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

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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. 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 $\Delta hum2$ cross. However, the reduction in aerial hyphae formation was much more dramatic in the $\Delta rep1$ cross. Moreover, colonies of the $\Delta rep1$ cross were completely wettable, while surface hydrophobicity was unaffected and only slightly reduced in the $\Delta hum2$ and the $\Delta hum2 \Delta hum3$ 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 $\Delta rep1$ 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.

Received 30 March 2006

Revised 3 August 2006

Accepted 20 September 2006

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 (see 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 *rep1* (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 aa and a larger peptide of 228 aa. The latter not only consists of the last two repeats of Rep1 but also contains a proline-rich region (Fig. 1). The 10 small repeats and the 228 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).

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Fig. 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. White arrowheads indicate a signal sequence; * 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 $\Delta rep1$ strain is thus 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). We studied here 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.

METHODS

Strains. Cloning was done in *Escherichia coli* DH5 α . 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).

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 r.p.m. or on solid potato dextrose agar (PDA, Sigma) either supplemented or not with 1% charcoal.

Molecular techniques. *U. maydis* chromosomal DNA was isolated as described by Hoffman & Winston (1987). DNA was blotted on Hybond-N⁺ (Amersham) and hybridized in 0.5 M sodium phosphate, 7% SDS, 10 mM EDTA, pH 7.2, at 60 °C with [α -³²P]CTP-labelled probes. RNA was isolated from ground cells (Microdismembrator, B. Braun) with Trizol (Invitrogen) according to the manufacturer's protocol.

Deletion of *hum2* and *hum3*. A 1.3 kb *SphI* fragment spanning the ORF 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, which contain *NotI* linkers at their 5' end (Table 1). The amplified fragment was digested with *NotI* and ligated to a hygromycin resistance cassette (Brachmann *et al.*, 2001). In the resulting plasmid p Δ Hum2 the region of *hum2* between the start and stop codons 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 *HindIII* and *BamHI* 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 an *EcoRI*–*HindIII* fragment between the corresponding sites of pUC19. This was followed by introduction of the downstream flank as a *BamHI*–*HindIII* fragment. A *NotI* carboxin resistance cassette taken from pNEB-Cbx (Brachmann *et al.*, 2001) was inserted in the *NotI* site at the 3' end of the upstream flank. This resulted in p Δ Hum3. The *EcoRI*–*HindIII* 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 $\Delta hum2$ strains to yield strains $\Delta hum2\Delta hum3$, $\Delta hum2\Delta rep1$ and $\Delta hum2\Delta hum3\Delta rep1$. In this case p Δ Rep1 (Wösten *et al.*, 1996) contained the nourseotricin (Brachmann *et al.*, 2001) instead of the

Table 1. Primers

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	CgACgTTgACgAgCTggTTTgAgCAACgggACgggCATC

hygromycin resistance cassette. *U. maydis* was transformed according to Brachmann *et al.* (2004). Transformants were selected on PDA plates supplemented with hygromycin, carboxin or nourseotricin at a final concentration of 200, 2 and 150 $\mu\text{g ml}^{-1}$, respectively.

Assessment 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 according to the instructions of the manufacturer. Attachment of hyphae was determined essentially as described previously (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 water was placed in the middle of the sheet. The droplet contained 50 000 cells of each mating type, isolated from a culture in exponential 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 *Z. mays* 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 $\mu\text{E m}^{-2} \text{s}^{-1}$.

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 *Schizophyllum commune* contains at least five hydrophobin genes (Wessels *et al.*, 1995; 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*, which encompasses 6801 ORFs

Fig. 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. 18S rRNA served as a loading control.

Fig. 3. Formation of aerial hyphae at the edge of colonies after crossing compatible partners. In contrast to hyphae that grow over the agar surface (thin arrow), aerial hyphae (thick arrow) give the colony a white fuzzy appearance.

(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 aa with eight conserved cysteine residues (Fig. 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 41–46 aa. Fourteen repeats are separated by KEX2 recognition sites (Fig. 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 endoplasmic reticulum into 15 repeats, and a hydrophobin of 117 aa. This hydrophobin shows 53 % identity to Hum2. The repeats, which we have named questellents, have no homology with proteins in the database but their hydropathy pattern shows a remarkable similarity to that of the repellents (Fig. 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 on PDA-charcoal medium (Fig. 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 strains FB1 and FB2 either in combination or not with an inactivation of the *rep1* gene (see 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; Fig. 3, Fig. 4A). In contrast, formation of aerial hyphae was only partially reduced when compatible Δ *hum2* strains were crossed (Fig. 3, Fig. 4A) and was almost unaffected in the Δ *hum3* cross. A Δ *hum2* Δ *hum3* cross formed fewer aerial hyphae than the Δ *hum2* cross. Inactivation of *hum2* and/or *hum3* in the Δ *rep1* background had little or no 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 fewer hyphae in the aqueous environment. The reason for this is not yet known.

Hydrophobicity of the colony surface correlated with formation of aerial hyphae. The surfaces of the wild-type and the Δ *hum2* and the Δ *hum3* cross were highly hydrophobic (Fig. 4A, B), 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 117 ± 2 degrees). The colony surfaces were completely wettable when *rep1* was inactivated (Fig. 4A, B; Wösten *et al.*, 1996). Since water droplets were

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

immediately absorbed by the colony, water contact angles could not be measured. Deletion of *hum2* and/or *hum3* had no additional effect in the $\Delta rep1$ background.

Taking these results together, we conclude that repellents have to a great extent replaced hydrophobins in formation of aerial hyphae in *U. maydis*. This contrasts with 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 & 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 $\Delta rep1$, $\Delta hum2$, $\Delta hum3$, $\Delta rep1\Delta hum2$, $\Delta rep1\Delta hum3$, $\Delta hum2\Delta hum3$ and $\Delta rep1\Delta hum2\Delta hum3$ were injected into 7-day-old maize plants. In all cases 90–100 % of the plants showed disease symptoms after 1 week (anthocyanin formation, chlorosis and tumour formation), demonstrating that repellents and hydrophobins are not involved in pathogenicity. In contrast, deleting the class I hydrophobin gene *MPG1* in *Magnaporthe grisea* did affect pathogenicity (Talbot *et al.*, 1996). This phenotype was explained by the inability of the *M. grisea* mutant strain to attach to the hydrophobic surface of the plant. Hydrophobin-mediated attachment to hydrophobic surfaces was also observed in *Schizophyllum 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 $\Delta hum2$, $\Delta hum3$ and $\Delta hum2\Delta hum3$ crosses (data not shown) but was reduced in the $\Delta rep1$ cross (Wösten *et al.*, 1996). No apparent further reduction of escape of hyphae was observed in $\Delta rep1$ 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 dry Teflon surface could not be removed by extensive washing with water (data not shown) or by stripping with 3 % agarose (Fig. 5). Similar results were obtained with the $\Delta hum2$, the $\Delta hum3$ and the $\Delta hum2\Delta hum3$ strains (data not shown). In contrast, 50 % of the hyphae of the $\Delta rep1$ cross were removed upon stripping with 3 % agarose (Fig. 5). Deleting either or both hydrophobin genes in the $\Delta rep1$ background did not further reduce attachment.

The reduction of attachment in the $\Delta rep1$ cross is much less dramatic than that observed after inactivation of the *SC3* gene of *Schizophyllum commune*. In this case, wild-type hyphae remained attached after stripping with 3 % agarose, whereas $\Delta SC3$ 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 suggests the involvement of an amphipathic molecule. Interestingly, the *U. maydis* genome contains two ORFs (um05708 and um06112) with a repeat structure like *rep1* and *hum3*. Both encode putative secreted proteins with 13 and 11 repeats, respectively, that are 21 aa in length. Most of these repeats end with KEX2 recognition sites. Future research should establish whether one of these genes mediates attachment.

Fig. 5. Hyphae of a wild-type cross (A, B) and the $\Delta rep1$ 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 $\Delta rep1$ cross before (A, C) and after (B, D) stripping with agarose are shown. The asterisks are marker positions on the Teflon sheets.

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