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# Significance of the class II hydrophobin FgHyd5p for the life cycle of *Fusarium graminearum*

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ABSTRACT

Hydrophobins are small secreted proteins ubiquitously found in filamentous fungi. Some hydrophobins were shown to have functions in fungal development, while others lack known function. Class II hydrophobins from *Fusarium graminearum* and *Fusarium culmorum* are characterized by formation of low stability aggregates and their solubility in organic solvents. They are economically relevant to the brewing industry because they can induce beer gushing. Since cellular functions of Hyd5p's are still unknown, we analyzed the influence of FgHyd5p on growth and morphology of *F. graminearum* using FgΔ*hyd5* knock-out mutants expressing sGFP under the control of the *hyd5* promoter and compared them with the performance of the parent wild type strain. Results demonstrate that FgHyd5p does not affect the colony and hyphal morphology. FgHyd5p affects the hydrophobicity of aerial mycelia but had no obvious function in penetration of hyphae through the water air interface. The hydrophobin affects the morphology of conidia, but not their fitness. Different sources of carbon and nitrogen as well as different pH have no effect on the expression of the *hyd5* gene, which was demonstrated to be expressed upon growth of *F. graminearum* on hydrophobic surfaces.



## Introduction

Filamentous fungi have been found to produce hydrophobins, a unique group of small amphiphilic proteins characterized by the presence of eight cysteine residues within their primary amino acid sequence. These cysteine residues are arranged with conserved spacing and are regularly found to form four pairs of disulphide bridges (Wösten 2001). Amino acid sequences in between cysteines are highly variable giving the hydrophobins a uniquely high degree of variability. Based on their hydropathy patterns and solubility characteristics, class I and class II hydrophobins are distinguished (Wessels 1994). Class I hydrophobin aggregates are extremely stable, and can be dissociated only in trifluoroacetic acid and formic acid, whereas class II hydrophobin aggregates can be solubilized in hot sodium dodecyl sulphate (SDS) or 60 % ethanol (Wösten 2001).

Hydrophobins may have different roles in the fungal life cycle. Hydrophobins play a pivotal role by decreasing the surface tension of water in the first step of forming an aerial mycelium following a phase of submerged growth. For example, the class II hydrophobin SC3 reduces the water surface tension from 72 to 24 J $m^{-2}$ at 50 μg $ml^{-1}$ (Wösten *et al.* 1999). Some hydrophobins form layers of rodlet-like structures on the surfaces of conidia and hyphae, which facilitate their dispersal by air or water depending on which part of the molecule

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faces the environment. Loss of this layer leads to reduction or even loss of surface hydrophobicity resulting in an 'easy wettable' phenotype (Wösten 2001). Some hydrophobins can affect the sporulation of fungi (Talbot *et al.* 1996; Askolin *et al.* 2005). A few hydrophobins have been shown to affect the dissemination of conidia and are involved in conidial germination (Talbot *et al.* 1996; Whiteford *et al.* 2004). Some hydrophobins are known to cover mushroom sporocarps (Lugones *et al.* 1999; van Wetter *et al.* 2000). Moreover, deletion of hydrophobin genes can lead to the complete inability to form sporocarps (Kazmierczak *et al.* 2005). Hydrophobins such as VDH1 (*Verticillium dahliae*) were found to be involved in the formation of microsclerotia (Klimes & Dobinson 2006).

*Fusarium graminearum* is an opportunistic plant pathogen, which causes Fusarium head blight of wheat, barley, and rice under optimum conditions, e.g. high humidity and temperature during anthesis (Goswami *et al.* 2004). The fungus is also capable of infecting other plant species without causing disease symptoms (Goswami *et al.* 2004). Utilization of brewing malt produced from *F. graminearum*-infected wheat and barley has been demonstrated to result in beer gushing (spontaneous over foaming of the beverage). Hydrophobins have an important role in gushing and hydrophobin research is still important for the brewing industry (Haikara *et al.* 2005; Sarlin *et al.* 2005; Zapf *et al.* 2006). Sequencing and annotation of the *F. graminearum* genome in 2007 enabled the prediction of five different hydrophobin genes in this particular species showing similarity with known hydrophobin sequences (Cuomo *et al.* 2007). Four of them (FG01763.1, FG01764.1, FG03960.1, FG09066.1) were predicted to be encoding class I hydrophobins. So far, FG01831.1 seems to be the only gene coding for a class II hydrophobin in *F. graminearum* (Sarlin *et al.* 2012).

Until now the function of the class II hydrophobin FgHyd5p in the life cycle of *F. graminearum* remains unknown. In order to elucidate the intra- and extracellular functions of Hyd5p proteins, we analyzed the influence of FgHyd5p on growth and morphology of *F. graminearum* using an *fg*Δ*hyd5* knock-out mutant which expresses the fluorescent reporter molecule sGFP under control of the *fghyd5* promoter. Results were compared with those obtained from parallel analysis of the parent wild type strain.

## Materials and methods

### *Maintenance of fungal and bacterial cultures*

Cultures of Escherichia *coli* DH5α (clone TMW 2.493), *Agrobacterium tumefaciens* AGL1 (clone TMW 2.1335) and *Fusarium graminearum* TMW 4.0157 (deposited in the CBS culture collection, Utrecht, The Netherlands under accession no. CBS 136009), TMW 4.0154, TMW 4.0148, and TMW 4.0133 were obtained from the strain collection of Lehrstuhl für Technische Mikrobiologie (TMW, Technische Universität München, Freising, Germany). The transformant strain of *A. tumefaciens* carrying plasmid pPK2-PkosGT was deposited in the TMW strain collection under accession number *A. tumefaciens* TMW 2.1484. The *hyd5* knock-out strain of *F. graminearum* used during the current study was deposited under collection number TMW 4.2468.

### *Nucleic acid manipulations*

Genomic DNA was isolated using the MasterPure™ Yeast DNA Purification Kit (Epicentre, Madison, United States). Plasmid DNA was isolated using the peqGold Plasmid Miniprep Kit II (Life Technologies GmbH, Darmstadt, Germany). DNA was isolated from agarose gels using the Qiaquick Gel Extraction Kit (Qiagen, Hilden, Germany). PCR was carried out using primer pairs as given below. PCR, DNA gel electrophoresis, ligation, and restriction digestions were performed using standard methods (Ausubel *et al.* 1993). All DNA sequencing was done by GATC Biotech AG (Konstanz, Germany).

### *Cloning strategy*

The cloning strategy used to build up the vector construct for the knock-out of the *hyd5* gene in *Fusarium graminearum* TMW 4.0157 (CBS 136009) was based on plasmid pHG which was available to the study from earlier work (unpublished data). The construct contained the intron-free *hyd5* gene from *F. graminearum* TMW 4.0157 and had previously been cloned in Escherichia *coli* DH5α. Clone TMW 2.493 was used in the current study. Since the sequence and structure of the *hyd5* promoter are still undefined, an 879 bp sequence upstream from the start codon of the *hyd5* gene was defined as the 'promoter area'. The sequence area was assigned as P*hyd5* and amplified by PCR using purified genomic DNA of *F. graminearum* TMW 4.0157 (CBS 136009) as template. Primers 5′PromHind2 (5′-GCGAAGCTTGTGGAGATTTTCTTGATTCTG-3′) and 3′Pro mHind2 (5′-CGCAAGCTTGATGAATAGAGTGATTGGTTTAG-3′) with integrated *Hind*III restriction sites in both primers were applied at an annealing temperature of 55 °C. The purified PCR product was integrated into plasmid pHG after restriction digestion with *Hind*III endonuclease to result in plasmid pPHG after ligation. Transformation of *E. coli* DH5α resulted in clone TMW 2.1479. Like the promoter sequence, also the structure of the *hyd5* terminator has not been elucidated yet. A 1002 bp sequence downstream from the stop codon of the *hyd5* gene was defined as 'terminator area'. The sequence was amplified by PCR using purified genomic DNA from *F. graminearum* TMW 4.0157 (CBS 136009). Primers 5′ThydHindIII (5′-GCGAAGCTTAGTGTCACGAGTTTGTGACCTTGTATAAT-3′) and 3′Thyd HindIII (5′-CGCAAGCTTTATCTCACTTTCACTCGGAAGCGCAT GAGTG-3′) with integrated *Hind*III restriction sites in both primers were applied at an annealing temperature of 60 °C. The purified terminator fragment T*hyd5* was integrated into plasmid pPK2 (Covert *et al.* 2001) after restriction digestion with *Hind*III restriction endonuclease to result in plasmid pPK2-Thyd5 after ligation. Transformation of *E. coli* DH5α with the vector resulted in clone TMW 2.1481. The P*hyd5* fragment was amplified by PCR using purified plasmid pPHG as template. Primers 5′PhydEcoRV-2 (5′-GCGGATATCGTGGA GATTTTCTTGATTCTGGGGCA-3′) and 3′Pknock out (5′-C GCGATATCGGGCCCATGAATAGAGTGATTGGTTT-3′) with integrated *Eco*RV restriction sites in both primers were applied at an annealing temperature of 55 °C. The purified PCR fragment was integrated into plasmid pPK2-Thyd5 after restriction digestion resulting in plasmid pPK2-PkoT. Transformation of *E. coli* DH5α with the vector resulted in