rep1* (Wösten et al., 1996) and the hydrophobin genes *hum2* and *hum3* were deleted in the haploid *U. maydis* strains FB1 (*a1b1*) and FB2 (*a2b2*) (Banuett & Herskowitz, 1989).

Primer	Sequence
hum2.5	TTTAGCGGCCGCAAAGGGATGCGAAGATGGTCTTGAACTGC
hum2.3	ATTTGCGGCCGCTTTGATCCAGACCTCGTGCACCCCCCTCTC
d-hum3f	GGATCCTTCGGTTTGGTTCGCATT
d-hum3r	AAGTACAAGCTTCGGCGTGCAG
u-pirhum3f	GAATTCGGCGTCAAGTAAGGCCACAG
u-pirhum3r	GGATCCGCGGCCGCCAGTTGATTG
mDF-Bf	CGGTTGGTGAGGCTAAGTGCTGTA
mDF-Br	GCGTCTTCTTCTTGCCTTCGTCTG
mUF-Hf	CCGTTGCTCAAAACCAGCTCGTCAACGTCGTGAGACACT
mUF-Hr	CGACGTTGACGAGCTGGTTTTGAGCAACGGGACGGGCATC

Table 1: Primers used in this study.

Growth conditions

U. maydis was routinely grown at 25 or 28 °C in YepsL (0.4 % yeast extract, 0.4 % peptone, 2 % sucrose) at 200 rpm or on solid potato dextrose agar (PDA, Sigma) either or not supplemented with 1 % charcoal.

Molecular techniques

U. maydis chromosomal DNA was isolated as described (Hoffman & Winston, 1987). DNA was blotted on Hybond N^+ (Amersham) and hybridized in 0.5 M $NaPO_4$, 7 % SDS, 10 mM EDTA, pH 7.2 at 60 °C with α-^{32}P-CTP labeled probes. RNA was isolated from ground cells (Microdismembrator, B.Braun) with Trizol (Invitrogen) according to the manufacturers protocol.

Deletion of *hum2* and *hum3*

A 1.3 kb *Sph*I fragment spanning the open reading frame of *hum2* (um05010; http://mips.gsf.de/genre/proj/ustilago/) was cloned in pUC19. The resulting plasmid pDik2S was used as a template in an inverse PCR reaction with primers hum2.5 and hum2.3 that contain *Not*1 linkers at their 5' end (Table 1). The amplified fragment was digested with *Not*I and ligated to a hygromycin resistance cassette (Brachmann *et al.*, 2001). In the resulting plasmid pΔHum2 the region of *hum2* between start and stop codon is replaced by the hygromycin resistance cassette.

Primer pairs u-pirhum3f/u-pirhum3r and d-hum3f/d-hum3r (Table 1) were used to amplify the 1 kb upstream and downstream region, respectively, of the coding sequence of *hum3* (um04433; http://mips.gsf.de/genre/proj/ustilago/). PCR products of both flanks were cloned in pGEM-T Easy (Promega). Internal *Hin*DIII and *Bam*HI sites were removed from the upstream and downstream flank, respectively, by Quikchange mutagenesis (Stratagene) using the primer pairs mUF-Hf/mUF-Hr and mDF-Bf/mDF-Br (Table 1). The upstream flank was cloned

as a *Eco*RI/*Hin*DIII fragment between the corresponding sites of pUC19. This was followed by introduction of the downstream flank as a *Bam*HI/*Hin*DIII fragment. A *Not*I carboxin resistance cassette taken from pNEB-Cbx (Brachmann *et al.*, 2001) was inserted in the *Not*I site at the 3' end of the upstream flank. This resulted in pΔHum3. The *Eco*RI/*Hin*DIII fragment of this vector encompassing both flanks and the resistance cassette was used to transform *U. maydis.*

The *hum3* and/or the *rep1* gene were deleted in compatible Δ*hum2* strains to yield strains Δ*hum2*Δ*hum3,* Δ*hum2*Δ*rep1*, and Δ*hum2*Δ*hum3*Δ*rep1.* In this case pΔRep1 (Wösten *et al*., 1996) contained the nourseotricin (Brachmann *et al.*, 2001) in stead of the hygromycin resistance cassette. *U. maydis* was transformed according to Brachmann *et al.* (2004). Transformants were selected on PDA plates supplemented with hygromcyin, carboxin or nourseotricin at a final concentration of 200, 2 and 150 μg ml^{-1}, respectively.

Assesment of surface hydrophobicity and hyphal attachment.
Surface hydrophobicity was assessed by determining the contact angle of water droplets of 1-5 μl with the Krüss Drop Shape Analysis System DSA10 Mk2. Attachment of hyphae was determined essentially as described (Wösten *et al.*, 1994a). Strains were grown on squares of Teflon sheet (FEP; 0,25 mm thick; Norton Fluorplast, Raamsdonkveer, The Netherlands). To this end, 30 μl of water was placed in the middle of the sheet. The droplet contained 50,000 cells of each mating type, isolated from a culture in logarithmic phase. Cells were grown under humid conditions. After 40 h, the water was removed with a pipette and the sheet was placed on top of a 200 μl droplet of low melting point agarose (40 °C; 0,5-3 %), which had been pipetted on a Petri-dish. After solidification of the agarose at 20 °C for 30 min the Teflon sheet was stripped from the gel. Presence of hyphae was scored in the agarose slab and on the Teflon sheet.

Pathogenicity assay
Overnight cultures (OD_{600} 0.4-2) were centrifuged and cells were taken up in water to a final OD_{600} of 3. Mating partners were mixed 1:1 and 0.5 ml of the mixture was injected in the leaf whorl of 12-30 1-week-old Early Golden Bantam plants. Injection was performed 1 cm above the potting soil. Plants were grown at 28 °C with 14 h of light with a minimum of 244 μEinstein m^{-2} sec^{-1}.

RESULTS AND DISCUSSION
Filamentous ascomycetes and basidiomycetes generally contain multiple hydrophobin genes (Wösten, 2001). For instance, 34 hydrophobin genes have been identified in the genome of *Coprinus cinereus* (R. Velagapudi & U. Kües, unpublished data), while *S. commune* contains at least 5 hydrophobin genes (Wessels *et al.*, 1995;

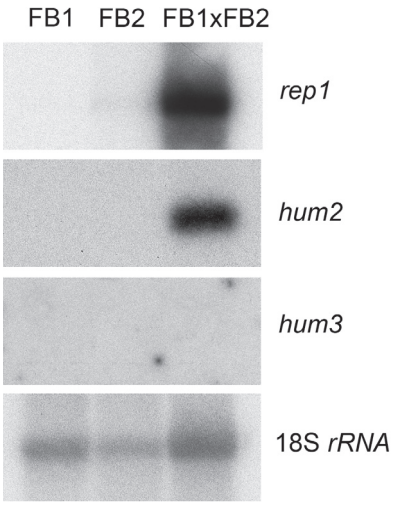

Figure 2: Northern analysis of expression of the repellent gene *rep1* and the hydrophobin genes *hum2* and *hum3* in the haploid strains FB1 and FB2 and their cross. Cultures were grown on PDA charcoal for 48 h. 18 S *rRNA* served as a loading control.

H.J. Deelstra & H.A.B. Wösten, unpublished results). In contrast, hydrophobin genes are absent in the genomes of the yeasts *Cryptococcus neoformans*, *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces lactis,* and *Yarrowia lipolytica*, and the dimorphic fungus *Candida albicans*. These organisms have probably lost their hydrophobin genes during evolution. We show here that *U. maydis* does have hydrophobin genes but that the encoded proteins have been functionally replaced, at least partially, by the repellents. *U. maydis* thus seems to be in between the filamentous fungi and the yeasts with respect to the role of hydrophobins in the life cycle.

U. maydis contains two hydrophobin genes

Two class I hydrophobin genes were identified in the genome of *U. maydis* that encompasses 6,801 open reading frames (MUMDB; http://mips.gsf.de/genre/proj/ustilago/). Since 99.7 % of the genome has been sequenced, it is likely that these are the only hydrophobin genes in this dimorphic fungus. The two hydrophobin genes were named *hum2* (um05010) and *hum3* (um04433). The *hum2* gene encodes a typical class I hydrophobin of 117 amino acids with eight conserved cysteine residues (Figure 1). The first cysteine residue is preceded by a signal sequence for secretion and 13 amino acids that are predicted to form the N-terminal part of the mature protein. The *hum3* gene encodes an atypical hydrophobin. The encoded protein consists of a class I hydrophobin domain preceded by a signal sequence and 17 imperfect repeats of 31 - 36 amino acids. Thirteen repeats are separated by KEX2 recognition sites (Figure 1). Two KEX2 processing sites are found in between the last repeat and the first cysteine of the hydrophobin domain. The protein is thus expected to be cleaved in the ER in 13 repeats, and a hydrophobin of 117 amino acids. This hydrophobin shows 53 % identity to Hum2. The repeats, which we named questellents, have no homology with proteins in the database but their hydropathy pattern shows a remarkable similarity to that of the repellents (Figure 1B).

Role of repellents and hydrophobins in formation of aerial hyphae

Both *rep1* (Wösten *et al.,* 1996) and *hum2* were shown to be expressed during aerial hyphae formation when the wild-type strains FB1 and FB2 were crossed

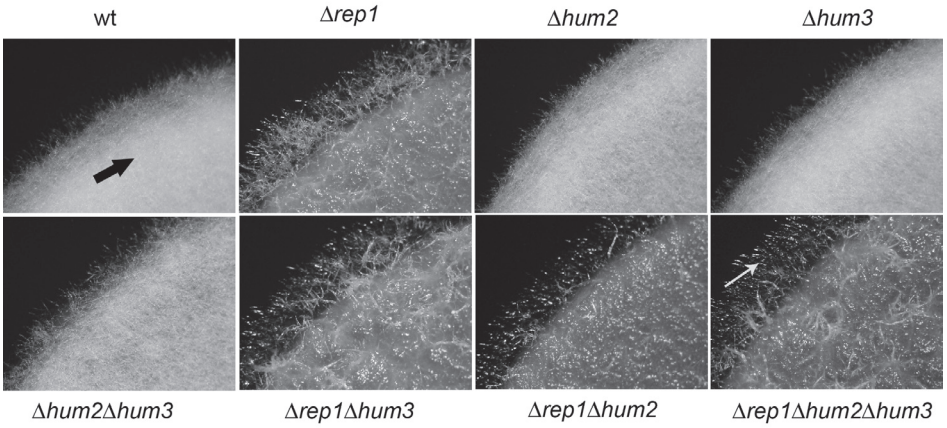

Figure 3: Formation of aerial hyphae at the edge of colonies after crossing compatible partners. Aerial hyphae give the colony a white fuzzy appearance. Aerial hyphae and hyphae that grow over the agar surface are indicated by a thick arrow and a thin arrow, respectively.

on PDA-charcoal medium (Figure 2). In contrast, expression of *hum2* and *rep1* was absent and low, respectively, in the non-crossed parental strains. In no case could expression be shown for *hum3*, implying that this hydrophobin gene has no significant role in aerial hyphae formation. To investigate this, genes *hum2* and/or *hum3* were deleted in FB1 and FB2 either or not in combination with an inactivation of the *rep1* gene (see Material and Methods). Southern analysis confirmed deletion of the genes (results not shown). Aerial hyphae formation was almost completely abolished when the repellent gene *rep1* was deleted (Wösten *et al.,* 1996; Figure 3 and Figure 4A). In contrast, formation of aerial hyphae was only partially reduced when compatible Δ*hum2* strains were crossed (Figure 3 and Figure 4A) and was almost unaffected in the Δ*hum3* cross. A Δ*hum2*Δ*hum3* cross formed less aerial hyphae than the Δ*hum2* cross. Inactivation of *hum2* and/or *hum3* in the Δ*rep1* background hardly, if at all, had an effect on aerial hyphae formation. The reduced formation of aerial hyphae in the Δ*hum2*Δ*hum3* cross compared to the Δ*hum2* cross is not explained by an up-regulation of *hum3* when *hum2* is inactivated (data not shown) but seems to be due to a defect in fusion of compatible Δ*hum2*Δ*hum3* partners. This is concluded from the fact that the cross of the hydrophobin double mutant also forms less hyphae in the aqueous environment. The reason for this is not yet known.

Hydrophobicity of the colony surface correlated with formation of aerial hyphae. Surfaces of the wild-type and the Δ*hum2* and the Δ*hum3* cross were highly hydrophobic (Figure 4AB) showing water contact angles of 127 ±5, 125 ± 4 and 127 ± 4 degrees, respectively. Surface hydrophobicity was slightly reduced in the Δ*hum2*Δ*hum3* cross (water contact angle of 117 ± 2 degrees). Colony surfaces were completely wettable when *rep1* was inactivated (Figure 4AB; Wösten *et al.,*

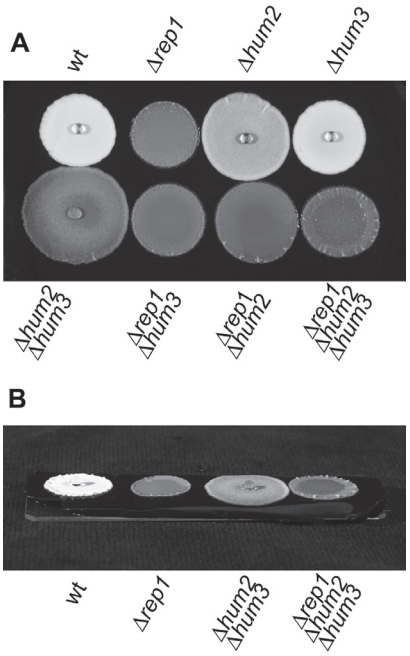

Figure 4: Formation of aerial hyphae by crosses of compatible partners correlates with surface hydrophobicity. White appearance of colonies is indicative for aerial hyphae formation. Surface hydrophobicity was assessed by placing a water droplet on the colony surface. The water droplet spreads completely on surfaces of crosses of strains in which the *rep1* gene has been inactivated, while surface hydrophobicity of the Δ*hum2*Δ*hum3* is only slightly affected. (A) Top view; (B) side view.

1996). In this case water contact angles could not be measured since water droplets were immediately absorbed by the colony. Deletion of *hum2* and/or *hum3* had no additional effect in the Δ*rep1* background.

Taken together, we conclude that repellents have to a great extent replaced hydrophobins in formation of aerial hyphae. This contrasts the suggestion that repellents would function by anchoring hydrophobins to the cell wall (Wösten *et al.*, 1996). The fact that we have been unable to identify hydrophobins in cell wall extracts (R. Bohlmann and H.A.B. Wösten, unpublished data) supports the conclusion that hydrophobins are not the main structural proteins that mediate aerial hyphae formation.

Role of repellents and hydrophobins in attachment and pathogenicity

Mating partners of the wild-type or those of Δ*rep1*, Δ*hum2*, Δ*hum3*, Δ*rep1*Δ*hum2,* Δ*rep1*Δ*hum3,* Δ*hum2*Δ*hum3*, and Δ*rep1*Δ*hum2*Δ*hum3* were injected in 7-day-old maize plants. In all cases 90-100 % of the plants showed disease symptoms after 1 week (anthocyanin formation, chlorosis and tumor formation). This demonstrates that repellents and hydrophobins are not involved in pathogenicity. In contrast, deleting the class I hydrophobin gene *MPG1* in *Magnaporthe grisae* did affect pathogenicity (Talbot *et al.*, 1996). This phenotype was explained by the inability of the *M. grisae* mutant strain to attach to the hydrophobic surface of the plant. Hydrophobin mediated attachment to hydrophobic surfaces was also observed in *S. commune* (Wösten *et al.*, 1994a). Attachment of *U. maydis* hyphae to a hydrophobic surface was studied by crossing compatible strains in a water droplet placed on a sheet of Teflon. Most hyphae were formed at the periphery of the water droplet and grew onto the dry hydrophobic Teflon. Outgrowth onto the dry Teflon surface was not affected in the Δ*hum2*, Δ*hum3* and Δ*hum2*Δ*hum3* crosses (data not shown) but was reduced in the Δ*rep1* cross (Wösten *et al.*, 1996). No apparent further reduction of escape of hyphae was observed in Δ*rep1* strains in which either or both hydrophobins had been deleted. Hyphae of the wild-type cross that had escaped the aqueous environment and had grown onto the

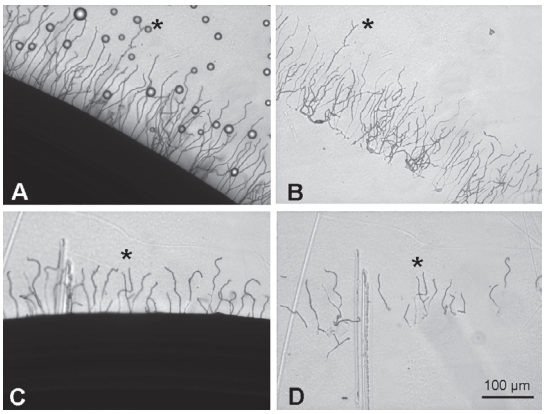

Figure 5: Hyphae of a wild-type cross (A, B) and the Δ*rep1* cross (C, D) of *U. maydis* that have grown out of a water droplet onto a dry Teflon sheet resist stripping with 3 % agarose. Hyphae of the wild-type cross and the Δ*rep1* cross before (A, C) and after (B, D) stripping with agarose. Asterisks are marker positions on the Teflon sheets.

dry Teflon surface could not be removed by extensive washing with water (data not shown) or by stripping with 3 % agarose (Figure 5). Similar results were obtained with the Δ*hum2*, the Δ*hum3* and the Δ*hum2*Δ*hum3* strains (data not shown). In contrast, 50 % of the hyphae of the Δ*rep1* cross were removed upon stripping with 3 % agarose (Figure 5). Deleting either or both hydrophobins genes in the Δ*rep1* background did not further reduce attachment.

The reduction of attachment in the Δ*rep1* cross is much less dramatic than that observed after inactivation of the *SC3* gene of *S. commune*. In this case, wild-type hyphae remained attached after stripping with 3 % agarose, whereas Δ*SC3* hyphae already detached at 1.5 % agarose. Apparently, other proteins are involved in attachment of *U. maydis*. The hydrophilic nature of the fungal cell wall and the hydrophobic surface of the host suggest the involvement of an amphipathic molecule.

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Repellents have replaced hydrophobins

CHAPTER 4

The filament specific Rep1-1 repellent of the phytopathogen *Ustilago maydis* forms functional surface-active amyloid-like fibrils

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ABSTRACT
Repellents of the maize pathogen *Ustilago maydis* are involved in formation of hydrophobic aerial hyphae and in cellular attachment. These peptides, called Rep1-1 to Rep1-11, are encoded by the *rep1* gene and result from cleavage of the precursor protein Rep1 during passage of the secretion pathway. Using GFP as a reporter, we here show that *rep1* is expressed in filaments and not in the yeast form of *U. maydis.* In situ hybridization localized *rep1* mRNA at the apex of the filament, which correlates with the expected site of secretion of the repellents into the cell wall. We also produced a synthetic peptide Rep1-1. This peptide reduced the water surface tension to as low as 36 mJ m^{-2}. In addition, it formed amyloid-like fibrils as was shown by negative staining, by Thioflavin T fluorescence, and by X-ray diffraction. These fibrils were not soluble in SDS but could be dissociated with trifluoroacetic acid. The repellents in the hyphal cell wall had a similar solubility and also stained with thioflavin T, strongly indicating that they are present as amyloid fibrils. However, such fibrils could not be observed at the hyphal surface. This can be explained by the fact that the Rep1-1 fibrils decrease in length at increasing concentrations. Taken together, we have identified the second class of fungal proteins that form functional amyloid-like fibrils at the hyphal surface.

INTRODUCTION
Ustilago maydis is the causal agent of smut in *Zea mays* (maize) and *Euchlaena mexicana* (Mexican teosinte). A filamentous pathogenic dikaryon is formed upon fusion of compatible yeast-like sporidia. Differentiation in the plant leads to the formation of diploid teliospores, which undergo meiosis ultimately resulting in haploid sporidia (Banuett & Herskowitz, 1988; Banuett, 1992; Christensen, 1963).

Fusion of haploid cells and development of an infectious dikaryon are controlled by the *a* and *b* mating type loci. The *a* locus regulates cell-fusion through a pheromone-based recognition system (Bölker *et al*., 1992) and the *b* locus controls post-fusion steps of pathogenic development including hyphal growth. The latter locus encodes two unrelated homeodomain proteins, bE and bW, that form heterodimers when they are derived from different alleles (Gillissen *et al*., 1992; Kämper *et al*., 1995). This heterodimer regulates a number of genes, among which the *rep1* gene (Bohlmann, 1996; Brachmann *et al*., 2001; Romeis *et al*., 2000; Wösten *et al*., 1996). This gene is highly expressed resulting in 2.5 % of the mRNA. It encodes a pre-pro-protein that, after processing at KEX2 recognition sites, results in 11 secreted peptides with a high sequence similarity. These peptides are localized in the cell wall of filaments, in a SDS insoluble, but trifluoroacetic acid (TFA) -extractable form (Wösten *et al*., 1996). They are involved in formation of hydrophobic aerial hyphae (Wösten *et al*., 1996) and in hyphal attachment to hydrophobic surfaces (**Chapter 3**; Teertstra *et al*., 2006) and as such they have functionally replaced hydrophobins in *U.maydis* (**Chapter 3**; Teertstra *et al*., 2006).

Hydrophobins, which are not related to the repellents, fulfill a wide spectrum of functions in fungal development (Wösten, 2001). They do so by forming an amphipatic protein film, which consists of amyloid-like fibrils (Butko *et al*., 2001; Kwan *et al*., 2006; Wösten & de Vocht, 2000). We here show that the repellent Rep1-1 also forms surface-active amyloid fibrils and that this peptide is specifically produced in filaments. Apparently, the amyloid structure is important for function in fungal development.

MATERIAL AND METHODS

Strains and Growth conditions

The *U. maydis* strains FB1 (*a1b1*), FB2 (*a2b2*) (Banuett & Herskowitz, 1989) and SG200 (*a1mfa2 bE1/bW2*) (Bölker *et al*., 1995) were used in this study. The non-isogenic strains FB1 and FB2 grow yeast-like in liquid medium. These strains are compatible and form a dikaryon upon mating that grows filamentous in contact with air. SG200 is a derivative of FB1 and harbors an active *b* mating type complex. As a consequence, this strain forms yeast-like cells in liquid medium and forms filaments at the water-air interface without the need for mating. *U. maydis* was grown at 25 °C using liquid or solidified (1.5 % agarose) nitrate minimal medium (Holliday, 1974) or liquid YEPS medium (0.4 % yeast extract, 0.4 % peptone, 2 % sucrose). For GFP expression analysis, 3 µl of cell suspension (2 10^7 cells ml^{-1}) was seeded in a cut away within a 0.25 mm thin layer of solidified medium that had been sandwiched between a glass slide and a cover slip (Figure 1A). A 0.17 mm layer of solidified medium was used for in situ hybridization.

Rep1 reporter construct and *rep1* constitutive expression

The promoter region of the *rep1* gene was amplified from genomic DNA of FB1 using Phusion™ polymerase (Finnzymes) with oligonucleotide primers prRep-fw (CGCTATGACCTGGCCTAAAG) and prRep-rev (GTCAGCACGCTGATGGAAAG). The resulting 1844 bp PCR fragment was used as a template in a PCR reaction with nested PCR primers prRep-*Kpn*I-fw (GGTACCGCAGCAATCACAGAG) and prRep-*Nco*I-rev (TGGAAGCCATGGTTGTAGTCGA), introducing *Kpn*I and *Nco*I restriction sites, respectively. The resulting 1688 bp fragment was introduced in the *Sma*I site of pUC19 and was amplified in *Escherichia coli* DH5α. Subsequently, the *Kpn*I /*Nco*I promoter fragment of *rep1* was introduced in the corresponding sites of pMF3c (Brachmann *et al*., 2004). As a result, *e-GFP* was placed under control of the *rep1* promoter, resulting in construct pMF3c-prRep.

The *O2tef* promoter (Brachmann *et al*., 2004) was amplified by PCR using primers Otef-fw (TGGGCCCGGTCGACTCTAGAACTAGTG) and Otef-rev (TACCATGGATCCCGTGGATGATGTTGTC), whereas the *rep1* coding sequence (Wösten *et al*., 1996) was amplified using primers Rep-fw (TACCATGGCTTCCAAGATCG) and

Rep-rev (AGGCGCGCCAGAGGTGTTTCTTC). The PCR products contained *Apa*I and *Nco*I and *Nco*I and *Asc*I sites at the 5' and 3' ends of the products, respectively. These fragments were cloned in vector pMF3c (Brachmann *et al*., 2004), thereby removing the *eGFP* coding sequence. The resulting vector was named pMF3c-OR.

Transformation of *U. maydis*

pMF3c-OR and pMF3c-prRep were introduced in *U. maydis* after digestion with *Age*I thus linearizing the plasmid in the carboxin resistance cassette. *U. maydis* was transformed (Brachmann *et al*., 2004) using a selection on carboxin containing PDA plates (2 µg ml^{-1}). Targeting to the succinate dehydrogenase gene *sdh2* (um00844) was confirmed by Southern analysis. To this end, *U. maydis* chromosomal DNA was isolated as described (Hoffman & Winston, 1987) and blotted onto Hybond N+ (Amersham). Hybridization was performed at 60 °C in 0.5 M phosphate buffer, pH 7.2, 7 % SDS, 10 mM EDTA, using a ^{32}P-α-dCTP labeled 1,9 kb *NotI* carboxin cassette as a probe.

***Rep1* expression analysis using in situ hybridization**

In situ hybridization was performed according to Teertstra *et al.* (**Chapter 2**; Teertstra *et al*., 2004). Cultures that had been grown in an agarose slab of 20 x 20 mm were fixed for 2 h at 20 °C with freshly prepared 4 % paraformaldehyde in PBS, pH 7.4. After washing with buffer, the agarose slab cultures were placed overnight at -20 °C in excess 50 % ethanol in PBS (v/v). The agarose slabs were placed on a cover slip (22 x 22 mm) and were stepwise rehydrated (30 % ethanol in PBS; 10 % ethanol in PBS; and twice in PBS, each step for 10 min). The remaining fluid was removed with a piece of filter paper and the cover slip with the agarose slab was placed in a hybridization-chamber (Corning Inc.). 50 µl of hybridization buffer (100 mM NaCl, 0.5 % SDS in 25 mM Tris-HCl, pH 9.0) was added, after which a Hybri-slip (22 x 22 mm; Molecular Probes) was placed on top of the buffer. The hybridization buffer contained 120 nM EuUni PNA probe and 300 nM *rep1* PNA probe. The eukaryotic 18S rRNA EuUni probe (ACCAGACTTGCCCTC) (Perry-O'Keefe *et al*., 2001) and the *rep1* mRNA probe (GATCAGCTTGTTCTC) were N-terminally labeled with fluorescein and Cy3, respectively. These probes had a Tm between 65 and 70 °C at the concentrations used (Applied Biosystems; Panagene). Hybridization was performed at 54 °C in a water bath for 1 h. The agarose slabs were washed 3 times 20 min with 10 mM Tris-HCl, pH 9.0, 1 mM EDTA (TE) at 54 °C and mounted on microscope slides using Vectashield mounting medium (Vector Laboratories).

Peptide synthesis

The N-acetyl carboxyamide form of the Rep1-1 peptide HYEYKSYNAGHNVESVVENKL VDASDLTLGVDIL-NH2 was assembled on an automatic ABI 433A Peptide Synthesizer (Applied Biosystems) using the ABI FastMoc 0.25 mMol protocols according

to the instructions of the manufacturer. Fmoc (9-fluorenylmethoxycarbonyl)-amino acid derivatives, activated in situ using O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate / N-hydroxy-benzotriazole and N,N'-diisopropylethylamine in N-methylpyrrolidone, were used in coupling steps of 45 min. The peptide was de-protected and cleaved from the resin by treatment with 10 ml TFA, 0.25 ml H_2O and 0.25 ml triisopropylsilane for 2 h at room temperature. Finally, the peptide was precipitated in methyl-tert-butylether / n-hexane (1/1, v/v), dissolved in 60 ml tert.-BuOH/water (1/1, v/v) and lyophilized. Preparative HPLC was performed using a semiautomatic Applied Biosystems HPLC system (Applied Biosystems) with a preparative reversed-phase column Luna C8 (100 Å, 10 µm, 250 x 21.2 mm) (Phenomenex), an UV detector operating at 214 nm, and a flow rate of 12 ml min^{-1}. Analytical HPLC was performed using an automatic HPLC system (Shimadzu) with an analytical reversed-phase column Phenomenex Luna C8 (100 Å, 5 µm, 250 x 4.6 mm), an UV detector operating at 214 nm, and a flow rate of 1 ml min^{-1}. The buffers used were 0.1 % TFA in water/acetonitrile (95/5, buffer A) and 0.1 % TFA in acetonitrile/water (60/40, buffer B). The peptide was eluted with a linear gradient from 100 % A to 100 % B over 48 min (analytical) or with a linear gradient from 25 % A to 77 % B over 81 min (preparative). The peptide was characterized using MALDI-TOF (Kratos AXIMA CFR) with bovine insulin oxidized B chain as external reference (monoisotopic [M+H]+ = 3,494.6513) and α-cyano-4-hydroxycinnamic acid as matrix. The calculated and detected monoisotopic mass [M+H]+ for Rep1-1 ($C_{170}H_{263}N_{44}O_{57}$) were determined as 3832.9034 and 3832.424, respectively.

SDS PAGE

Proteins were separated on 12 % SDS-PAGE gels and stained with colloidal Coomassie R250 (Neuhoff *et al*., 1988).

Surface activity

Surface activity was measured with the Krüss Drop Shape Analysis system DSA10 Mk2 (Krüss) using 18 µl droplets of Rep1-1 solution.

Thioflavin T fluorescence

Strains were grown on square siliconized cover slips of 22 x 22 mm (Hampton research) to induce filamentous growth. To this end, a 30 µl droplet of water containing 10^6 cells from a culture in logarithmic phase was placed in the middle of the cover slip. Cells were grown under humid conditions for 40 h (**Chapter 3**; Teertstra *et al*., 2006). The water was removed with a pipette and the cover slips with attached hyphae were submerged in 7.5 µM thioflavin T (ThT) in PBS for 30 min in the dark. For yeast-like growth, strains were grown in YEPS at 28 °C. After washing with H_2O once, cells were resuspended in 7.5 µM ThT in PBS and incubated

for 30 min in the dark. 5 µl of the suspension (containing 80.000 cells) was spotted on a cover slip that was coated with a thin layer of 1.5 % agarose to adhere the cells. Filaments and yeasts were examined with the confocal microscope immediately after staining (see below). To study the interaction of ThT with synthetic Rep1-1, fluorescence of 25 µM of the dye in 50 mM glycine buffer pH 9.0 was recorded at 485 nm in the presence or absence of 30 µg ml^{-1} peptide. Fluorescence in the absence of ThT or protein was used for background subtraction and 2.5 µg ml^{-1} human amyloid-β (Aβ) was used as a positive control. Samples were analyzed after 10 min of incubation at room temperature, using an Ascent Fluoroskan fluorescence spectrophotometer (Thermo Scientific).

Fluorescence microscopy

Fluorescein and GFP fluorescence were monitored using a FITC filter set, while a Cy3 filter was used for detection of Cy3. Fluorescence microscopy was done using a Zeiss Axioscope 2PLUS equipped with a HBO 100 W mercury lamp and a Photometrics Cool SNAP camera (1392 x 1024 pixels) connected to a computer. For expression studies, confocal laser scanning microscopy was performed at a resolution of 1024 x 1024 pixels with a Zeiss LSM 5 Pascal equipped with an argon 488 nm and a helium/neon 543 laser line. Maximal intensity in the Z-axis was projected. Sequential scans were made to visualize fluorescein and Cy3 in co-hybridisation experiments. For ThT staining, confocal laser scanning microscopy was performed at a resolution of 1024 x 1024 pixels with a Zeiss LSM 5 Pascal equipped with an argon 488 nm laser line. Single images were taken using a BP filter of 470-500 nm.

Electron microscopy

5 µl of an aqueous solution of Rep1-1 was dried down on carbon-coated grids and negatively stained with 1 % uranyl acetate. Samples were viewed using transmission electron microscopy (Technai 10, FEI) at 100 kV. To analyze the ultrastructure of filaments of the FB1 x FB2 dikaryon, 50.000 cells of each of the parental strains were seeded in a 30 µl droplet of water placed in the middle of a square of Teflon sheet (FEP; 0.25 mm thick; Norton Fluorplast, Raamsdonkveer, The Netherlands). Cells were grown under humid conditions and after 40 h the droplet was removed. Hyphae that had attached to the Teflon were fixed in 3 % glutaraldehyde/PBS and postfixed in 1 % osmium tetroxide. After acetone dehydration the hyphae were critical point dried followed by Pt/Pd sputter coating. Cells were viewed with a field emission scanning electron microscope at 5 kV (FEI). Spores of *Aspergillus niger* served as a positive control.

X-ray diffraction

A solution of 300 µg ml^{-1} Rep1-1 was vortexed for 3 min. The sample was centrifuged for 10 min at 10.000 g and the pellet was taken up in $1/10^{th}$ volume.

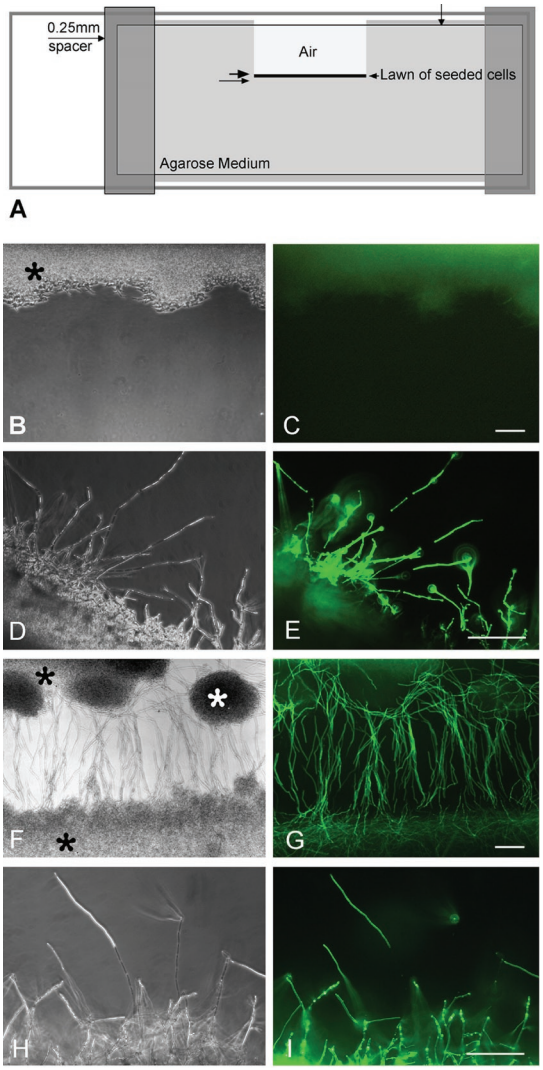

Figure 1: Localization of *rep1* expression. (A) Cells of strain FB1 (B-E) and SG200 (F-I) were seeded in an air slot on top of a thin layer of solidified medium sandwiched between a glass slide and a cover slip, allowing the cells to grow into the medium as well as into the air. Cells were monitored by phase contrast (B, D, F, H) and by fluorescence microscopy (C, E, G, I). Cells of B, C, F, G were growing in the agar medium (position indicated by a thin arrow in A) and cells of D, E, H, I were growing in the air (position indicated by a thick arrow in A). Bar indicates 100 µm. Asterisks in B and F indicate clumps of yeast cells.

After introducing an aliquot in a glass capillary, the sample was allowed to dry at ambient conditions. X-ray diffraction data were recorded on a Mar Image Plate using CuKα radiation from a Bruker AXS FR591 generator with rotating anode and Montel 200 mirrors. The capillaries were placed in a position perpendicular to the X-ray beam at a distance of 250 mm from the detector. Scattering of air and the capillary was subtracted by using in-house software (VIEW/EVAL) (Duisenberg *et al*., 2003).

RESULTS

Repellents are specifically expressed in filaments of U. maydis

Strains FB1 and SG200 were transformed with pMF3c-prRep, which contains the *e-GFP* gene behind the 1688 bp *rep1* promoter. Southern analysis showed that the plasmid had been targeted into the *sdh2* gene in transformants WR1 and WR2, which have a FB1 and a SG200 background, respectively (data not shown; see Material and Methods for details). These strains were grown on slices of nitrate minimal medium (NM) in between an object glass and cover slip (Figure 1A). Cells of the FB1 derivative WR1 grew mainly yeast-like on the agar layer (Figure 1B). Some filaments were formed that grew into the air (Figure 1D). These filaments, but not the yeast-like cells, showed GFP fluorescence (Figure 1CE). Cells of the SG200 derivative WR2, which encompasses active *a* and *b*-mating

type complexes, grew yeast-like on the agarose medium (Figure 1F). From this layer, hyphae grew into the air (Figure 1H) as well as into the medium (Figure 1F). At a depth of 0.5 mm the filaments adopted a yeast-like morphology again (Figure 1F). Both the aerial and substrate hyphae expressed *eGFP*, whereas expression was not observed in the yeasts (Figure 1GI).

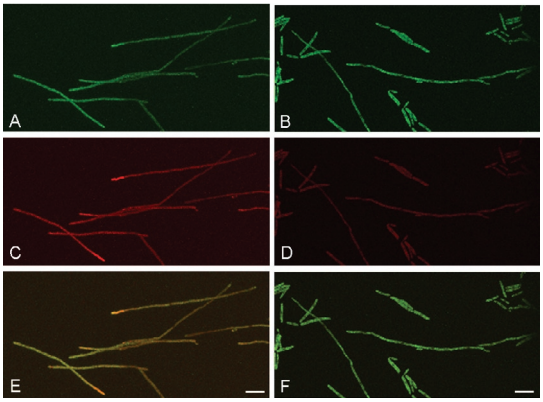

Figure 2: In situ co-hybridization of 18S *rRNA* (A, B), and *rep1* mRNA (C, D) in SG200 (A, C, E) and SG200Δ*rep1* (B, D, F). E and F represent overlays of A and C and B and D, respectively. Bar represents 20 µm.

GFP fluorescence correlated with in situ hybridization using a *rep1* PNA probe that binds 5 out of the 12 repeats within this gene. The PNA probe was found to hybridize in filaments of SG200, whereas no hybridization was observed in the SG200Δ*rep1* strain (Figure 2CD). The 18S PNA probe was detected both in yeasts and filaments of these strains in a co-hybridization experiment (Figure 2AB). Interestingly, the *rep1* probe was mainly observed in the apical part of hyphae of the SG200 strain.

The repellent peptide 1-1 forms amyloid fibrils in vitro

Peptide Rep1-1 was synthesized chemically (see Material and Methods). Similar to the peptide residing in the cell wall (Wösten *et al.*, 1996) synthetic Rep1-1 was only soluble in water or 2 % SDS after treatment with TFA. The peptide of 3.7 kDa ran at an apparent molecular weight of 21 kDa (data not shown), indicating that Rep1-1 forms oligomers.

Water surface activity of the peptide at a concentration of 100 µg ml^{-1} was hard to measure. The shape of the drop hanging at the tip of a syringe changed readily (Figure 3B) and often detached after a few minutes. In those cases that the droplet remained attached, surface activity was reduced from 72 to 36 mN m^{-1} in a 1 h period (Figure 3A). A similar decrease was observed at 30 µg ml^{-1} but in this case it took 90 min to reach this surface tension. Reduction in the water surface tension was accompanied with the formation of a rigid protein film, as was shown by sucking back the water droplet into the syringe (Figure 3C). Taken together, the Rep1-1 peptide self-assembles into a rigid highly surface-active protein membrane at the water-air interface.

Rep1-1 did not only assemble at the water-air interface, it also aggregated in the aqueous solution, as was shown by the reduction in the amount of soluble peptide

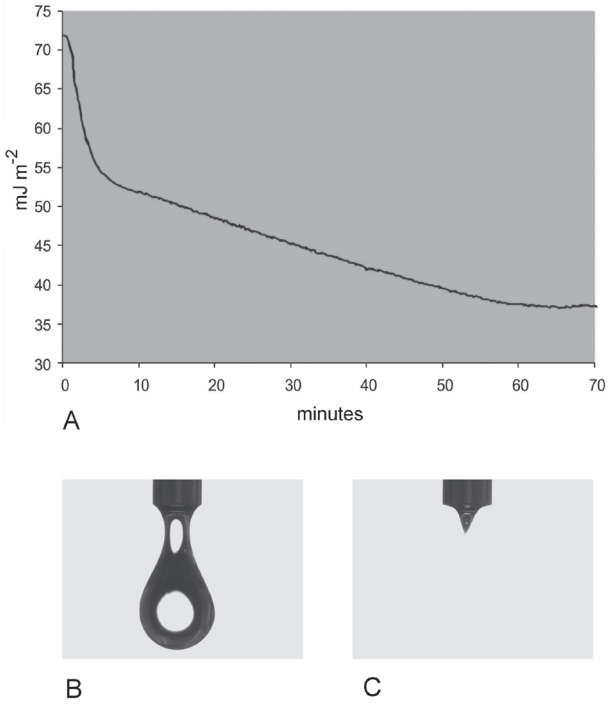

Figure 3: Rep1-1 (100 µg ml^{-1}) forms a highly surface-active protein film at the water-air interface. (A) Temporal reduction of the water surface tension of the aqueous Rep1-1 solution. (B) Reduction of the water surface tension is accompanied by a change of the shape of the hanging droplet. (C) A protein film remains attached to the tip of a syringe after sucking back the aqueous solution into the pipette.

as monitored by SDS-PAGE (Figure 4A). The aggregates that had already formed within a 30 min period could be pelleted upon centrifugation and were soluble again in water after TFA treatment (data not shown). Aggregate formation could be accelerated by shaking, probably explained by creating a large water-air interface (Figure 4A). Fluorescence of ThT was measured to address whether Rep1-1 forms amyloid-like fibrils upon aggregation. 30 µg ml^{-1} of Rep1-1 in water was incubated with ThT 30 min after solubilization with or without additional vortexing for 3 min. In both samples an increase in fluorescence at OD 485 was measured (Figure 4B). Amyloid fibril formation was also indicated by negative staining. Long fibrils were observed when 3 µl of a solution of 50 µg ml^{-1} Rep1-1 was dried down on a grid (Figure 4D). In contrast, a more amorphous structure was observed at 100 µg ml^{-1} (Figure 4E). Finally, an amyloid-like nature was indicated by X-ray diffraction (Figure 4C). Precipitated Rep1-1 showed a diffraction at 4.7 and 10 Å. These diffractions represent the inter β-strand distance, resulting from the main chain hydrogen bond, and the intersheet spacing between β-sheets (Dobson, 1999, Sunde & Blake, 1997), respectively. The diffraction at 3.7 Å is also often found in diffraction patterns of amyloids (Kirschner *et al*., 1987; Sikorski *et al*., 2003).

Repellents form amyloid fibrils in vivo

Scanning electron microscopy did not show amyloid fibrils at the surface of dikaryotic hyphae that grew out of a water droplet (Figure 5A). In contrast, hydrophobin amyloids could be clearly visualized at the surface of *A. niger* spores (Figure 5B). This result could be explained by absence of amyloid fibrils in vivo or by the high concentration of the repellents in the cell wall (see Figure 4E). To address this, cells of SG200 were stained with ThT. Yeast cells that had grown in the liquid medium did not stain with ThT (Figure 6F). In contrast, hyphae that had grown out of the

water droplet did stain (Figure 6B). However, this was also observed with a SG200Δ*rep1* strain (Figure 6D). This suggests that other cell wall proteins also form amyloids when exposed to air. Indeed, yeast cells of SG200 and SG200Δ*rep1* stained with ThT when they were air-dried (data not shown). Since these experiments did not indicate that repellents form amyloid in vivo, *rep1* was placed under regulation of the strong constitutive *O2tef* promoter in vector pMF3c-OR. Formation of hydrophobic aerial hyphae was restored when this vector was introduced in SG200Δ*rep1,* showing that the gene was functionally expressed. In contrast to yeast cells of SG200 (Figure 6F) or SG200Δ*rep1* (data not shown), yeast cells harboring pMF3-OR did stain with ThT when they had been grown in liquid (Figure 6H). Staining correlated with the presence of *rep1* mRNA as detected by Northern hybridization (data not shown). Taken together, it is concluded that repellents form amyloid in vivo but that also other cell wall proteins do so when they contact air.

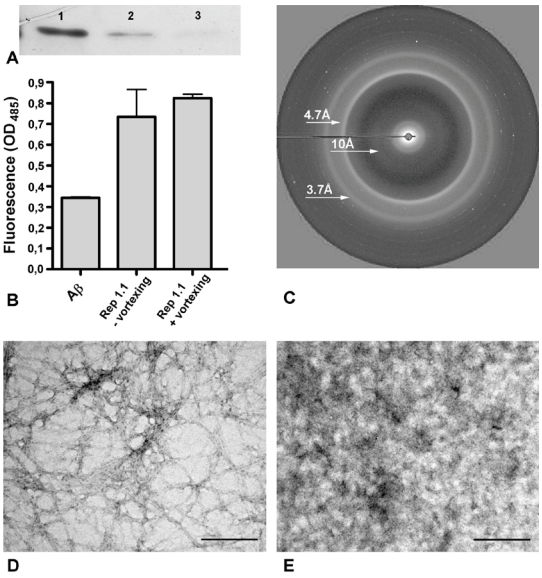

Figure 4: Rep1-1 forms amyloid-like fibrils. (A) SDS PAGE analysis of TFA treated Rep1-1 after dissolving in SDS sample buffer (lane 1), and after dissolving in PBS for 30 min (lane 2) followed by tumbling on a rotary table for 1 h (lane 3). (B) ThT fluorescence of Rep1-1 after dissolving for 30 min in water with and without subsequent vortexing for 3 min. β Alzheimer peptide Aß served as a positive control. (C) X-ray diffraction of Rep1-1 dissolved in water and vortexed for 3 min. (D, E) Negative staining of 50 (D) and 100 (E) µg ml^{-1} of Rep1-1 dissolved in water and dried down on a carbon-coated nickel grid. Bar indicates 200 nm.

DISCUSSION

Class I hydrophobins fulfill a wide spectrum of functions in filamentous fungi. These proteins function by forming a rigid, surface-active film consisting of amyloid-like fibrils (Wösten & de Vocht, 2000; Wösten, 2001). Recently, it was shown that repellents of *U. maydis* have replaced class I hydrophobins in forming hydrophobic aerial hyphae and in hyphal attachment to hydrophobic surfaces (**Chapter 3**; Teertstra *et al*., 2006). We here showed that repellents, like class I hydrophobins, are filament specific and that they form a rigid, surface active amyloid-like layer.

Previously, it was shown that *rep1* is 50-fold up-regulated in a compatible cross between FB1 and FB2 when compared to the parental strains (Wösten *et al*., 1996). However, expression in the monokaryons was still considerable (± 0.05 % of the mRNA). Therefore, we addressed whether yeast cells do express *rep1.* Using GFP as

a reporter, it was shown that *rep1* was only expressed in the few filaments formed by the FB1 strain. Expression was observed from the moment yeasts switched to filamentous growth. Hyphae in the FB1 strain were formed at the water-air interface and most of them grew out into the air. Formation of filaments was much more pronounced in the co-isogenic SG200 strain that contains a compatible combination of *a* and *b* mating-types genes. In this case, hyphae did not only grow into the air but also into the agar medium. Both the aerial and vegetative hyphae were shown to express GFP from the *rep1* promoter and the fluorescence intensity was similar to that of hyphae of FB1. Taken together, it is concluded that *rep1* is filament specific rather than mating type regulated as was concluded previously (Wösten *et al*., 1996).

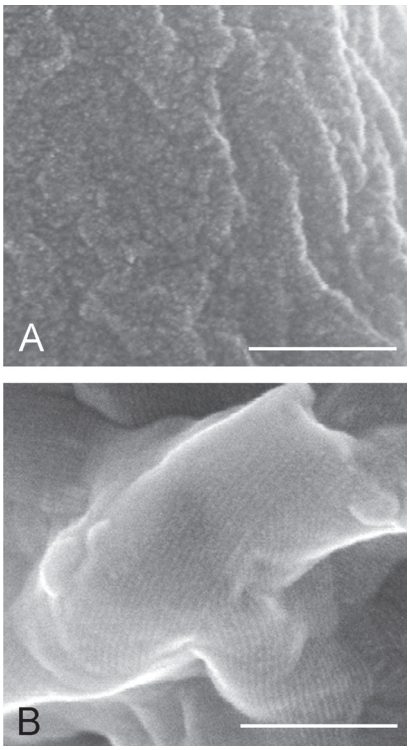

Figure 5: Scanning electron microscopy does not reveal rodlets at the surface of dikaryotic hyphae of *U. maydis* that have grown out of a water droplet (A). In contrast, hydrophobin rodlets were clearly visible at the surface of spores of *A. niger* (B). Bar indicates 200 nm.

In situ hybridization correlated with GFP fluorescence. Fluorescence was observed in filaments and not in yeast cells. Interestingly, in contrast to GFP, the *rep1* probe was mainly observed in the apical part of the hypha. This difference can be explained by the stability of the GFP protein and/or its diffusion in the cytoplasm. Our results indicate that the *rep1* mRNA is synthesized in the apex or is transported to this site. Trafficking of mRNA to the apex has been proposed to occur in *U. maydis* (Becht *et al*., 2006). In the case of the *rep1* mRNA this would make sense since the encoding protein is expected to leave the hyphae at the apex (Moukha *et al*., 1993; Wösten *et al*., 1991; 1994).

Repellents were originally identified by adopting the procedure to purify class I hydrophobins from cell walls (Wösten *et al*., 1996). Like the hydrophobins, repellents could only be extracted from the cell wall with TFA, after which these peptides were soluble in water or aqueous detergent (de Vries *et al*., 1993; Wösten *et al*., 1993; 1996). Synthetically produced Rep1-1 showed similar solubility as repellents and hydrophobins in the cell wall. It was only soluble in water or SDS after TFA treatment. Like the class I hydrophobin, the Rep1-1 peptide assembled into a surface-active rigid membrane at the water-air interface. The decrease in water

surface tension to 36 mJ m^{-2} is similar to that of hydrophobins and other microbial surface-active peptides involved in aerial growth and attachment (Claessen *et al*., 2003; Lugones *et al*., 2004; Tillotson *et al*., 1998; Wösten *et al*., 1999ab). The water-soluble form of the peptide, which seems to be an oligomer based on its behavior in SDS PAGE, also aggregated readily in a SDS-insoluble form in the aqueous solution. This contrasts the class I hydrophobins (Wösten *et al*., 1993), which have four disulfide bridges which prevent premature assembly in the aqueous environment (de Vocht *et al*., 2000). How the repellent prevents self-assembly in the cell is not yet known. The unprocessed pro-protein Rep1 may have a function in this.

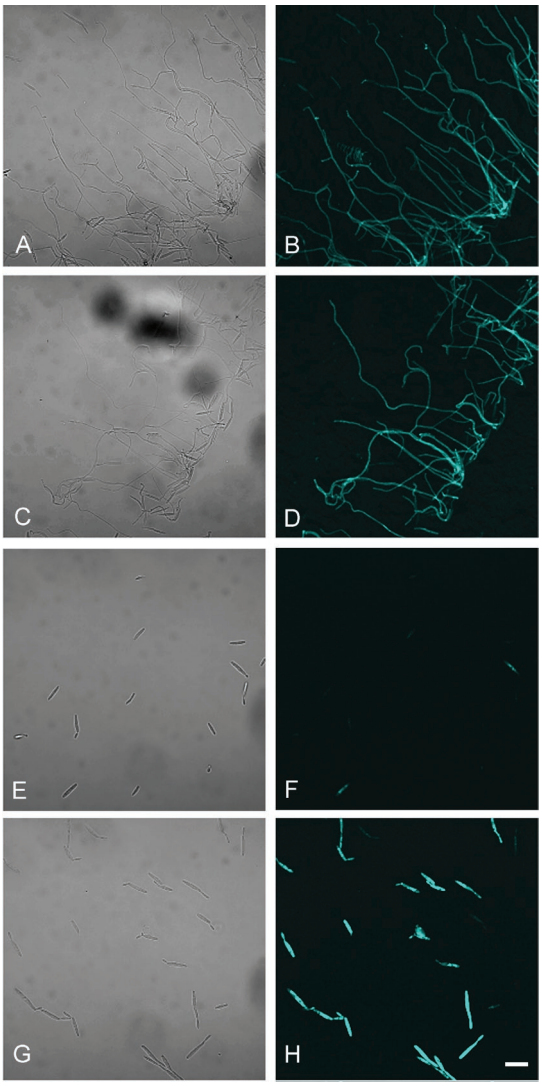

Figure 6: Visualization of amyloid fibrils by ThT fluorescence at the surface of yeast cells and hyphae of *U. maydis*. Bright field microscopy (A, C, E, G) and ThT staining (B, D, F, H) of SG200 (A, B, E, F), SG200Δ*rep1* (C, D) and SG200Δ*rep1*OR (G, H). Amyloid fibrils are detected at the surface of aerial hyphae of strain SG-200 (B) and its derivative SG200Δ*rep1*(D). In contrast, amyloid fibrils are not observed at the surface of yeast cells of strain SG200 grown in liquid culture (F). However, they are detected at yeast cells of SG200Δ*rep1*OR that express *rep1* from the constitutive *O2tef* promoter (H). Bar indicates 20 μm.

The SDS-insoluble form of the synthetic repellent was shown to be of an amyloid-like nature. It stained with ThT and it showed the typical X-ray diffraction pattern of amyloids. Scanning electron microscopy (SEM) did not reveal amyloid fibrils at the surface of filaments. This suggested that repellents do not form amyloid in vivo. However, Rep1-1 was shown not to aggregate in detectable long fibrils at a high concentration, as is assumed to be the case in the cell wall (Wösten *et al*., 1996). The absence of detectable fibrils at a high protein concentration can be explained by a high number of nucleation points, as was reported previously for hydrophobins (Wösten *et al*., 1994). ThT

staining was performed to strengthen the hypothesis that repellents do form amyloid in vivo. Yeast cells that do not express *rep1* in liquid medium did not stain with ThT. In contrast, these cells did stain when the gene was expressed from the constitutive *O2tef* promoter. Taken together, we conclude that at least Rep1-1 forms amyloid-like fibrils in vitro as well as in vivo. The similarity between the repellents (Wösten *et al*., 1996) suggests that other peptides of the family participate in this process. This is strengthened by the prediction that Rep1-9 and Rep1-10 have an aggregation tendency (Fernandez-Escamilla *et al*., 2004). Amyloid formation by a number of homologous peptides has previously been reported for the chaplins in the filamentous bacterium *Streptomyces coelicolor* (Claessen *et al*., 2003).

ThT staining was also observed at the surface of hyphae of the wild-type strain that express *rep1.* However, hyphae of a strain in which *rep1* was inactivated were also ThT-positive. This suggests that other cell wall proteins can also form amyloid-like structures. From the fact that wild-type yeasts became ThT positive after air-drying we conclude that contact with air is inductive for amyloid formation of one or more other cell wall proteins of *U. maydis*. Indeed, contact with hydrophobic surfaces has been shown to promote formation of these fibrils (Kowalewski & Holtzman, 1999; Schladitz *et al*., 1999; Wösten & de Vocht, 2000). The repellents do not seem to need contact with a hydrophilic-hydrophobic interface as is concluded from the properties of the synthetic Rep1-1 peptide and the staining of yeast cells that express *rep1* in liquid medium.

Amyloid-like fibrils also occur on bacterial cell surfaces. Chaplin fibrils coat filaments of *Streptomyces coelicolor* (Claessen *et al*., 2003), whereas curli and Tafi fibrils are present at the surface of *Escherichia* and *Salmonella* species (Chapman *et al*., 2002). The repellents are thus the fourth microbial class of surface proteins (and the second fungal class) that form functional amyloids. In addition, it seems that amyloid fibrils of the adhesive Als proteins cover the yeast cells of *Candida albicans* (Otoo *et al*., 2008; Rauceo *et al*., 2004). It has been proposed that formation of amyloids is a generic property of proteins (Chiti *et al*., 1999; Guijarro *et al*., 1998). This would explain why non-related proteins have evolved to serve similar functions in microbial development such as in formation of aerial hyphae and in attachment of cells.

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CHAPTER 5

Absence of repellents induces genes encoding small secreted proteins in *Ustilago maydis*

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To be submitted for publication

ABSTRACT
The *rep1* gene of the maize pathogen *Ustilago maydis* encodes a preproprotein that is processed in the secretory pathway into 11 peptides, called repellents. Strains in which the *rep1* gene is inactivated (Δ*rep1* strain) are affected in aerial hyphae formation. This makes these strains instrumental to assess changes in global gene expression as a consequence of aerial growth. Microarray analysis revealed that only 31 genes in the Δ*rep1* strain had a fold change in expression of ≥ 2. Twenty-two of these genes are up-regulated and half of them encode small secreted proteins (SSP's) with unknown functions. Seven of the SSP genes and two other genes that are over-expressed in the Δ*rep1* strain encode secreted cysteine-rich proteins (SCRP's). Such proteins are likely to represent apoplastic effectors that interact with the environment. The SCRP gene *um00792* showed the highest up-regulation in the Δ*rep1* strain. Using GFP as a reporter, it was shown that this gene is over-expressed in the layer of hyphae at the medium-air interface. These hyphae seem unable to escape from the agar medium to grow into the air. Taken together, only minor changes occur in the expression profile when *U. maydis* forms aerial structures. This contrasts the situation in *Schizophyllum commune* and *Streptomyces coelicolor*.

INTRODUCTION
The lifecycle of the pathogenic heterobasidiomycete *Ustilago maydis* is characterized by distinct morphological and nuclear states. Fusion of compatible yeast-like sporidia results in the formation of a pathogenic filamentous dikaryon. Upon formation of an appressorium the host (*Zea mays* [maize] and *Euchlaena mexicana* [Mexican teosinte]) is invaded. The fungus proliferates and branches inside the host, resulting in the formation of diploid teliospores. These spores are dispersed into the environment. After germination, meiosis occurs resulting in the formation of haploid sporidia (Banuett & Herskowitz, 1988, Banuett, 1992, Christensen, 1963).

Only when two partners harbor different alleles of the *a*- and *b*-loci, mating and pathogenic development can occur. Cell fusion is controlled by the *a1* and *a*2 mating-type loci (Bölker *et al*., 1992). These loci encode a pheromone and a receptor for the pheromone of the opposite mating type. The multi-allelic *b* locus regulates the post-mating processes of filament formation and pathogenic development. The *b* locus encodes two unrelated homeodomain proteins, bE and bW, that form active hetero-dimers when derived from different alleles (Gillissen *et al*., 1992, Kämper *et al*., 1995).

Rep1 is one of the genes that are regulated by the bE/bW heterodimer (Bohlmann, 1996, Brachmann *et al*., 2001, Romeis *et al*., 2000, Wösten *et al*., 1996). The *rep1* gene encodes a pre-pro-protein, which consists of a signal sequence for secretion and 12 repeated sequences. Processing at KEX2 recognition sites in the secretory

pathway results in 10 peptides of 34 - 53 amino acids and one larger protein of 229 amino acids (Wösten *et al*., 1996). These cleavage products that are collectively known as repellents are secreted into the cell wall of filaments. Here they form amyloid fibrils (**Chapter 4**; Teertstra *et al*., 2009), which are involved in attachment to hydrophobic surfaces and in formation of hydrophobic aerial hyphae (**Chapter 3,4**; Teertstra *et al*., 2006; 2009, Wösten *et al*., 1996).

Strains in which the *rep1* gene has been inactivated form colonies with only few aerial hyphae (**Chapter 3**; Teertstra *et al*., 2006, Wösten *et al*., 1996). Such strains can be used to assess global changes in gene expression as a consequence of formation of aerial hyphae. We here show that expression of only 31 genes is changed at least two-fold in the Δ*rep1* strain when compared with the parental strain under conditions of aerial growth. Most of these genes are up-regulated and encode for small secreted proteins without a predicted enzymatic function. Taking together, it is concluded that only minor changes occur in the expression profile when *U. maydis* forms aerial structures.

Primer Name	Sequence
RepMUF-fw	TTTGCGTATTCCACCTGCAGTAGCC
RepMDF-rev	CAACTACTGGGAAAAGTATGGAGCGG
pRep-fw	GGTACCGCAGCAATCACAGAG
cRep-rev	GCGGCCGCATGAGGAAACCCTAAC
pr792-fw	AAACTTGGGCCCGCTACCAG
pr792-rev	GGAGGAACAACGAGGATGAC
RepUF-fw	GGATGTAGCTGTCGTGCTTCCA
RepUF-rev	GGCCATCTAGGCCGTGATAATGT

Table 1: Primers used in this study.

MATERIAL AND METHODS

Strains and growth conditions

U. maydis strain SG200 (*a1mfa2 bE1/bW2*) (Bölker *et al*., 1995) and its derivatives (see below) were used in this study. SG200 is a haploid FB1 strain, which harbors an active b mating type complex. Consequently, the strain grows yeast-like in liquid medium and forms filaments at the water-air interface without the need for mating. *U. maydis* strains were routinely grown at 25 °C using liquid YEPSL medium (0.4 % yeast extract, 0.4 % peptone, 2 % sucrose) or potato dextrose agar (PDA, Sigma) that had either or not been supplemented with 1 % charcoal. For isolation of RNA for microarray analysis, cells were grown at 22 °C on nitrate minimal medium (NM^+) (Holliday, 1974) containing 2 % agar, 1 % charcoal, 20 mg l^{-1} histidine, 380 mg l^{-1} leucine, 20 mg l^{-1} tryptophan, 50 mg l^{-1} uracil and 76 mg l^{-1} Yeast Synthetic Drop-out Medium Supplements (Sigma, Y2001). For GFP expression analysis, 1 µl

of cell suspension (2 10^7 cells ml^{-1}) was seeded on each side of a 0.25 mm thin 20 x 20 mm square of solidified (1.5 % agarose) NM^+ medium that was sandwiched between a glass slide and a cover slip. Cells were grown at 25 °C under humid conditions.

Constructs

SG200Δ*rep1* was generated as described (Müller *et al*., 2008a). The PCR fragment that was used to inactivate *rep1* was amplified with oligonucleotide primers RepMUF-fw and RepMDF-rev (Table 1) using Phusion™ polymerase (Finnzymes). The resulting PCR fragment, consisting of a nourseothricin resistance cassette (Brachmann *et al*., 2004) flanked by the upstream and downstream sequences of the *rep1* gene, was used to transform the SG200 strain.

Vector pUC19-Rep-c was used to complement the SG200Δ*rep1* strain. A 3674 bp PCR fragment was amplified for its construction. This fragment consisted of a 1680 bp *rep1* promoter region and the coding sequence of this gene. For this, Phusion™ polymerase (Finnzymes) was used with genomic FB1 DNA as a template and oligonucleotide primers pRep-fw and cRep-rev (Table 1). The latter primer introduces a *Not*I-site at the 3' end. The 3674 bp PCR-fragment was introduced into pUC19 in the *SmaI*-site. In the next step, a *Not*I fragment encompassing a carboxin-resistance cassette (Brachmann *et al*., 2004) was introduced into the respective site of the pUC19 derivative, resulting in pUC19-Rep-c.

The promoter region of gene *um00792* was amplified from genomic DNA of FB1 using Phusion™ polymerase (Finnzymes) with oligonucleotide primers pr792-fw and pr792-rev (Table 1). The resulting 1837 bp fragment was introduced in the *Sma*I site of pUC19, resulting in vector pUC19-pr792. The 1791 bp *Apa*I /*Nco*I promoter fragment of *um00792* was digested from pUC19-pr792 and introduced in the corresponding sites of pMF3c (Brachmann *et al*., 2004). As a result, *eGFP* was placed under control of the *um00792* promoter. The resulting construct pMF3c-pr792 was linearized in the carboxin resistance cassette with *Age*I, thus targeting the DNA to the *sdh2*-locus of *U. maydis* upon transformation.

Transformation of *U. maydis*

U. maydis was transformed according to Brachmann et al. (Brachmann *et al*., 2004). Transformants were selected on PDA plates containing nourseothricin and carboxin at a final concentration of 150 µg and 2 µg ml^{-1}, respectively. Deletion of the *rep1* gene was confirmed by Southern analysis. *U. maydis* chromosomal DNA was isolated as described (Hoffman & Winston, 1987) and blotted onto Hybond N+ (Amersham). Hybridization was performed at 60 °C in 0.5 M phosphate buffer, pH 7.2, 7 % SDS, 10 mM EDTA. A 936-bp ^{32}P-α-dCTP labeled PCR-fragment was used as

a probe, which was derived from the *rep1* promoter region of FB1 using the primers RepUF-fw and RepUF-rev (Table 1).

Northern analysis
U. maydis cultures were homogenized with a microdismembrator (B.Braun). RNA was extracted with Trizol (Invitrogen) according to the manufacturer's protocol. Hybridization was performed using standard protocols (Sambrook *et al*., 1989). Internal ^{32}P-α-dCTP labeled fragments of genes *um00792*, *um00913* and *um03817* were used as a probe.

Microarray analysis
2 x10^7 cells of exponentially growing cultures of strains SG200 and SG200Δ*rep1* were seeded on solid NM$^+$-charcoal medium and grown for 48 h. Cells were harvested, frozen in liquid nitrogen, and homogenized with a dismembrator (Retsch). RNA was isolated with Trizol, after which double stranded cDNA was made according to the Affimetrix protocol (Affimetrix). The cDNA was purified over a cDNA clean up spin column, after which biotin labeled antisense cRNA was obtained using the Enzo BioArray HighYield RNA transcript labeling kit (ENZO Diagnostics). Concentration of the cRNA was determined using the NanoDrop N-1000 (Thermo Scientific) and quality of the cRNA was determined using the 2100 Bioanalyzer (Agilent Technologies). cRNA was fragmented according to the Affimetrix protocol and the resulting 30-200bp fragments were hybridized to the Affimetrix custom array (MIPIUstilagoA). Data analysis of biological triplicates was performed as described previously (Eichhorn *et al*., 2006). Genes with a P value of <0.01, whose expression was at least 2-fold changed, were filtered in Excel.

Analysis of protein sequences
Protein sequences of *U. maydis* were obtained from the MUMDB database (http://mips.gsf.de/genre/proj/ustilago) and analyzed for functional domains with SCOP (Murzin *et al*., 1995), SMART (Ponting *et al*., 1999), PFAM (Bateman *et al*., 2002), SignalP (Bendtsen *et al*., 2004), TANGO (Fernandez-Escamilla *et al*., 2004) and Waltz (Rousseau *et al*., 2008).

Fluorescence microscopy
Fluorescence microscopy was carried out using a Zeiss Axioscope 2PLUS equipped with a HBO 100 W mercury lamp and a Leica LFC 420C camera (2592x1944 pixels). GFP fluorescence was monitored using a FITC filter set.

RESULTS
Absence of repellents has a minor effect on overall gene expression in *U. maydis*
Gene *rep1* was deleted in strain SG200 by transformation with a PCR-fragment

consisting of a nourseothricin resistance cassette flanked by upstream and downstream sequences of *rep1*. Five out of 12 transformants showed loss of surface hydrophobicity and reduction of aerial hyphae formation on PDA charcoal. Southern analysis confirmed deletion of *rep1* in these strains. Introduction of pUC19-Rep-c encompassing the *rep1* gene restored formation of aerial hyphae, showing that the phenotype was solely caused by the gene deletion.

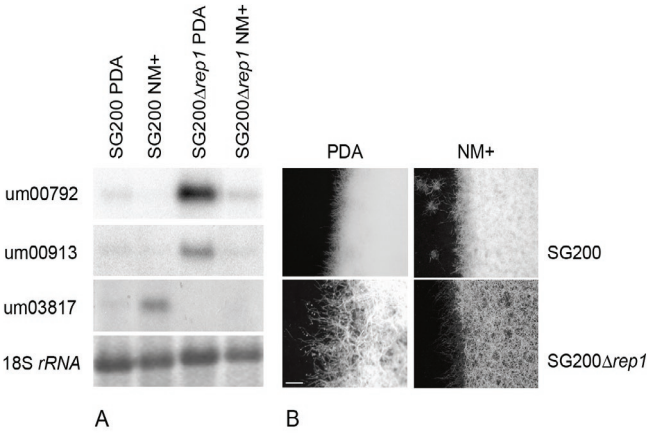

Figure 1: Inactivation of *rep1* results in reduced aerial hyphae formation and in changes in gene expression. (A) Northern analysis of genes *um00792*, *um00913* and *um03817* in strains SG200 and SG200Δ*rep1,* 18S *rRNA* serving as a control. Cultures were grown on PDA-charcoal and NM+-charcoal for 48 h. (B) Detail of colonies of the SG200 and SG200Δ*rep1* strain grown on PDA and NM+-charcoal plates. Colonies of strain SG200 form abundant aerial hyphae, whereas in the *rep1* deletion strain aerial hyphae formation is severely reduced. Bar represents 0.5 mm.

To monitor the effect of the *rep1* deletion on overall gene expression during aerial hyphae formation, DNA microarrays (representing ~90 % of the genes of *U. maydis*) were hybridized with RNA from SG200 and a SG200Δ*rep1* strain. To this end, both strains were grown for 2 days on a synthetic medium containing charcoal. SG200 formed abundant aerial hyphae, whereas aerial hyphae formation in the mutant strain was severely affected (Figure 1B). Thirty-one genes showed a fold change ≥2 when expression of the two strains was compared. Of these, 22 genes were up-regulated and 9 down-regulated in the SG200Δ*rep1* strain (Table 2). The microarray data were confirmed with Northern analysis for 3 genes (Figure 1A). This showed that expression of genes *um00792* and *um00913* was increased in the SG200Δ*rep1* strain compared to the parental strain, whereas expression of gene *um03817* was decreased. Expression of genes *um00792* and *um00913* was higher on PDA than on NM+-charcoal, which correlated with the number of aerial hyphae that were formed. In contrast, expression of *um03817* was more pronounced on solidified NM^+-charcoal. This indicates that expression of at least *um03817* is not only influenced by the *rep1* deletion, but also by the composition of the medium.

Of the genes that were down-regulated in the SG200Δ*rep1* strain, 2 encode secreted proteins. One of these genes is predicted to have enzymatic activity. This gene, *um11112*, is related to genes encoding Versicolorin B synthase. The *U. maydis* gene is proposed to encode a protein with aryl-alcohol oxidase activity (Müller *et al*., 2008b). The other gene, *um04248*, encodes a repetitive protein with no similarity to known proteins (Müller *et al*., 2008a). Eighteen out of the 22 genes with an increased expression in the SG200Δ*rep1* strain encode secreted proteins,

Table 2: Genes that change their expression 2-fold or more when *rep1* is inactivated. Properties of the secreted proteins are indicated.

um number	description	fold change	aa	TargetP secretion prediction	secretome* enzymatic function	secretome* SCRP cys class	TANGO** secreted protein b-aggregation score	WALTZ *** number amylogenic regions	gene deletion mutant	phenotype deletion mutant	references
um03924	**repellent precursor**	**0,01**	**652**	**RC2**				**14**	**single gene**	**reduced aerial hyphae, virulence unaffected**	**Teertstra et al., 2006**
genes up	**secreted**										
um00792	cons hyp U-spec protein	8,76	174	RC1	no	cys II	588	2	single gene	virulence unaffected	Vraneš., 2006
um00913	glyoxaloxidase	5,28	625	RC2	yes			7	single gene	virulence unaffected	Leuthner *et al*., 2005
um12258	hypothetical protein	4,60	142	RC1	no	cys V	515	1			
um01239	cons hyp U-spec protein	4,41	283	RC1	no		619	2	cluster2A	increased virulence	Kämper *et al*., 2006
um01377	hyp pr expansin	4,03	532	RC1	no		243	2			
um00793	cons hyp U-spec protein	3,72	160	RC1	no	cys II	620	1			
um03614	cons hyp pr	3,01	489	RC4	no	cys III	1225	6	cluster 9A	virulence unaffected	Kämper *et al*., 2006
um10418	hypothetical protein	2,74	146	RC1	no	cys III	1136	2			
um05495	cons hyp pr expansin	2,70	385	RC2	no			4			
um01375	hypothetical protein	2,55	118	RC2	no			2			
um01820	hypothetical protein	2,47	240	RC2	no		337	3	single gene	virulence unaffected	Vraneš., 2006
um06126	Mig2-6	2,38	404	RC2	no	cys IX		0			
um01240	cons hyp U-spec protein	2,32	279	RC1	no		797	1	single gene/ cluster2A	virulence unaffected / increased virulence	Vraneš., 2006; Kämper et al., 2006
um04422	rel xylanase	2,23	631	RC5	yes			2			
um03202	cons hyp U-spec protein	2,15	126	RC1	no	cys VI	15	1	cluster 8A	virulence unaffected	Kämper *et al*., 2006
um00081	put protein GPI	2,04	261	RC1	no	cys X	1018	5			
um01300	cons hyp U-spec protein	2,02	136	RC2	no	cys V	303	2	cluster 2B	virulence unaffected	Kämper *et al*., 2006
um05781.2	hypothetical protein	2,00	329	RC1	no		425	1			
	non-secreted										
um01374	hypothetical protein	2,88	435	no							
um03881	rel Hsp80	2,83	215	no							
um11883	put pr mitochondrion	2,37	621	RC4 no							
um11935	cons hyp U-spec protein	2,37	571	no							
genes down	**secreted**										
um11112	rel.versicolorin B synthase	0,27	599	RC3	yes						
um04248	putative protein	0,34	407	RC1	no		34	1			
	non-secreted										
um03817	putative protein	0,16	185	no							
um04106	transferase	0,20	465	no	yes						
um02050	cons hyp protein	0,29	3743	no							
um03398	rel esterase	0,41	401	no	yes						
um03117-2	cons hyp protein	0,43	615	no							
um04482	cons hyp protein	0,47	460	no							
um06459	rel Cyt P450	0,48	589	no							

* secretome: classification according to Müller et al., 2008b

** TANGO b-aggregation scores at pH 7.0 (Fernandez-Escamilla et al., 2004) //tango.crg.es/

*** Waltz amylogenic regions at pH7.0 (Rousseau et al., 2008) //switpc7.vub.ac.be/cgi-bin/submit.cgi

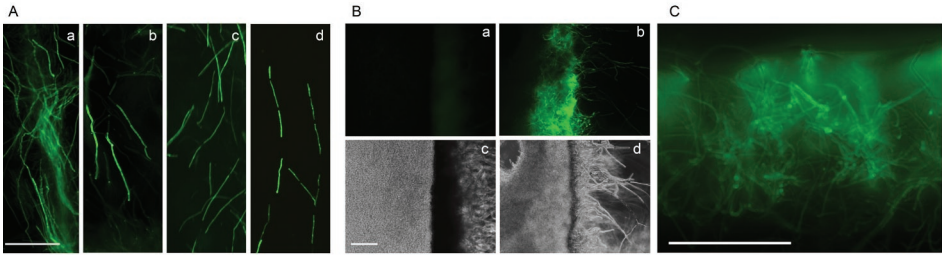

Figure 2: Localization of expression of *um00792* in the SG200 derivative uG114 and the SG200Δ*rep1* derivative uG115 using GFP as a reporter. Cells were seeded along a thin layer of solidified medium between a glass slide and a cover slip and grown for 40 h. Cells were monitored by phase contrast and fluorescence microscopy. (A) Individual hyphae of strain 114uG (a,b) and uG115 (c,d) growing in the substrate (a,c) or in the air (b,d) show similar fluorescence. (B) Strain uG114 (a,c) and strain uG115 (b,d) growing along the solidified medium. Total fluorescence of the top layer, consisting of yeasts and developing hyphae, is highly increased in the uG115 strain (b). (C) Detail of (B, b). Bar represents 100 µm.

as predicted by SignalP and TargetP (see Müller *et al*., 2008b). Several of these genes have already been deleted in *U. maydis*, either as a single or as a multiple gene deletion (Table 2; Kämper *et al*., 2006, Leuthner *et al*., 2005, Vraneš, 2006). Two upregulates genes encode secreted proteins with an enzymatic function. Gene *um00913* encodes glyoxaloxidase 2 (Leuthner *et al*., 2005), whereas *um04422* is predicted to encode an endo-xylanase (Müller *et al*., 2008b). Remarkably, the other 16 genes encode secreted proteins with no apparent enzymatic function. Eleven of them belong to the small secreted proteins (SSP's), which have an arbitrary size limit of 300 amino acids. Of these genes, 7 encode secreted cysteine-rich proteins (SCRP's), whereas another 2 upregulated SCRP's exeed 300 aa (Müller *et al*., 2008b). The TANGO algorithm (Fernandez-Escamilla *et al*., 2004) predicts that all these SCRP's except for *um06126*, (Mig2-6; Zheng *et al*., 2008) have aggregation-prone regions, whereas the Waltz algorithm (Rousseau *et al*., 2008) predicts that these SSP's have amylogenic regions as well. These properties are also predicted for the SSP's encoded by genes *um01239* and *um01240*.

Gene um00792 is highly induced in the SG200Δ*rep1* strain in the filaments growing on the substrate

The SCRP gene *um00792* shows the highest change in expression when strains SG200 and SG200Δ*rep1* are compared. Its expression is 8.75 fold increased in the SG200Δ*rep1* strain. Expression of this gene was monitored using GFP as a reporter. To this end, strains SG200 and SG200Δ*rep1* were transformed with construct pMF3c-pr792. This vector contains the *e-GFP* gene, which is under control of the *um00792* promoter. Transformants uG114 and uG115, which are derivatives of SG200 and SG200Δ*rep1* respectively, showed a fluorescence pattern illustrative for the majority of the fluorescent transformants and were therefore selected for further study. They were grown along a thin slab of nitrate minimal medium, sandwiched between an object glass and a cover slip. Aerial hyphae and hyphae growing in the substrate showed similar GFP fluorescence in strains uG114 and uG115 (Figure 2A). Gene

expression was not detected in yeast cells of both strains growing in liquid shaken cultures (data not shown), but yeast cells growing in and on the solid substrate did show fluorescence. At the medium-air interface, a 10-fold increase in fluorescence was shown in the SG200Δ*rep1* derivative uG115. This layer consists of yeast cells and filaments that have not yet escaped into the air (Figure 2BC).

DISCUSSION

Strains in which the *rep1* gene is inactivated form few aerial hyphae (**Chapter 3**; Teertstra *et al*., 2006, Wösten *et al*., 1996). We here used this phenotype to assess global changes in gene expression as a consequence of this developmental process. It is shown that deletion of this gene has a minor effect on global gene expression under conditions of aerial growth in the wild-type. Interestingly, a majority of the genes that are up-regulated at least two-fold encode small secreted proteins.

Gene expression at the stage of aerial hyphae formation was monitored with microarray analysis. Only 31 genes had a fold change in expression of at least two when expression profiles of strains SG200 and SG200Δ*rep1* were compared. Of these genes, 7 had a fold change of at least 4 and only 1 gene had a fold change of at least 8. This is a striking difference with gene regulation in the basidiomycete *Schizophyllum commune* and the filamentous bacterium *Streptomyces coelicolor*. These microorganisms produce repellent-like proteins; i.e. the hydrophobins in *S. commune* (Wösten *et al*., 1993, Wösten *et al*., 1994; 1999) and the chaplins in *S. coelicolor* (Claessen *et al*., 2003; 2004). Like repellents, hydrophobins and chaplins form functional amyloid fibrils at the hyphal surface (Butko *et al*., 2001, Claessen *et al*., 2003, Wösten & de Vocht, 2000). Inactivation of the genes encoding these proteins results in a phenotype similar to that of the Δ*rep1* strain (Claessen *et al*., 2003, van Wetter *et al*., 1996; 2000). Over 4000 genes had changed their expression at least two-fold under conditions of aerial growth when expression of a wild-type strain and the hydrophobin knockout strain Δ*SC3*Δ*SC4* was compared (R.A.Ohm, H.A.B. Wösten, unpublished results). In *S. coelicolor* 244 genes had a changed expression in the Δ*chp* strain (de Jong *et al*., 2009). Transcriptional regulators were among the genes with an affected expression in both *S. commune* and *S. coelicolor*. In contrast, genes encoding transcriptional activators were not affected in the Δ*rep1* strain. How can we explain this result? In *S. commune* and *S. coelicolor,* aerial hyphae formation is the first step in a differentiation process leading to the formation of fruiting bodies and spore chains, respectively. In contrast, aerial hyphae of *U. maydis* may only represent vegetative hyphae that happen to grow into the air.

Remarkably, most of the genes that changed their expression in the Δ*rep1* strain encode secreted proteins without an enzymatic function. Eleven out of 22 genes that are up-regulated encode small secreted proteins (SSP's) and 9 of them belong to the

class of secreted cysteine-rich proteins (SCRP's). These proteins harbor no common protein domains. For these proteins, similarity in three-dimensional structure may be more important than sequence similarity. This is emphasized by the TANGO and Waltz algorithms (Fernandez-Escamilla *et al*., 2004, Rousseau *et al*., 2008) that predict β-aggregation and amylogenic regions in more than 80 % of the SSP's and SCRP's. We previously showed that the repellents form amyloids in the cell wall of hyphae of *U. maydis* (**Chapter 4**; Teertstra *et al*., 2009). Interestingly, filaments of the SG200Δ*rep1* strain also stained with ThT, from which it was concluded that other secreted proteins also have the capability to form amyloid fibrils. The SSP's that are up-regulated in SG200Δ*rep1* could be candidates for such proteins.

Twelve clusters are found in the genome of *U. maydis* that contain genes encoding SSP's (Kämper *et al*., 2006). Deletion of five of them changed virulence (Kämper *et al*., 2006). Four of these clusters contain SSP's that are up-regulated in the Δ*rep1* strain (see Table 2). The symbiotic basidiomycete *Laccaria bicolor* also harbors a high number of genes encoding SSP's. Ten percent of the secreted proteins belong to this class, many of which are classified as SCRP's. Part of these genes is up-regulated during mycorrhizal interactions, whereas others are down-regulated. This suggests an important role for these proteins in the symbiosis (Martin *et al*., 2008). SCRP's of fungi and oomycetes have been described to function as apoplastic effectors (Kamoun, 2006). Such effectors include toxins, elicitors, virulence and avirulence proteins. The cysteine residues have been proposed to enhance stability of the proteins and yield protection to plant proteases (Joosten *et al*., 1997, Luderer *et al*., 2002). Why the *U.maydis* SSP and SCRP genes change their expression in the absence of repellents remains to be investigated.

The highest up-regulated gene in the Δ*rep1* strain is gene *um00792*. It encodes a SCRP of 144 amino acids with 7 cysteines. The gene is clustered in the genome with genes *um00793*, *um00794* and *um00795.* It has 28 % sequence homology with gene *um00793*, which is also up-regulated in the SG200Δ*rep1* strain. Deletion of gene *um00792* has no influence on pathogenicity (Vraneš, 2006) but its expression depends on *biz1*. This gene encodes a transcription factor, which functions at the stage of filament and appressorium formation (Flor-Parra *et al*., 2006). It down-regulates *clb1*, the mitotic cyclin, (Flor-Parra *et al*., 2006) and thereby causes G2 cell cycle arrest prior to plant invasion. Of interest, expression of *um06126, um01240, um01820* and *um01377* is also increased in the absence of repellents and decreased in the absence of Biz1 (This study; Vraneš, 2006). The latter indicates a role for these genes in the early of stages of pathogenic development. At the moment we cannot explain why these genes are up-regulated in the Δ*rep1* strain.

Expression of gene um00792 was followed using GFP as a reporter. Expression

of this gene was detected in strains SG200 and SG200Δ*rep1* in aerial hyphae and hyphae growing in the substrate. Gene expression was not detected in yeast cells growing in liquid culture, but yeast cells growing in and on the solid substrate, where hyphal growth is initiated, did show fluorescence. Strikingly, over-expression of um00792 was only detected in the Δ*rep1* strain in the layer of seeded cells that lay on the solid substrate. This layer contains many hyphae that seem unable to escape from the extracellular matrix. Possibly, the fungus senses absence of the repellents in the extracellular matrix, causing up-regulation of gene *um00792*. The protein encoded by this gene, however, is not able to complement repellents in formation of aerial hyphae. Future studies should reveal the role of the SSP's and SCRP's that are upregulated in *U. maydis* upon inactivation of the *rep1* gene. It may well be that the genes are redundant, requiring multiple knock-outs before a phenotype will be observed.

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CHAPTER 6

Summary and general discussion

The fungus *Ustilago maydis* is an important model organism to study fungal pathogenicity and fungal dimorphism. *U. maydis* is the causal agent of smut in maize. Infected corn causes severe economic losses but is also considered a delicacy. The life cycle of *U. maydis* starts with mating of haploid sporidia with opposite mating types. As a result, a dikaryon is formed that grows filamentous on an artificial medium or on the surface of the host. These filaments can also grow into the air and can invade the host. The latter is needed for the completion of the life cycle. Within the plant, tumors are being produced in which *U. maydis* proliferates. This is followed by formation of diploid teliospores. These spores are dispersed and give rise to sporidia following meioses.

In this Thesis the expression and function of hydrophobins and repellents of *U. maydis* were studied. Hydrophobins are produced by the higher fungi (ascomycetes and basidiomycetes) where they fulfill a wide spectrum of functions (Wösten, 2001). These proteins enable hyphae to grow out of a moist environment into the air and make aerial structures hydrophobic. The hydrophobic coating prevents aerial hyphae to fall back into the moist substrate, prevent collapse of air channels in fruiting bodies, and function in dispersal of spores via the air. Hydrophobins also attach filaments and spores to hydrophobic surfaces such as that of a host, and mediate signaling thereof. SC3 of the basidiomycete *Schizophyllum commune* is the best-studied hydrophobin. It mediates escape of hyphae from the aqueous environment into the air and it coats aerial hyphae. Moreover, SC3 attaches hyphae of *S. commune* to a hydrophobic surface. Hydrophobins can be isolated from SDS-treated cell walls of aerial hyphae with trifluoroacetic acid. Using a similar protocol, repellents, but not hydrophobins, were isolated from cell walls of aerial hyphae of *U. maydis* (Wösten *et al*., 1996). This novel class of peptides was absent in cell walls of sporidia.

Repellents are encoded by a single gene called *rep1.* This gene encodes a pre-pro-protein that is processed in the endoplasmic reticulum into 10 small proteins with a size of 34 - 55 amino acids, called Rep1-1 to Rep1-10 and one larger peptide, Rep1-C, of 229 aa. The phenotype of a strain in which the *rep1* gene is inactivated was partially characterized. It was shown that formation of aerial hyphae and surface hydrophobicity were severely affected. This phenotype is similar to that of the *SC*3 deletion strain of *S. commune* (van Wetter *et al*., 1996, Wösten *et al*., 1994). These data suggest that repellents have partially replaced hydrophobins in *U. maydis.* Alternatively, repellents might function indirectly for instance by anchoring hydrophobins to the cell wall (Wösten *et al*., 1996). The latter could not be excluded since two hydrophobins were identified in the genome of *U. maydis* (**Chapter 3**).

Expression of hydrophobins and repellents in *S. commune and U. maydis*

Fluorescent in situ hybridization (FISH) is a powerful tool to study localization of gene expression. In contrast to reporter proteins, this technique is independent of translation of the RNA. However, DNA probes that were previously used in in situ hybridizations have major disadvantages in filamentous fungi. Since DNA probes are unable to diffuse through the fungal cell wall, experiments were performed on sections (de Groot *et al*., 1999, Peñas *et al*., 1998). In this way, only the sectioned part of the hypha is accessible; a longitudinal overview cannot be obtained. Permeabilization of the cell wall prior to hybridization is an alternative way to introduce the DNA probe into the cell. This method, however, is elaborate and hard to reproduce. Peptide nucleic acid (PNA) probes offer a good alternative for DNA probes in in situ hybridizations. PNA is a DNA mimic with a peptide backbone (Egholm *et al*., 1993, Nielsen *et al*., 1994). PNA is uncharged and thus allows diffusion through cell walls of bacteria and yeasts without the need for prior treatment (Rigby *et al*., 2002, Stender *et al*., 2001). Moreover, PNA probes have a more rapid and stronger binding with complementary strands of DNA (Egholm *et al*., 1993) and are not prone to degradation by nucleases and proteases (Demidov *et al*., 1994). In **Chapter 2** an easy and reproducible in situ hybridization protocol is described based on PNA probes for detection of RNA in fungal hyphae. Transcripts of the hydrophobin gene *SC3* were detected in all hyphae at the periphery of a 4-day-old colony of *S. commune*. Interestingly, differences in *SC3* mRNA levels were observed between hyphae. This suggests the existence of heterogeneity in gene expression similar to that observed in *A. niger* (Vinck *et al*., 2005). Signals of a probe for 18S rRNA were also variable and the intensity did not correlate with that of the *SC3* probe in a co-hybridization experiment. This raises the question whether *SC3* mRNA is translated with the same efficiency in the different hyphae. The findings imply that high accumulation of mRNA, as assessed by Northern analysis, not necessarily correlates with a similar increase in protein.

Transcripts of 18S rDNA were more abundant in apical compartments of hyphae of *S. commune*. Since co-hybridization experiments showed that *SC*3 mRNA was more evenly distributed in the hyphae, it was concluded that these differences did not result from uneven penetration of the probe. This suggests that the apical compartment of hyphae of *S. commune* is most active in protein synthesis. Distribution of 18S rRNA was even heterogenic within the apical compartment. Signals for this RNA, but not for that of *SC3*, were much weaker in the extreme apex. This indicates that ribosomes are not evenly distributed in the apical compartment. This finding is consistent with transmission electron microscopy studies. Heath (1994) showed that ribosomes are less abundant at the apex of fungal filaments when compared to sub-apical parts.

The in situ hybridization protocol developed in **Chapter 2** was used to localize *rep1* mRNA and 18S rRNA in *U. maydis* (**Chapter 4**). 18S rRNA was detected in the yeast form as well as in the hyphae. Similar to *S. commune*, apical compartments of filaments of *U. maydis* showed a stronger labeling with the 18S rRNA probe. However, in *U. maydis* labeling increased towards the tip. These results imply that in both *S. commune* and *U. maydis* rRNA is more abundant in the apical compartment. However, the spatial distribution of the ribosomes within this compartment seems to be different in these basidiomycetes.

In *U. maydis*, *rep1* mRNA was exclusively observed in filaments. The highest concentration was detected at the apex of the first hyphal compartment. Considering the fact that the nuclei are located more subapically, this implies that *rep1* mRNA is transported to the apex. Active transport of mRNA to the apex has been shown to occur in *U. maydis* by RNA recognition motif proteins (Rrm) (Becht *et al*., 2006, König *et al*., 2009), such as Rrm4. However, experimental evidence so far indicates that the cargo of the Rrm4 protein consists of mRNA's encoding intracellular proteins. Translation of mRNA's of proteins destined for secretion is localized at the ER. The signal recognition particle (SRP) recognizes the N-terminal signal peptide in a ribosome-nascent chain complex (RNC) and targets this complex to the translocon of the ER, where further translation takes place (Egea *et al*., 2005). Since secretion of SC3 and repellents is expected to occur at the apex (Moukha *et al*., 1993, Wösten *et al*., 1991, Wösten *et al*., 1994), translation of the mRNA in its vicinity makes sense.

Repellents have replaced hydrophobins in *U. maydis* during aerial growth

The genome of *U. maydis* contains two hydrophobin genes (**Chapter 3**). Gene *hum*2 (um05010) encodes a typical class I hydrophobin of 117 amino acids with eight conserved cysteine residues. In contrast, the hydrophobin encoded by *hum*3 (um04433) is atypical. The encoded protein consists of a class I hydrophobin, which is preceded by a signal sequence and 17 imperfect repeats of 31 - 36 amino acids. Thirteen of these end with a KEX2 recognition site, indicating that they are processed in the ER. Processing would result in a hydrophobin that is 53% identical to Hum2 and 13 peptides, named questellents. The hydropathy pattern of these peptides is highly similar to that of the repellents. Gene *hum*2, but not *hum3* was expressed during aerial hyphae formation. Deletion of *hum2* only slightly reduced formation of aerial hyphae and surface hydrophobicity, while inactivation of *hum3* in the wild-type or in the Δ*hum2* strain had no effect. In contrast, inactivation of *rep1* dramatically affected surface hydrophobicity and aerial hyphae formation.

Deletion of the hydrophobin genes had no effect on attachment of hyphae to a hydrophobic substrate but attachment was reduced by 50% in a cross of *rep1* deletion strains. Additional deletion of either or both the hydrophobin genes did not

further reduce attachment. From these data it was concluded that hydrophobins have been functionally replaced in *U. maydis* by repellents. Thus, repellents do not function by anchoring hydrophobins to the cell wall. Also in *S. commune* a protein has been identified, called SC15, that is not a hydrophobin but that does fulfill a role in aerial hyphae formation and in attachment. Disruption of the *SC*3 gene in *S. commune* reduced aerial hyphae formation and attachment of hyphae to a hydrophobic substrate (van Wetter *et al*., 1996, Wösten *et al*., 1994). These processes were completely abolished when the *SC15* gene was also inactivated (Lugones *et al*., 2004). Deletion of *SC15* in a wild-type strain had no effect on aerial hyphae formation on synthetic medium. However, aerial hyphae formation was diminished during growth on wood. Like SC3, SC15 is a surface-active protein, albeit to a lower extent. This explains why SC15 can fulfill functions similar to that of SC3. Taken together, it is concluded that repellents, hydrophobins and SC15 function in formation of aerial hyphae and in attachment. Hydrophobins are the most important proteins for these processes in *S. commune*, while repellents are the most important in *U. maydis.*

Inactivation of the hydrophobin and repellent genes individually or in combination did not affect pathogenicity (**Chapter 3**). Similarly, deletion of *rsp1* (repetitive secreted protein 1) had no effect on the infection process (Müller *et al*., 2008). However, deletion of both *hum3* and *rsp1* completely abolished pathogenicity. The double mutant was able to penetrate the host, but hyphal growth stopped early in infection (Müller *et al*., 2008). Like *hum3* and *rep1*, *rsp1* encodes a secreted protein that consists of repeats that are separated by KEX2 sites (Müller *et al*., 2008). The eleven repeats have a hydrophilic nature rather than the amphipathic nature as found in repellents and questellents. These data indicate that hydrophobins and repetitive proteins may act in concert or fulfill redundant functions in *U. maydis* during pathogenicity.

It should be noted that the highly effective infection method that was used to assess the role of the hydrophobin genes and *rsp1* and *rep1* in pathogenicity might not reveal the natural situation. In the infection assay, strains are introduced by injection in the stem or leaf whorl or by adding droplets on the leaf using 10^5 to10^7 cells. In nature, cells may be far less abundant at the plant surface. Especially penetration defects may not be noticed when the place of infection is saturated with fungal cells (Flor-Parra *et al*., 2007). Taken together, it would be worthwhile to assess pathogenicity of the hydrophobin, repellent and *rsp1* deletion strains under more natural conditions. Also the Δ*rep1*Δ*rsp1* strains should be tested. Future studies should also reveal whether hydrophobins and repellents have a role in spore dispersal and survival.

Repellents form functional amyloids

The class I hydrophobins SC3 and EAS of *S. commune* and *Neurospora crassa* respectively, self-assemble into amyloid fibrils at hydrophobic-hydrophilic interfaces (Butko *et al*., 2001, Kwan *et al*., 2006, Wösten & de Vocht, 2000). Amyloids represent an ordered fibrillar structure of proteins in a stacked β-sheet conformation. The β-strands have a characteristic distance of 4.7 Å. Since repellents and hydrophobins have similar functions and solubility characteristics (**Chapter 3**), I hypothesized that repellents may also form amyloids in the cell wall. In **Chapter 4** this was tested using the synthetic peptide Rep1-1. This peptide of 34 amino acids had similar solubility characteristics as repellents isolated from cell walls. This suggests that its behavior is representative for these peptides under natural conditions. Rep1-1 could only be solubilized in water after TFA treatment. In contrast to hydrophobins, Rep1-1 rapidly aggregated in the aqueous solution without shaking or vortexing. This difference may be explained by the absence of intra-cellular disulfide bridges in the repellents. These bridges prevent premature self-assembly of hydrophobins in aqueous solution (de Vocht *et al*., 2000). This raises the question how premature self-assembly of repellents is prevented in the cell. Possibly, chaperones play a protective role.

Like SC3, Rep1-1 formed a highly surface-active protein film that lowered the water-surface tension to as low as 36 mJ m^{-2}. Within the rigid protein film, Rep 1-1 formed fibrils with an amyloid nature, as was shown by thioflavinT (ThT) fluorescence, negative staining and X-ray diffraction. Repellents were also shown to form amyloids in vivo. Yeast cells from a liquid culture that expressed the *rep1* gene from a constitutive promoter stained positive with ThT. In contrast, wild type yeast cells, which do not express *rep1*, did not stain. Repellents thus represent the second fungal class of proteins that forms functional amphipathic amyloid fibrils. This property explains why repellents can fulfill hydrophobin-like functions.

Filaments of *U. maydis* that express *rep1* stained with ThT. Remarkably, air-exposed wild-type yeasts, but not yeast cells from a liquid medium, also stained with ThT. Moreover, filaments of Δ*rep1* strains were ThT-positive. These data indicate that other cell wall proteins also form amyloids when *U. maydis* cells contact a hydrophobic substrate like air. It has been proposed (Kowalewski & Holtzman, 1999, Schladitz *et al*., 1999) that the water-air interface acts as a denaturing surface, which increases the chance of amyloid formation. Amyloids also have been shown to occur on bacterial cell surfaces (Chapman *et al*., 2002, Claessen *et al*., 2003, Larsen *et al*., 2007) and at the surface of yeast cells of *Candida albicans* (Otoo *et al*., 2008, Rauceo *et al*., 2004). These data indicate that amyloid fibrils generally occur on microbial surfaces and that a wide variety of proteins can form these structures at these surfaces.

At the moment, it is not clear whether repellents solely form amyloids at the surface of filaments of *U. maydis* or that a mixture of proteins forms these structures. The TANGO (Fernandez-Escamilla *et al*., 2004) and Waltz algorithm (Rousseau *et al*., 2008) predict that a variety of putative secreted proteins are prone to form amyloid fibrils (see below; **Chapter 5**). Many of these proteins do not have a predicted enzymatic function. Future experiments should reveal whether these proteins indeed form amyloid fibrils in the presence of repellent peptides. It may be that the high concentration of repellents fills the cell wall-air interface, thus preventing other proteins to become dysfunctional by adopting the amyloid conformation.

Absence of repellents induces expression of SSP's

In **Chapter 5** gene expression of SG200 was compared to that of a strain with a deletion of *rep1* (SG200Δ*rep1*). RNA was isolated at the moment SG200, but not SG200Δ*rep1*, formed aerial hyphae. Whole genome micro-array analysis showed that only 31 genes had a ≥ 2-fold change in expression. Of these, 7 genes had a fold change of at least 4, whereas only 1 gene had a fold change of at least 8. From these results it is concluded that gene expression is hardly changed at the moment aerial hyphae are formed. This contrasts the situation in *S. commune* and *S. coelicolor* (R.A.Ohm, H.A.B. Wösten, unpublished results; de Jong *et al*., 2009). Expression of a few thousand and few hundred genes, respectively, changed upon deletion of hydrophobin and chaplin genes. These deletions cause a similar phenotype as the inactivation of *rep1* deletion in *U. maydis.* Transcriptional regulators were among the genes with an affected expression in *S. commune* and *S. coelicolor*. This was not observed in the Δ*rep1* strain. How can this result be explained? In *S. commune* and *S. coelicolor,* aerial hyphae formation is the first step in formation of reproductive structures. In contrast, aerial hyphae of *U. maydis* may not result from a differentiation process. Rather, they may represent vegetative hyphae that happen to grow in the air.

Of the 31 genes with a changed gene expression in SG200Δ*rep1*, 22 genes were up-regulated. Of these, 18 are predicted to encode secreted proteins, of which 16 represent proteins with no apparent enzymatic function. Eleven of them belong to the small secreted proteins (SSP's), which have an arbitrary size limit of 300 amino acids. The genome of *U. maydis* harbors twelve clusters that contain genes for SSP's (Kämper *et al*., 2006). Deletion of five of these clusters altered the virulence of *U. maydis* (Kämper *et al*., 2006). For instance, inactivation of cluster 2A, containing 2 SSP's that are up-regulated in the Δ*rep1* strain, increased the virulence of the smut. The Waltz algorithm (Rousseau *et al*., 2008) predicts that more than 80% of the up-regulated SSP's have amylogenic regions. These proteins are excellent candidates to form amyloid fibrils together with or in the absence of repellents.

The SSP gene um00792 showed the highest up-regulation in Δ*rep1* strain. Interestingly, expression of this gene and 4 other up-regulated genes depends on Biz1. This transcription factor acts at the stage of infection and appressorium formation. Using GFP as a reporter, it was shown that gene um00792 is over-expressed in the layer of hyphae at the medium-air interface. These hyphae seem unable to escape from the agar medium to grow into the air, once more illustrating the function of *rep1*. Future studies should reveal the role of the SSP's that are up-regulated in *U. maydis* upon inactivation of the *rep1* gene.

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Summary and general discussion

APPENDIX

- Samenvatting
- Dankwoord
- Curriculum vitae
- Publications

SAMENVATTING

De schimmel *Ustilago maydis* veroorzaakt de ziekte builenbrand bij maïs. Maïs die door *U. maydis* geïnfecteerd is zorgt voor grote economische verliezen, maar tegelijkertijd wordt de geïnfecteerde maïskolf als een delicatesse beschouwd. In Mexico worden dergelijke kolven als huitlacoche gegeten en er bestaat een markt voor verkoop van opzettelijk geïnfecteerde maïs, ook in sommige staten van de USA. In het laboratorium is *U. maydis* een belangrijk modelorganisme voor onderzoek aan ziekteverwekkende schimmels van planten. De levenscyclus van *U. maydis* begint als twee haploïde gist-cellen versmelten die elk een ander sex-type hebben. Dit resulteert in een cel met twee kernen, het zogenaamde dikaryon. Dit dikaryon groeit niet langer als een gist maar vormt schimmeldraden. Deze schimmeldraden kunnen de lucht in groeien, maar ook zijn ze in staat de gastheer te infecteren. Dit laatste is een vereiste om de levenscyclus geheel te doorlopen. Eenmaal in de plant veroorzaakt de schimmel de vorming van tumoren, waarbinnen de schimmel zich vermeerdert. In de tumoren worden uiteindelijk diploïde teliosporen gemaakt. Deze sporen worden in de omgeving verspreid en vormen na meiose weer gistcellen met een enkele kern.

In dit proefschrift werd de aanmaak en de functie van hydrofobines en repellents in *U. maydis* bestudeerd. Hydrofobines worden door de meeste schimmels gemaakt. Het zijn kleine eiwitten van ongeveer 100 aminozuren die tal van functies vervullen. Zo zorgen zij ervoor dat schimmeldraden in staat zijn om vanuit een waterige omgeving de lucht in te groeien, voorkomen zij dat luchtkanaaltjes in paddenstoelen dichtslaan onder natte omstandigheden en zorgen zij ervoor dat sporen zich kunnen verspreiden door de lucht. Ook zorgen hydrofobines ervoor dat schimmels en sporen zich kunnen hechten aan het waterafstotende oppervlak van de gastheer en geven zij het signaal van hechting door. SC3 van *Schizophyllum commune* (het waaiertje in het nederlands) is het best bestudeerde hydrofobine. Dit eiwit verandert van vorm op een grensvlak van water en een waterafstotend oppervlak (zoals lucht, olie of het oppervlak van de mens en veel planten). Hierdoor ontstaat een laagje dat aan de ene kant waterminnend is en aan de andere kant waterafstotend. Dit zogenaamde amfipathische membraan verlaagt de oppervlaktespanning van water enorm en zorgt er hierdoor voor dat de schimmel kan ontsnappen uit een waterige omgeving. Voorts maakt dit laagje schimmeldraden die in de lucht groeien waterafstotend en zorgt het voor hechting van schimmeldraden aan een waterafstotend oppervlak. Het hydrofobine laagje is zeer onoplosbaar en kan slechts met het oplosmiddel trifluorazijnzuur (TFA) worden opgelost. Gebruikmakend van hetzelfde protocol werden geen hydrofobines maar repellents van het oppervlak van de schimmeldraden (de zogenaamde celwanden) van *U. maydis* geïsoleerd.

Deze nieuwe klasse van peptiden (peptiden zijn hele kleine eiwitten, in dit geval van 34-55 aminozuren) was niet aanwezig in celwanden van de gisten van deze schimmel.

Eén enkel gen, genaamd *rep1*, codeert voor alle repellents. In de cel wordt de code voor transport na gebruik verwijderd en vervolgens wordt het eiwit in 10 kleine peptiden en één groter eiwit gesplitst, waarna de eiwitten worden uitgescheiden in de celwand. Het uiterlijk van een *U. maydis* stam waarin de repellents niet meer werden gevormd leek erg op dat van een *S. commune* stam waarin het *SC3* gen was uitgeschakeld. In beide gevallen was de waterafstotendheid van het kolonieoppervlak sterk verminderd en groeiden schimmeldraden nog nauwelijks de lucht in. Dit suggereert dat in *U. maydis* de repellents de hydrofobines (gedeeltelijk) vervangen hebben. Een alternatieve verklaring was dat repellents hydrofobines verankeren in de celwand.

De expressie van hydrofobines en repellents in *S. commune* en *U. maydis*
Om de lokalisatie van genexpressie te bestuderen is gereedschap nodig. Met behulp van de techniek FISH (fluorescente in situ hybridisatie) wordt boodschapper RNA in een cel aangetoond door fluorescent DNA te laten binden aan dit RNA. Probes van DNA zijn in schimmels echter niet bruikbaar. Doordat zij geladen zijn kunnen zij de celwand niet doordringen. Hun gebruik is daarom alleen mogelijk op doorgesneden cellen. Deze optie is voor schimmels niet aantrekkelijk omdat een schimmeldraad is opgebouwd uit zeer langgerekte draden die zich moeilijk over de volle lengte laten snijden. Een goed alternatief voor DNA probes zijn de PNA probes. Deze lijken op DNA, met dezelfde GATC codes, maar hebben als ruggengraat geen geladen suikers en fosfaat maar een peptide. Doordat PNA probes niet geladen zijn kunnen zij wel door de celwand van schimmels dringen. In **Hoofdstuk 2** wordt een nieuw protocol beschreven om met behulp van fluorescente PNA probes boodschapper RNA in schimmels aan te tonen. In *S. commune* wordt dit protocol gebruikt om de hoeveelheid en plaats van het *SC3* boodschapper RNA te vergelijken met dat van het ribosomale 18S RNA, dat een deel van het ribosoom is. Ribosomen zijn de machines binnen de cel waarmee boodschapper RNA wordt vertaald naar eiwit. Het bleek dat de verdeling van beide RNA's in de cel niet hetzelfde was en dat de onderlinge hoeveelheden ook per cel verschilde. Dit resultaat suggereert dat er binnen de kolonie differentiatie bestaat, waarbij bepaalde schimmeldraden zich toeleggen op bepaalde functies.

Hetzelfde protocol dat ontwikkeld werd in **Hoofdstuk 2** werd in **Hoofdstuk 4** gebruikt om repellent boodschapper RNA en 18S rRNA aan te tonen in *U. maydis*. Het ribosomale 18S RNA werd in zowel gistcellen als schimmeldraden aangetoond, terwijl boodschapper RNA voor repellents alleen aangetoond werd

in schimmeldraden. Net als in *S. commune* bond de probe voor 18S rRNA beter in topcellen, maar in tegenstelling tot *S. commune* nam het signaal in *U. maydis* toe naar de uiterste top. Dit laat zien dat de verdeling van de ribosomen in de topcel van beide schimmels verschilt. Ook het boodschapper RNA voor de repellents werd voornamelijk in de uiterste top aangetoond. Dit suggereert dat op deze plaats de repellents worden gevormd, wat efficiënt zou zijn omdat aangetoond is dat eiwitten aan de uiterste top in de celwand worden uitgescheiden.

Repellents vervangen hydrofobines tijdens hechting en tijdens groei in de lucht
In **Hoofdstuk 3** wordt beschreven dat het genoom van *U. maydis* twee hydrofobine genen bevat. Het gen *hum2* codeert voor een klassiek hydrofobine met 117 aminozuren, terwijl het hydrofobine gen *hum3* afwijkend is. Dit gen codeert voor een eiwit dat bestaat uit een klassiek hydrofobine, wat vooraf gegaan wordt door een secretiesignaal en 17 onvolmaakte repeats (herhalende aminozuurvolgorden) van 31-36 aminozuren. Dertien van deze repeats eindigen met een KEX2 herkenningssequentie. Dit impliceert dat deze repeats worden geknipt in 13 kleine peptiden (de zogenaamde questellents) en een klassiek hydrofobine voordat ze in de celwand worden uitgescheiden. De aminozuurvolgorden van de questellents bestaan uit een waterminnend en een waterafstotend deel en lijken sterk op dat van de repellents.

Het gen *hum2* komt tot expressie tijdens vorming van schimmeldraden in de lucht, maar activiteit van het *hum3* gen kon niet worden aangetoond. Verwijdering van *hum2* had een klein effect op de vorming van luchtdraden, terwijl verwijdering van *hum3* geen effect had. Uitschakeling van de hydrofobine genen had ook geen effect op aanhechting op een waterafstotend oppervlak. Verwijdering van het repellent gen resulteerde echter in een sterk verminderde waterafstoting op het kolonieoppervlak, een sterk verminderde vorming van luchtdraden, en een sterk gereduceerde hechting van de schimmeldraden aan een waterafstotend oppervlak. Hieruit werd de conclusie getrokken dat in *U. maydis* repellents de functie van hydrofobines overgenomen hebben en dat repellents niet functioneren in verankering van hydrofobines in de celwand. Mutanten waarin repellents, al dan niet in combinatie met hydrofobines, afwezig waren, waren nog steeds pathogeen. Dit werd echter getest door veel gistcellen te injecteren in de plant, waardoor het niet opvalt als mutanten eventueel verminderd infectieus zijn. Evenmin is er gekeken naar de rol van deze eiwitten in de verspreiding en overleving van de teliosporen.

Repellents vormen functionele amyloïden
Vouwing van eiwitten in een amyloïde vorm veroorzaakt in mens en dier ernstige ziekten zoals die van Huntington, Alzheimer, en Creutzfeld-Jakob. In micro-organismen is deze eiwitvouwing echter uiterst functioneel. In **Hoofdstuk 4**

wordt aangetoond dat repellents, net als hydrofobines, als functionele amyloïde in de celwand voorkomen. Dit werd ondermeer met Thioflavine (ThT) kleuring en Röntgen diffractie aangetoond. Amyloïde eiwitten zijn in een geordende vezelachtige structuur gevouwen met een regelmatige afstand van 4.7 Å tussen de onderlinge fibrillen. In **Hoofdstuk 4** werd één van de repellents, Rep1-1, synthetisch gemaakt om voldoende materiaal te hebben om de eigenschappen van dit peptide te onderzoeken. De oplosbaarheid van het synthetische Rep1-1 bleek identiek aan dat van repellents in de celwand van *U. maydis*. Het was enkel na TFA extractie in water op te lossen. Echter, het peptide bleek al snel weer over te gaan in de amyloïde vorm. Hoe voorkomen wordt dat repellents in de cel te snel aggregeren is nog onbekend. Rep1-1 bleek ook op het grensvlak van water en lucht amyloïde fibrillen te vormen, waardoor de oppervlaktespanning sterk werd verlaagd. Dat repellents ook in levende cellen amyloïden vormen werd aangetoond door een mutant te maken die repellents vormt wanneer het als een gistcel groeit. Deze gistcellen, maar niet het wilde type, kleurden positief met ThT als ze in een vloeistofculture waren gegroeid. Dit laat zien dat repellents functionele amyloïden vormen, en dat zij dus soortgelijke eigenschappen hebben als hydrofobines. Het verklaart waarom repellents dezelfde functies kunnen vervullen als hydrofobines.

Niet alleen luchtdraden van een stam waarin repellents gemaakt worden kleurden positief met ThT, maar ook die van een stam waarin het repellent gen verwijderd was. Ook gistcellen, waarin het *rep1* gen niet actief is, kleurden met ThT indien zij aan de lucht werden gedroogd. Dit betekent dat ook andere celwandeiwitten amyloïden kunnen vormen als ze aan de lucht blootgesteld worden. Dit wordt verklaard door het feit dat lucht waterafstotende eigenschappen heeft en hiermee de vouwing van eiwitten beïnvloedt (denk maar eens aan het effect van het kloppen van lucht door room of eiwit van een ei).

Afwezigheid van repellents zorgt voor aanmaak van SSP's

In **hoofdstuk 5** wordt de genexpressie van de stam SG200 vergeleken met eenzelfde stam waarin het *rep1* gen verwijderd is. SG200 is een haploïde stam die zich gedraagt als een dikaryon. Deze stam maakt dus schimmeldraden. Boodschapper RNA uit beide stammen werd geïsoleerd op het moment dat de SG200 stam luchtdraden maakte. Doormiddel van microarray analyse werd aangetoond dat in de stam die geen repellents maakte slechts 31 genen hun expressie met een factor 2 of meer veranderde ten opzichte van SG200. Dit getal is verrassend laag en ik concludeer dan ook dat de vorming van luchtdraden niet een stap is in de ontwikkeling van *U. maydis.* In veel andere schimmels, zoals de paddenstoelvormende schimmels, is dit juist wel het geval. In *U. maydis* lijkt de vorming van luchtdraden een willekeurig proces te zijn waarbij draden vanuit het medium per ongeluk de lucht in groeien.

Van de 31 genen met veranderde expressie in de stam waarin het repellent gen was geïnactiveerd komen er 22 hoger tot expressie. Van achttien hiervan wordt voorspeld dat ze uitgescheiden worden. Van deze genen blijken er 11 te coderen voor zogenaamde kleine gesecreteerde eiwitten, SSP's genoemd, die niet groter zijn dan 300 aminozuren. Volgens het Waltz algoritme bezit meer dan 80 % van deze eiwitten amyloïde gebieden en zijn dus potentieel kandidaat om net als Rep1 amyloïde te vormen in de celwand.

Het gen um00792, dat codeert voor een SSP eiwit, had de hoogste veranderde activiteit in de stam waarin het repellent gen was verwijderd. Door gebruik te maken van het groen fluorescerende eiwit GFP werd aangetoond dat dit gen vooral hoog tot expressie komt in de laag van schimmeldraden die aan het water-lucht grensvlak groeit. Deze schimmeldraden lijken in de stam waarin het repellent gen was verwijderd niet te kunnen ontsnappen aan dit grensvlak, wat eens te meer de functie van repellents in het wilde type illustreert.

DANKWOORD

Het ontstaan van dit proefschrift was niet alleen een logisch vervolg op een studie, maar ook een doel op zich. De weg die heeft geleid tot dit proefschrift was lang, kijk maar naar mijn C.V.. Dus begin ik gewoon bij het begin.

Het begon op een donderdag in 1954, vanaf die dag heeft mijn vader mij de natuur laten zien. Dit deed hij direct en met alle details, of het nu eetbaar was of niet. Zijn antwoorden op mijn waarom-vragen waren zelden gevat in een pasklaar antwoord, zo bleef er steeds iets te vragen over. Daar ligt de basis voor wat ik nu officieel word: een bioloog.

Daar waar mijn wetenschappelijke carrière zich nu afspeelt begon het ook, bij de Universiteit van Utrecht in het Kruijtgebouw, dat toen nog Trans III heette, op de 4e verdieping. Het werk bij de Fysiologische Chemie is vervat in meerdere publicaties. Dat wat nu heel gewoon is, werd toen ontwikkeld: werken met synthetische oligo's en restrictie-enzymen. Maar het belangrijkste was de wetenschappelijke voortgang en ik wil met name Piet Baas hartelijk danken voor de bijzondere samenwerking die wij hadden. Ik heb veel geleerd tijdens die lange tijd, dank ook voor je waardering van mijn inbreng en de interpretatie van de experimenten. Elly, jij bevolkte later hetzelfde lab, weliswaar op een andere plaats, en met jouw niet aflatend enthousiasme bracht je mij weer op het wetenschappelijke spoor. Van jou leerde ik ook het belang van vrouwen in de wetenschap. Audrey, ook jou wil ik bedanken, jij was de eerste AIO waar ik volledig mee samenwerkte. Dat resulteerde niet alleen in mooie artikelen, het was ook heel erg prettig.

Later startte ik mijn werk bij de afdeling Microbiologie van de faculteit Biologie. Eerst kort bij de bacteriegroep, waar kwalitatief goed werk verricht werd. Daar heb ik zeker van geprofiteerd, en ik wil met name Jan en Hendrik daarvoor danken. Bij de schimmelgroep begon het allemaal. Ik was vanaf het begin bij de groep betrokken, en het werk daar verricht is de basis van mijn proefschrift.

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mijn hiërogliefen geïnterpreteerd worden. Dank voor je vertrouwen in mij!

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De mensen van de fotografie afdeling, Ronald en met name Frits, dank ik voor hun inbreng in de figuren, die gebruikt worden in de artikelen in dit proefschrift en voor de hulp bij het gebruik van de CLSM.

Hier wil ik ook mijn paranimfen bedanken.
Pauline, jij was mijn eerste *Ustilago* student, onze samenwerking wordt nog een mooi artikel. En jouw overleg vanuit Duitsland was erg memorabel, persoonlijk en goed. Gelukkig werk je nu als AIO bij de schimmels, zodat ik je elke dag zie.
Ina, al meer dan 20 jaar lang, sinds S.F., zijn wij vriendinnen. Jij hebt mij nog nooit in de steek gelaten. En je bent bioloog, dus wie kan er beter mijn paranimf zijn!

Het thuisfront was natuurlijk van groot belang, niet chronologisch, maar altijd. Mijn ouders dank ik voor mijn opvoeding. Henk, mijn maatje, dank zij jou was wetenschap er altijd, aan tafel en tijdens lange wandelingen. Gelukkig wist jij ook altijd voor ontspanning te zorgen met muziek en bijzondere vakanties. Onze dochters Annika, Karlijn en Marjolein, ik heb jullie wel eens verwaarloosd voor een hoger doel. Maar zelf zijn jullie nu alle drie wetenschappers, daar ben ik trots op.

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En voor iedereen die denkt: “Waar ben ik?”. Jullie staan er misschien niet bij, maar iedereen zit in mijn hoofd en in mijn hart. Bedankt.

CURRICULUM VITAE

Willemina Renske Teertstra was born April 8, 1954 in Groningen, the Netherlands. She finished her secondary education in 1971 with a diploma HBS-B. The same year she started to study at the Laboratoriumschool in Amersfoort, followed by two years at the Laboratoriumschool in Groningen, the Netherlands, where she graduated in 1974 with a diploma HBO-B Biochemistry. She worked as a research technician at the department of Physiological Chemistry at the University of Utrecht from 1974-1984. She studied DNA replication in the virus model φX174 under supervision of prof. dr. H.S. Jansz and dr. P.D. Baas. From 1984-1986 she worked at the Hubrecht laboratorium, Utrecht, the Netherlands, under supervision of dr. O.H.J. Destrée. The aim of the research was to determine the DNA sequence of histon genes of *Xenopus leavis*. From 1986 to 1988 she lived with her family in San Francisco, USA. She returned in 1991 to the department of Physiological Chemistry, University of Utrecht, to work on expression of IGF-II under supervision of prof. dr. J.S. Sussenbach and dr. P.E. Holthuizen. From 1993 to 1997 she worked in the group of prof. dr. P.C. van der Vliet, supervised by drs. A.J. King, on DNA-protein interactions in the adenoviral model system. This was followed by a position as a research technician at the RITOX (Research institute for toxicology; department of Veterinary Medicine of the University of Utrecht). Here, she studied induction of cytochrome-P450 by toxic compounds under supervision of dr. M. van den Berg. From 1999 to 2001 she worked on phage-shock proteins of *E.coli* at the Department of Microbiology of the Faculty of Biology under supervision of dr. J.P.M. Tommassen and drs. H. Adams. In 2001, she switched to the fungal research group of the same department where she started her own research line. This PhD thesis is the result of the work she did between 2004 and 2009.

PUBLICATIONS

Teertstra W. R., van der Velden G. J., de Jong J. F., Kruijtzer J. A., Liskamp R. M., Kroon-Batenburg L. M., Müller W. H., Gebbink M. F., Wösten H. A. B. (2009). The filament-specific Rep1-1 repellent of the phytopathogen *Ustilago maydis* forms functional surface-active amyloid-like fibrils. *J Biol Chem* **284**, 9153-9159.

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