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Hydrophobins and Fungispumins – surface active fungal proteins with a role in foam stability of carbonated beverages and fungus-plant interaction

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Abbreviations

AfpA	Alkaline foam protein A
AfpAi	AfpA - interfered
AOX	Alcohol oxygenase
APS	Ammonium persulfate
ATMT	*Agrobacterium tumefaciens*-mediated transformation
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BLAST	Basic local alignment search tool
bp	Base pairs
BSA	Bovine serum albumine
CAPS	*N*-cyclohexyl-3-aminopropanesulfonic acid
cDNA	Complementary DNA
CIA	Chloroform and isoamylalcohol
CM	Complete medium
CTAB	Cetyl trimethylammonium bromide
ΔAfpA	Knock out of AfpA
DMSO	Dimethyl sulfoxide
dNTP	Desoxynucleotidtriphosphate
dsRNA	Double-stranded RNA
DTT	Dithiothreitol
EDTA	Ethylendiaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
FcHyd5(p)	*Fusarium culmorum* Hydrophobin 5 (protein)
FG	*Fusarium graminearum*
FgHyd5(p)	*Fusarium graminearum* Hydrophobin 5 (protein)
FHB	*Fusarium* head blight
FPLC	Fast protein liquid chromatography
FvHyd5(p)	*Fusarium verticillioides* Hydrophobin 5 (protein)
GFP	Green fluorescent protein
HFB2	Class II hydrophobin Hfb2 from *Trichoderma reesei*
IEF	Isoelectric focussing
IgG	Immunoglobulin G
IPTG	Isopropyl β-D-1-thiogalactopyranoside

kb	Kilo base pairs
LB	Lysogeny broth
MES	2-(N-Morpholino)ethanesulfonic
MHP1	*Magnaporthe grisea* hydrophobin 1
miRNA	MicroRNA
Mut, Mut^S/ Mut^+	Methanol utilisation, Methanol utilisation slow / plus
MW	Molecular weight
NBT	Nitro blue tetrazolium chloride
nsLTP1	Non-specific lipid transfer protein 1
OD	Optical density
onc	Oncogene
PAGE	Polyacrylamide gel electrophoresis
PCNB	Pentachloronitrobenzene
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pI	Isoelectric point
PKS12	Polyketide synthase 12
PKS12i	PKS12 - interfered
PTGS	Posttranscriptional gene silencing process
RdRP	RNA-dependent RNA polymerase
RISC	RNA-induced silencing complexes
RNAi	RNA interference
RNase	Ribonulease
SC3	*Schizophyllum commune* hydrophobin 3
SDS	Sodium dodecyl sulphate
siRNA	Small interfering RNA
SOC	Super optimal broth with catabolite repression
spp.	Species (plural)
TCEP	Tris(2-carboxyethyl)phosphine
T-DNA	Transfer DNA
TE	Tris, EDTA
TEMED	Tetramethylethylendiamine
TF	Transformant

TGS	Transcriptional gene silencing
Ti	Tumour inducing
TMW	Technische Mikrobiologie Weihenstephan
Tricin	N-(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine
Tris	Tris (hydroxymethyl) aminomethan
UTR	Untranslated region
UV	Ultra violet
v	Volume
vir	Virulence
w	Weight
WT	Wild type
YNB	Yeast Nitrogen Base

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1 Introduction

1.1 Properties of *Fusarium* spp.

The fungal genus *Fusarium* belongs to the 'true fungi' (eumycota). In contrast to the 'lower fungi' (myxomycota, oomycota), the cell walls of eumycetes contain mainly glucans and chitin and the mycelial cells are separated periodically by septa in the ascomycetes and the basidiomycetes (Müller and Loeffler, 1992). True fungi are divided into four phyla: Chytridiomycota, zygomycota, ascomycota and basidiomycota. The deuteromycetes, also known as 'fungi imperfecti', are a pseudo class erected within the ascomycota to contain filamentous fungi with unknown sexual reproduction. *Fusarium* spp. have also been classified in the deuteromycetes. Because they have no known teleomorphic state, they cannot be placed with complete confidence in any of the phyla. Most of the deuteromycetes can be deduced to have phylogenetic affinities to the ascomycota by DNA sequence analysis. Nowadays, *Fusarium* is widely considered an anamorphic genus affiliated with order hypocreales within the ascomycota (Rubella et al., 2004). The defining feature of ascomycota is the 'ascus', a microscopic sexual structure, in which ascospores are formed. However, some species of the ascomycota are asexual, meaning that they do not have a sexual cycle and thus do not form asci or ascospores. The mycelia of ascomycota are haploid and form mostly uninucleate hyphae (Dörfelt, 1994; Schlegel, 2006).

Where associations are known, the sexual stages of many *Fusaria* are in the ascomycetous genus *Gibberella;* some species also have teleomorphs in *Nectria*. In contrast to *F. graminearum* (teleomorphic state: *Gibberella zeae*), sexual reproduction of *F. culmorum* has never been observed. Hence, *F. culmorum* has no known teleomorphic stage; it has never been shown to produce sexual ascospores. *Fusarium* spp. produce slimy, septate, canoe-shaped macroconidia which in most species are produced in fruiting-structures called sporodochia (Figure 1). Furthermore, some species also produce distinctly different conidia in the aerial mycelium (microconidia). Chlamydospores, thick-walled resting spores, which survive under unfavourable conditions, are also produced by some species. Persistent chlamydospores as soil-borne inoculum are important, for example, in *F. culmorum*.

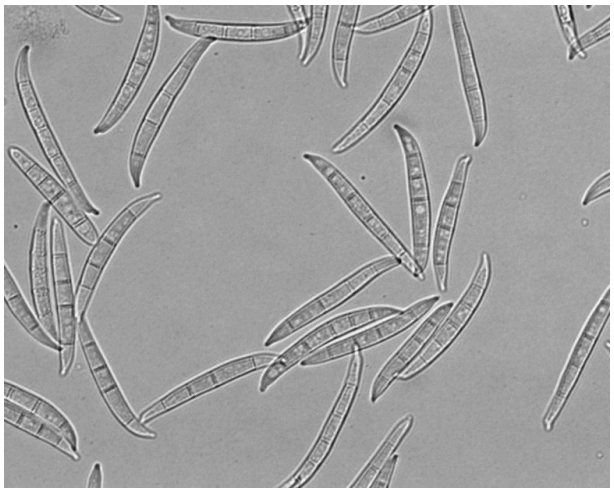

Figure 1. Macroconidia of *F. graminearum* visualised by light microscopy (1000x). Macroconidia of *F. graminearum* are septate, canoe-shaped and multi-cellular and are important for the asexual reproduction of the fungus.

Most *Fusarium* spp. are common in soil and many live in association with plants. However, some species are important plant pathogens and produce mycotoxins that can affect human and animal health. *F. graminearum* and *F. culmorum* are predominantly associated with infection of wheat and other small cereals (Bottalico, 1998) and are the most prominent producers of the wide spread mycotoxin deoxynivelenol (Laitila et al., 2002) (chapter 1.2). *Fusarium* infections in humans with a weakened immune system are sometimes caused by *F. solani, F. oxysporum, F. verticillioides* and *F. proliferatum* (Dekker, 2003). *F. venenatum* is produced industrially for use as a human food (mycoprotein "Quorn").

Prior to 1999, homothallic fungi causing *Fusarium* head blight of wheat and barley were known as *F. graminearum* 'group 2' to distinguish them from the heterothallic fungus causing crown and foot rot on cereals, which was called *F. graminearum* 'group 1' (Francis and Burgess, 1977). It is now known that these fungi are separate biological and phylogenetic species, each producing their own characteristic sexual state. The former 'group 1' is now known as *F. pseudograminearum* and has a sexual stage called *G. coronicola* (Aoki and O'Donnell, 1999). Aside from considering *F. pseudograminearum* as a separate species, *F. graminearum* 'group 2' is now known to be a monophyletic species complex consisting of at least nine separate phylogenetic species, some of which are localised on particular continents or geographical regions (O'Donnell et al., 2000; O'Donnell et al., 2004). Lineages 1 to 5 coming from the Southern Hemisphere (two in South/Central America, three in Africa) and lineage 6 and 7 from the Northern Hemisphere, lineage 6 being restricted to Asia (Japan). An eighth lineage from Brazil has

been discovered (Ward et al., 2002). Lineage 7 (*F. graminearum* sensu stricto), as the most dominant, is the only lineage that has been so far reported in Europe and North America.

1.2 Pathogenicity of *Fusarium* spp. and production of mycotoxins

Fusarium is one of the most important genera of plant pathogenic fungi, with a record of devastating infections in various economically important crops (Chelkowski, 1989). In addition to causing yield and quality reduction of cereals, fusaria have caused severe problems for the consumer (Steffenson, 1998). Certain species of *Fusarium* are capable of producing toxic secondary metabolites, so-called mycotoxins. Mycotoxins can be accumulated in the infected plants and in stored material. Zearalenone, various trichothecenes, including deoxynivelenol, T-2 toxin, diacetoxyscirpenol, fusarenone X, and nivalenol, and fumonisins, in particular fumonisin B1 can be detected in cereals such as maize, rice and barley. In addition, moniliformin, beauvericin, and fusaproliferin were also found in *Fusarium* infected cereal ears (Bottalico, 1998; Chelkowski, 1989). It has been reported that cereal-based products are major sources of intake of these toxins by consumers (Eriksen and Alexander, 1998). In addition to grain quality deterioration, Schwarz et al. (1995) and Scott et al. (1993) showed that *Fusarium* toxins could be produced during the malting process of barley. Niessen et al. (1993) showed that the toxin is transferred through the brewing process and can be found in retail beer samples. The abundance of *Fusarium* contamination and the diversity of the species are particularly depending on crop susceptibility, agricultural practices, climate and geographic location (Chelkowski, 1991; Steffenson, 1998). Contamination of the barley crop by *Fusaria* is of concern particularly in years when poor weather conditions favour the growth of toxigenic *Fusarium* species (Laitila et al., 2007).

Fusarium mycotoxins are capable of inducing both acute and chronic effects. The effects observed are often related to dose levels and duration of exposure. Although acute and chronic effects in farm livestock are readily demonstrated under experimental conditions, similar manifestations have been reported in natural outbreaks of *Fusarium* mycotoxicosis in Europe, Asia, New Zealand and South America (Fazekas and Bajmocy, 1996; Galhardo et al., 1997; Kramer et al., 1997; Prathapkumar et al., 1997). Chronic exposure of farm animals to deoxynivelenol is a continuing hazard in Canada, the USA and continental Europe. In Japan, several cases of mycotoxicosis in animals have been

attributed to consumption of cereals contaminated with deoxynivelenol and nivalenol (Yoshizawa, 1991). A number of specific syndromes in farm livestock have now been positively linked with exposure to certain trichothecenes, zearalenone, and fumonisins. These include feed refusal, emesis and anorexia; oral and gastro-intestinal lesions; ill-thrift; reproductive dysfunction; equine leukoencephalomalacia; and porcine pulmonary edema (D'Mello et al., 1999).

The species of *Fusarium* can cause several plant diseases generally recognised according to the affected part of the host, as *Fusarium* seedling blight, foot rot, and head blight (scab) of small grain cereals (wheat, oats, barley, rye, triticale); and *Fusarium* stalk and ear rot, and seedling blight of maize (Bottalico, 1998; Nicolaisen et al., 2009). Characteristically for the infection is the co-occurrence of several species of *Fusarium* usually referred to as a 'complex'. In fact, it is quite common to isolate up to nine different *Fusarium* species, from a single fragment of infected plant tissue or up to seventeen different species from freshly harvested wheat samples collected in a limited area (Bottalico, 1998). However, only a restricted number of species have been regarded as pathogenic and generally only very few of them are predominant. Especially *Fusarium* head blight epidemics (FHB) of wheat (Figure 2) and barley cause heavy economic losses to farmers due to yield decrease and production of mycotoxins, which render the grain useless for flour and malt production (Jansen et al., 2005). Maize, wheat and barley constitute almost two-thirds of the world production of cereals and almost 80% of the European grain production and are causing thus great concern because of the extent of infections and the contamination by mycotoxins (Bottalico, 1998). The *Fusarium* species predominantly found in association with FHB in small-grain cereals all over Europe are *F. graminearum* (*Gibberella zeae*), *F. avenaceum,* and *F. culmorum* (Bottalico and Perrone, 2002). However, *F. poae*, *F. tricinctum*, *F. sporotrichioides*, *F. equiseti* and *F. langsethiae* are also very common (Bottalico and Perrone, 2002; Kosiak et al., 2003).

F. graminearum overwinters and survives in infected grain, grass stubble, and cornstalk residue as mycelia, conidia, chlamydospores and perithecial initials (Sutton, 1982). Also this fungus can contaminate seeds internally and externally (Markell and Francl, 2003). The conidia are produced abundantly during warm and moist conditions on corn and small grain residue and ascospores are discharged into air under humid conditions during spring (Sutton, 1982; Trail et al., 2002). The infection is initiated by ascospores or conidia in wheat florets during the short period (10-20 days) from anthesis through the soft dough stage of kernel development (Schroeder and Christensen, 1963). Recent

studies found that hyphae may directly penetrate ovaries or the inner walls of lemmas, paleas, or glumes (Bushnell et al., 2003). Infections are most serious when the anthers are exposed during flowering (Sutton, 1982). Symptoms develop within three days after infection when the temperature is between 25°C and 30°C with high humidity. Symptoms of FHB start at the point of infection with water-soaked brown spots (Figure 2). Bleaching of the original spikelet becomes apparent within a few days (Parry et al., 1995). Subsequently, the fungus may colonize adjacent spikelets via the rachis and kill all or part of the spike. In seven to ten days after infection, salmon-pink conidia form on the bases of the infected spikelets and these conidia may cause secondary infections.

Figure 2. Field-grown wheat inflorescence showing symptoms of *Fusarium* head blight (Rubella et al., 2004). The third spikelet from the bottom shows a darkened necrotic lesion ('scab') whereas the second and fifth spikelets demonstrate tissue-bleaching ('blight') symptoms.

Despite the importance of this pathogen, the molecular basis of its pathogenicity is only poorly understood. Up to now, only four pathogenicity traits are known. Evidence has been presented that the mycotoxins resulting from the trichothecene pathway contribute to virulence (Bai et al., 2002; Proctor et al., 2002; Proctor et al., 1995). Additionally, the signalling pathways involving the mitogen-activated protein kinases pmk1, map1, and mgv1 are regulating important fitness and virulence traits (Hou et al., 2002; Jenczmionka et al., 2003; Urban et al., 2003). So far, no virulence gene controlled

by these pathways is known. A further general virulence factor of *Cochliobolus heterostrophus* has been identified recently by the analysis of restriction enzyme-mediated integration mutants. The function is not yet clarified but disruption of the homologous genes in *F. graminearum* and other cereal pathogenic fungi revealed a strong reduction of pathogenicity in all cases (Lu et al., 2003).

1.3 Surface-active proteins from *Fusarium* spp.

Surface-active proteins have a wide range of different functions for filamentous fungi and thus are very important to this group of organisms. Also as putative factors of secondary gushing surface-active proteins play an important role (chapter 1.4). Three major groups were distinguished in fungi, hydrophobins, repellents, and fungispumins. The group of hydrophobins is the largest and most important group of surface-active proteins in filamentous fungi. A lot of different studies were accomplished since Wessels *et al.* (1991a) described hydrophobins for the first time. On the other hand, the function of repellents and fungispumins in fungi are unknown and need to be elucidated.

1.3.1 Hydrophobins

Hydrophobins are small proteins of about 10 kDa in size, which are produced ubiquitously by filamentous fungi. Hydrophobins occur exclusively in mycelial fungi. These proteins and their encoding genes have been isolated from ascomycetes and basidiomycetes. Some evidence indicates that hydrophobins occur in zygomycetes as well (de Vries et al., 1993), but it is not yet clear whether they occur in the chytridiomycetes. Hydrophobins show a characteristic pattern of eight cysteine residues at conserved positions (Schuren and Wessels, 1990) forming four disulphide bridges. Based on their hydropathy patterns and solubility characteristics, class I and class II hydrophobins were identified (Wessels, 1994). Class I hydrophobins have been identified in both ascomycetes and basidiomycetes. Until now, class II hydrophobins have been found in ascomycetes only. Despite the fact that the amino acid sequences of hydrophobins within these classes are diverse (Wessels, 1997), at least class I hydrophobins seem to be functionally related, i.e. they can (partially) substitute for each other (Kershaw et al., 1998; van Wetter et al., 2000). Both classes assemble into amphipathic layers on hydrophilic-hydrophobic interfaces (Wösten and de Vocht, 2000).

The layers formed by class II hydrophobins easily dissolve (e.g. by adding detergent or by applying pressure). In contrast, membranes formed by class I hydrophobins are very stable. The latter membranes are typified by an ultrastructure consisting of a mosaic of parallel rods, referred to as rodlet layer (Wösten et al., 1993) (Figure 3).

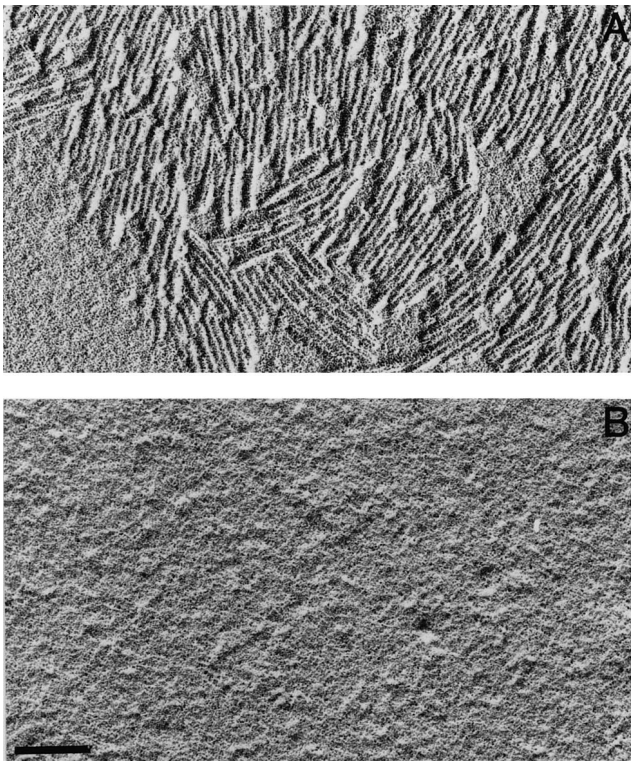

Figure 3. Ultrastructure of assembled hydrophobins visualised by atomic force microscopy according to Wösten et al. (2000). The hydrophobic side of class I hydrophobin SC3 of *Schizophyllum commune* is characterised by a mosaic of rodlets as shown by surface shadowing (A). In contrast, no rodlets are observed in the case of the class II hydrophobin cryparin of *Cryphonectria parasitica* (B).

All hydrophobins that have been isolated hitherto assemble at hydrophilic-hydrophobic interfaces into amphipathic membranes (Lugones et al., 1996; Lugones et al., 1999; Lugones et al., 1998). By self-assembly, hydrophobins stabilise air bubbles in water and change the property of surfaces by forming amphipathic membranes (Wessels, 1996). One side of the hydrophobin membrane is moderately to highly hydrophilic (water contact angles ranging between 22° and 63°), while the other side exposes a surface as hydrophobic as Teflon™ or paraffin (water contact angle 110°). The membranes formed by class I hydrophobins are highly insoluble (resisting 2% SDS at 100°C) and can only be dissociated by agents such as formic acid or trifluoroacetic acid (de Vries et al., 1993; Wessels et al., 1991a; Wessels et al., 1991b). In contrast, membranes of the class II hydrophobins cerato-ulmin of *Ophiostoma ulmi* and cryparin of *Cryphonectria parasitica*

readily dissociate in 60% ethanol and in 2% SDS (Carpenter et al., 1992). Self-assembly of hydrophobins is accompanied by conformational changes. Monomeric class I and class II hydrophobins are rich in β-sheet structure (de Vocht et al., 1998) (Figure 4). Class I hydrophobins attain more β-sheet structure at the water-air interface (β-sheet state), while at the interface between water and a hydrophobic solid, a form with increased α-helix is observed (α-helical state) (de Vocht et al., 1998). The α-helical state seems to be an intermediate of self-assembly, whereas the β-sheet state is the stable end-form.

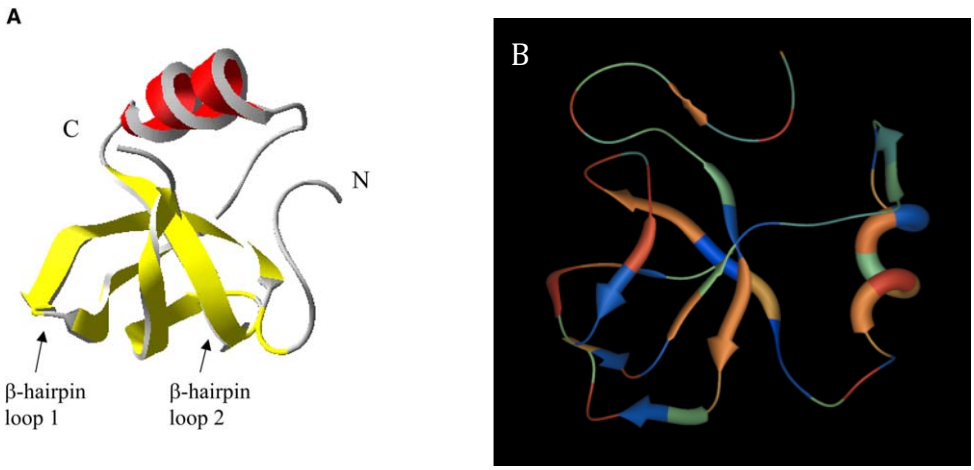

Figure 4. Comparison of the structure of the *T. reesei* HFB2 hydrophobin according to Linder et al. (2005) (A) and the predicted secondary structure of the *F. culmorum* Hydrophobin 5 calculated with Swiss model (http://swissmodel.expasy.org/) (B). The hydrophobins offer a similar secondary structure and are rich in β-sheets. The models show amphiphilic molecules with one hydrophilic and one hydrophobic part.

Hydrophobins belong to the most surface-active molecules. With a maximal lowering of the water surface tension from 72 to 24 mJ/m² at 50 µg/ml, the hydrophobin SC3 of *Schizophyllum commune* is the most surface-active protein known (Wösten et al., 1999). Other hydrophobins are also highly surface-active (de Vries et al., 1999; Lugones et al., 1999; Lugones et al., 1998). Their surface-lowering activities are at least similar to those of traditional biosurfactants. In contrast to these surfactants, surface activity is not dependent on a lipid conjugate but is solely caused by the amino acid sequence. Moreover, while the maximal lowering of the surface tension by the traditional surfactants is attained within seconds, it takes minutes to hours in the case of class I hydrophobins. This is explained by the fact that hydrophobins lower the water surface only after self-assembly that is accompanied by conformational changes in the molecule (Wösten, 2001).

Hydrophobins are secreted into liquid media and are present at the surface of aerial fungal mycelia (Wessels et al., 1991a). These proteins have a high surface activity (Askolin et al., 2006) and fulfil a broad spectrum of functions in fungal growth and development. They are involved in formation of hydrophobic aerial structures like aerial hyphae, spores and fruiting bodies (e.g. mushrooms or brackets) (Wessels, 1997) and mediate the escape of the hyphae from the liquid growth medium into the air (Wösten et al., 1999) or attachment of hyphae to hydrophobic surfaces (Wösten et al., 1994c) and signalling thereof (Talbot et al., 1996). The latter is important in initial steps of fungal pathogenesis where the fungus must attach to the hydrophobic surface of the host before penetration and infection can occur (Talbot et al., 1996). Moreover, hydrophobins seem to function in cases of symbiosis between fungi and plants (ectomycorrhizae) (Tagu et al., 1996) or algae and/or cyanobacteria (lichens) (Scherrer et al., 2000). The mechanism is based on the characteristics of hydrophobins to self-assemble at a hydrophilic/hydrophobic interface into an amphipathic membrane (Wösten et al., 1994b; Wösten et al., 1995). Upon self-assembly at the interface between the hydrophilic cell wall and a hydrophobic environment, the hydrophilic side of the amphipathic membrane orients and attaches itself to the cell wall, while the hydrophobic side becomes exposed to the hydrophobic environment. Aerial hyphae and spores thus become hydrophobic, whereas hyphae that grow over a hydrophobic substrate attach themselves (Wösten, 2001). Moreover, hydrophobins are useful for a multitude of applications including their application as surfactants (Linder et al., 2005), for biomedical applications (Haas Jimoh Akanbi et al., 2010; Janssen et al., 2002; Janssen et al., 2004), as stabilisers of food foam (Cox et al., 2009), and even as indicator for gushing (Linder et al., 2005) (see chapter 1.4).

1.3.2 Repellents

The repellents, a class of small SDS-insoluble and amphipathic cell wall proteins, affect hyphal growth and surface hydrophobicity in the corn smut pathogen *Ustilago maydis* (Wösten et al., 1996). These peptides with a length of 35 to 53 amino acids are secreted and linked with cell surface hydrophobicity. Despite of common properties, repellents and hydrophobins display no similarities at the amino acid level.

1.3.3 Fungispumins

A new class of proteins was purified after foam fractionation of a cell free culture supernatant of *F. culmorum* (Zapf et al., 2007). It was characterised as a surface-active and foam-stabilising protein with a molecular weight of 20 kDa and a calculated pI of 9. Because of its strong alkaline character and the presence in foam, this protein was termed 'Alkaline foam protein A' (AfpA) (DQ336148). Analysis of the gene revealed a signal peptide sequence preceding the protein coding region and a homologous sequence was also found in the closely related fungus *F. graminearum* (HM185071). Additional PCR analysis with specific oligonucleotide primers for AfpA resulted in PCR products with expected size in five different isolates each of *F. culmorum* and *F. graminearum* as well as in two strains of *F. cerealis*, one strain of *F. lunulosporum*, and a strain of *F. oxysporum* f. sp. *dianthi* (Zapf et al., 2007).

Although AfpA has similar properties to hydrophobins such as surface activity, incorporation into gas-liquid interphases, heat stability, and the ability to form aggregates, the protein does not belong to this protein family. However, several other fungal proteins, most of them hypothetically deduced from RNA sequences, were found to share structural similarities with AfpA. All these proteins have in common the amino acid motifs 'SGMGQG' and 'ACP' in their highly conserved 'core region'. In reference to the distribution of the aligned proteins throughout fungal species and the ability of AfpA to form stable foams (*spuma* in Latin), the new protein family was termed 'fungispumins' (Zapf et al., 2007).

Up to now, members of this group of proteins have only been described in a few fungal species and the function of most fungispumin proteins in the fungal organism is still unknown. One representative of the fungispumins, PhiA, has been described in more detail. PhiA, also known as BinB, was found to be strongly upregulated in the presence of bafilomycin, an antibiotic produced by *Streptomyces* spp., which reduces radial growth rate and induces morphological changes in fungi, indicating a modification of the cell wall composition as a response to the compound (Melin et al., 1999). Furthermore, PhiA was described as a cell wall protein with a function in phialide and conidium development. *PhiA* knock out mutants displayed reduced growth and severely reduced sporulation (Melin et al., 2003).

PhiA and AfpA as representatives of the fungispumins share an analogues secondary structure indicating a similar function of these proteins. They were predicted to have a high content of β-sheets separated by loop regions. The proportion of α-helices within

the protein is insignificant. Interestingly, the hydrophobin SC3 of *S. commune* is predicted to have a similar secondary structure, a close succession of extended and loop regions with almost no share of α-helix (Zapf et al., 2007). The extended regions are predicted to be hydrophobic and the distribution of extended, hydrophobic regions may be related with aggregation of these proteins, since they can possibly form β-stacks, as observed with hydrophobins and other proteins rich in β-sheet secondary structure.

1.4 Gushing of beverages

The term "gushing" describes a condition, in which bubbles of carbon dioxide are released unexpectedly and vigorously upon opening of a bottle of a carbonated beverage such as sparkling fruit juice, beer, or sparkling wine resulting in over-foaming of the liquid from the bottle. This phenomenon is unpleasant for the consumer and a financial and image problem for the producer. The problem is known for more than a hundred years. Copious hypotheses about the reasons for the phenomenon have been developed. However, the mechanisms involved are still not fully understood. It was demonstrated that gushing is not a monocausal problem, but is rather a result of a variety of factors acting together. Regarding possible factors leading to gushing in beer, the research group at Carlsberg Research (Gjertsen, 1967; Gjertsen et al., 1963) divided the phenomenon into two types: 'primary gushing', which occurs periodically and appears to be related to the quality of malt, and 'secondary gushing', which is due to faults during beer production or to the incorrect treatment of packaged beer.

1.4.1 Primary gushing

Primary gushing appears to be related to the quality of malt used for beer brewing (Hippeli and Elstner, 2002). Mould growth in brewing malt may cause contamination with fungal metabolites, i.e. gushing factors (Sarlin et al., 2005). It has been shown that the primary gushing is most probably caused by mould growth, either in the field (field fungi like *Fusarium, Alternaria, Stemphylium, Cladosporium*), during storage (storage fungi like *Aspergillus, Penicillium* or *Rhizopus*) or during malting (Amaha et al., 1973; Gjertsen et al., 1965; Gyllang and Martinson, 1976; Haikara, 1980; Niessen, 1993). Experimental evidence was found, that especially *Fusarium* species have an adverse effect on the quality of malt and possibly cause gushing in beer produced from

contaminated batches (Haikara, 1983; Munar and Sebree, 1997; Schwarz et al., 1996). Gjertsen et al. (1965) confirmed in experimental malting tests that inoculation of several *Fusarium* cultures onto barley grains during steeping could yield gushing malts but addition of mycelial extracts or culture filtrate of *Fusarium* to the mash failed to cause gushing in beer produced from such mashes. The authors concluded that gushing is not caused by the *Fusarium* itself but as result of an interaction between *Fusarium* and the germinating grain. Potential gushing factors produced by fungi have been studied for decades. They have been reported to be polypeptides or peptide-containing substances. Very small amounts of these substances have been reported to induce gushing of beer. Aastrup et al. (1996) observed that addition of proteolytic enzymes to gushing-inducing malt extract significantly reduced gushing tendency, suggesting that the gushing-inducing factors present in malt were proteins or polypeptides. It has been assumed that gushing factors are surface active molecules that stabilise carbon dioxide bubbles by forming a stable layer at the water/gas interface (Draeger, 1996; Pellaud, 2002). Hydrophobins share most of those properties and have therefore been under discussion as gushing inducing factors (Haikara, 2000; Laitila et al., 2007). Actually, hydrophobin levels in malts could be related with gushing volume (Haikara, 2000).

1.4.2 Secondary gushing

Secondary gushing depends on technological failure appearing during beer production. The reason for secondary gushing is the introduction or generation of particles in beer, which act as nucleation initials for carbon dioxide phase transition. Typical substances are impurities in bottles, calcium oxalate crystals, metal ions, cleansing agent residues or residual filter aids (Rammert and Pahl, 1992). An increased CO_2 pressure or an uncontrolled fermentation in the bottle may also be a reason for secondary gushing. However, this type of over-foaming can be managed by using proper technological processes, which eventually will eliminate all this factors of secondary gushing.

1.4.3 Putative gushing mechanisms

Carbonated beverages in closed bottles are under elevated pressure. As long as such a bottle is closed, the dissolved carbon dioxide in the liquid and the gaseous carbon dioxide in the headspace of the bottle are in balance. Immediately upon opening the

system, pressure in the bottle is reduced to atmospheric pressure. This leads to a carbon dioxide supersaturation of the beverage, which forces the release of carbon dioxide at the liquid surface. At this border, equilibrium is generated and the liquid is saturated with carbon dioxide. For the rest of the liquid this balance is not generated immediately, since this process is limited by diffusion of carbon dioxide molecules to the surface. However, when carbonated beverages are opened, gas release can also be observed in the form of bubbles, which ascended from the bulk liquid. In a pure liquid, however, a spontaneous formation of gas bubbles is very unlikely (Draeger, 1996). Also, no formation of bubbles was observed when pure water was saturated with carbon dioxide at 100 atm and the pressure was decreased to 1 atm (Kenrick et al., 1924). Furthermore, in pure fluids at thermo dynamical equilibrium no bubbles can exist (Fischer, 2001). According to the bubble, nucleation theory homogeneous nucleation in low supersaturated carbonated drinks is not possible because of the very large critical radius requirement (Liger-Belair, 2005; Wilt, 1986). Also, heterogeneous nucleation at walls is unlikely because the length-scale and angle of surface imperfections in general do not match the required values (Carr et al., 1995; Wilt, 1986). These differences can only be bridged if the presence of microbubbles is possible (Brennen, 1995). For carbonated beverages there are some experimental indications for the presence of stabilised bubbles (Strasberg, 1959). Several hypotheses have been put forward to explain the presence of these microbubbles, most notable are organic molecules, gas inclusion in crevices on the container wall or on solid impurities in the liquid (Leighton, 1997). Beer consists of many different ingredients, some of which are surface-active compounds, and thus cannot be seen as pure fluid. Surface-active proteins can assemble on hydrophilic-hydrophobic surfaces and exert a stabilising effect on microbubbles in the closed bottle. Such stabilising bubble skins may limit the solubilisation of carbon dioxide and prevent bubbles from completely disappearing in the pressurised bottle. Those bubbles could occur at the filling step of the bottles or during the transport. In these steps, the liquid is mixed with the gas phase of the headspace and the generated bubbles are immediately covered with surface-active molecules. Pressure release at opening may enable the diffusion of carbon dioxide into stabilised microbubbles leading to bubble growth and ascension. However, the presence of stabilised microbubbles in carbonated beverages, does not necessarily lead to gushing, since in non-gushing beer about 2500 microbubbles per millilitre can be found (Curtis and Martindale, 1961). In non-gushing beer, the bubble layer is made up from surface-active substances, which are

present there. In this context, nsLTP1 and Z-proteins are known as the two major foam-active proteins (Leisegang and Stahl, 2005). However, other amphiphilic substance may also play a role. Considering the occurrence of gushing in beer, hydrophobins are discussed as inducing agents in current research activities. These proteins may also be able to occupy the bubble skin and stabilise microbubbles. Since these proteins are known to form highly ordered and stable films at interfaces (Kisko et al., 2009; Wösten et al., 1993), they might impede the growth of bubbles upon opening of a bottle and cause a burst of the bubble. A bursting bubble would provide several new bubble nuclei leading to a greatly increased number of ascending bubbles, which manifests it as over-foaming of the beverage.

1.5 Discovering gene functions in filamentous fungi

Recently, genomic sequences have been released for a variety of filamentous fungi, e.g. *F. graminearum* (Cuomo et al., 2007; Gale et al., 2005). In combination with other published genomic libraries or homologous sequences of known genes in closely related taxa, many genes can now easily be identified and it can be theoretically speculated about the function of a gene. For analysis and verification of the functions, an established approach is to inhibit the expression of the gene of interest and to compare the resulting mutant with the wild type strain. In order to accomplish inhibited gene expression, several different methods have been described. The methods most frequently used are gene disruption and gene silencing. Both technologies will be described in the following chapters.

1.5.1 Gene disruption by homologous recombination

In order to define the function of an uncharacterised gene of interest, targeted disruption by transformation-mediated homologous integration is a valuable method (Schäfer, 1994; Struhl, 1983) since in many cases the phenotype of such a mutant provides essential information for understanding the role of the gene. This deletion should be as precise as possible, ideally just deleting the coding region, although this is not always possible in practice. With insertional inactivation or partial deletion there remains the possibility of partial gene function being retained, while deletion of flanking sequences has the potential to cause additional phenotypes (Hynes, 1996). Targeted

gene disruption was also applied in other studies as a powerful tool for examining the role of genes in the pathogenicity of fungi on plants (Bowyer et al., 1995; Herrmann et al., 1996; Rogers et al., 1994). Gene disruption involves homologous integration of an internal fragment of a gene yielding two versions of the gene, one with 3' sequences deleted and one with 5' sequences deleted (Figure 5A). This has the potential to yield partially functional gene fragments. Another possibility is to use the one-step disruption procedure developed for *Saccharomyces cerevisiae* by Rothstein (1983). A linear fragment, in which gene sequences are replaced with a selectable marker, allows the selection of disruptants generated by homologous recombination (Figure 5B). In filamentous fungi the flanking homologous sequences usually need to be at least 1 kb long to yield the desired replacement at a reasonable frequency (Hynes, 1996). If the selectable marker gene has a homologue in the genome then some transformants can arise by gene conversion or integration of this sequence. There are advantages therefore in using heterologously selectable markers such as bacterial hygromycin B and Neomycin resistances.

It is generally important to be able to determine whether a cloned gene is essential for viability. Therefore, methods for demonstrating that a gene knockout is lethal are required. In *S. cerevisiae* this is readily done by generating the knockout in a diploid strain, allowing sporulation, dissecting asci, and determining whether all four ascospores are viable. Inactivation of essential genes in haploid strains by transformation is capable of yielding heterokaryons, which cannot be broken down into the constituent homokaryons. The use of this method is particularly favourable in *A. nidulans* where the asexual spores (conidia) are uninucleate. Therefore, the inability to recover a marker genotype in normal colonies arising from streaked conidia is indicative of an essential gene. Careful examination of the defect in poorly germinating conidia carrying the inactivated gene is then possible. This method has been of particular use in analysing mutations affecting the cell cycle (Osmani et al., 1988; Rasmussen et al., 1994).

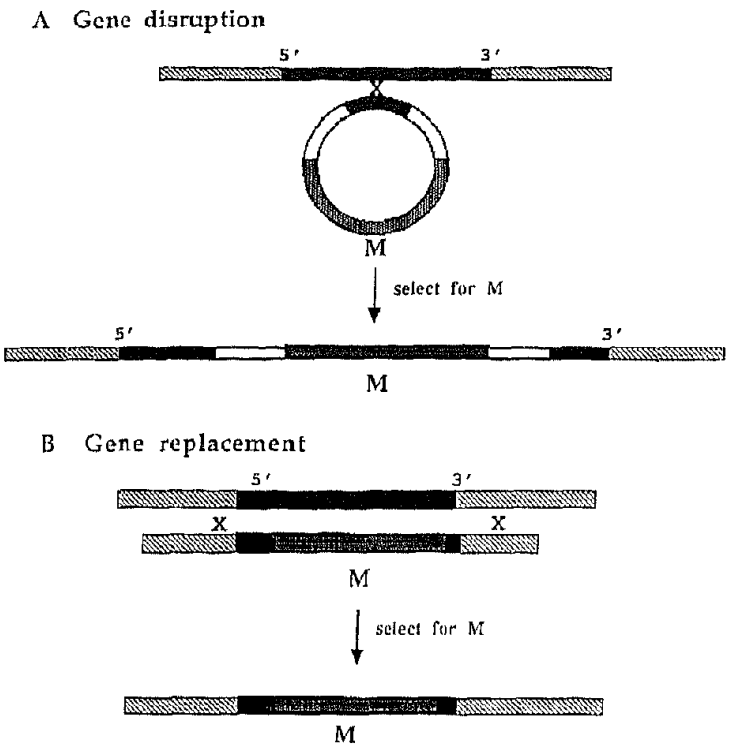

Figure 5. Strategies for generation of gene knockouts according to Hynes (1996). Gene disruption by homologous integration of an internal gene fragment (A). Gene deletion and replacement by integration of a linear fragment carrying homologous flanking sequences (B). In both cases, transformants are selected by the marker M.

1.5.2 Gene silencing by RNA interference

RNA silencing is a general term for a particular collection of phenomena, in which short RNA molecules trigger repression of homologous sequences (Almeida and Allshire, 2005; Hammond et al., 2001; Meister and Tuschl, 2004; Parrish et al., 2000). It is a highly conserved pathway, found in a large variety of eukaryotic organisms, and its main characteristic is the use of small RNA molecules of 21–28 nucleotides that confer high specificity to the target sequence. Originally, it was described as part of a 'co-suppression' phenomenon in plants (Matzke et al., 1989; Napoli et al., 1990; van der Krol et al., 1990) or 'quelling' in *Neurospora crassa* (Cogoni et al., 1996). It was later attributed to a posttranscriptional gene silencing process (PTGS) occurring in the presence of complementary RNA molecules that would bind and form double-stranded RNA (Metzlaff et al., 1997). RNA silencing provides organisms with a defence against mobile DNA elements, which cause mutations when they insert themselves within, or close to, a gene (Plasterk and Ketting, 2000). In plants, RNA silencing may also offer protection against viruses (Mourrain et al., 2000). The existence of RNA silencing, at least in animals, has been inferred from experiments in which double-stranded RNA

(dsRNA) was introduced into cells (Fire et al., 1998). Most RNA in a cell is single-stranded, so the presence of dsRNA might signal to the cell that it is being invaded by mobile DNA or viruses. It is now evident that the core machinery required for RNA silencing plays also crucial roles in cellular processes as diverse as regulation of gene expression, protection against the proliferation of transposable elements and modifying chromatin structure (Almeida and Allshire, 2005). In RNA silencing, double-stranded RNA enters the 'canonical pathway' after cleavage small (21–28 nt) RNA duplexes by the helicase/RNase-like III Dicer (Bernstein et al., 2001). Following unwinding, a single-stranded small RNA (small interfering RNA: siRNA) becomes part of protein complexes in which Argonaute is a central player (Fagard et al., 2000; Hammond et al., 2000) (Figure 6a, b). These RNA-induced silencing complexes (RISC) then target homologous mRNAs and exert silencing either by inducing cleavage ('slicing') or, as in the case of micro-RNA-loaded RISC, by also eliciting a block to translation (Figure 6c, e). RNA-dependent RNA polymerase (RdRP) also plays a role in nematodes (Smardon et al., 2000), plants (Dalmay et al., 2000; Mourrain et al., 2000) and fungi (Cogoni and Macino, 1999; Volpe et al., 2002), but is apparently not required or detectable in the genomes of flies and vertebrates. RdRP amplifies the RNA interference (RNAi) and PTGS response by generating more double-stranded RNA from single-stranded targets that can then enter and continue to stimulate the RNA silencing pathway (Figure 6a).

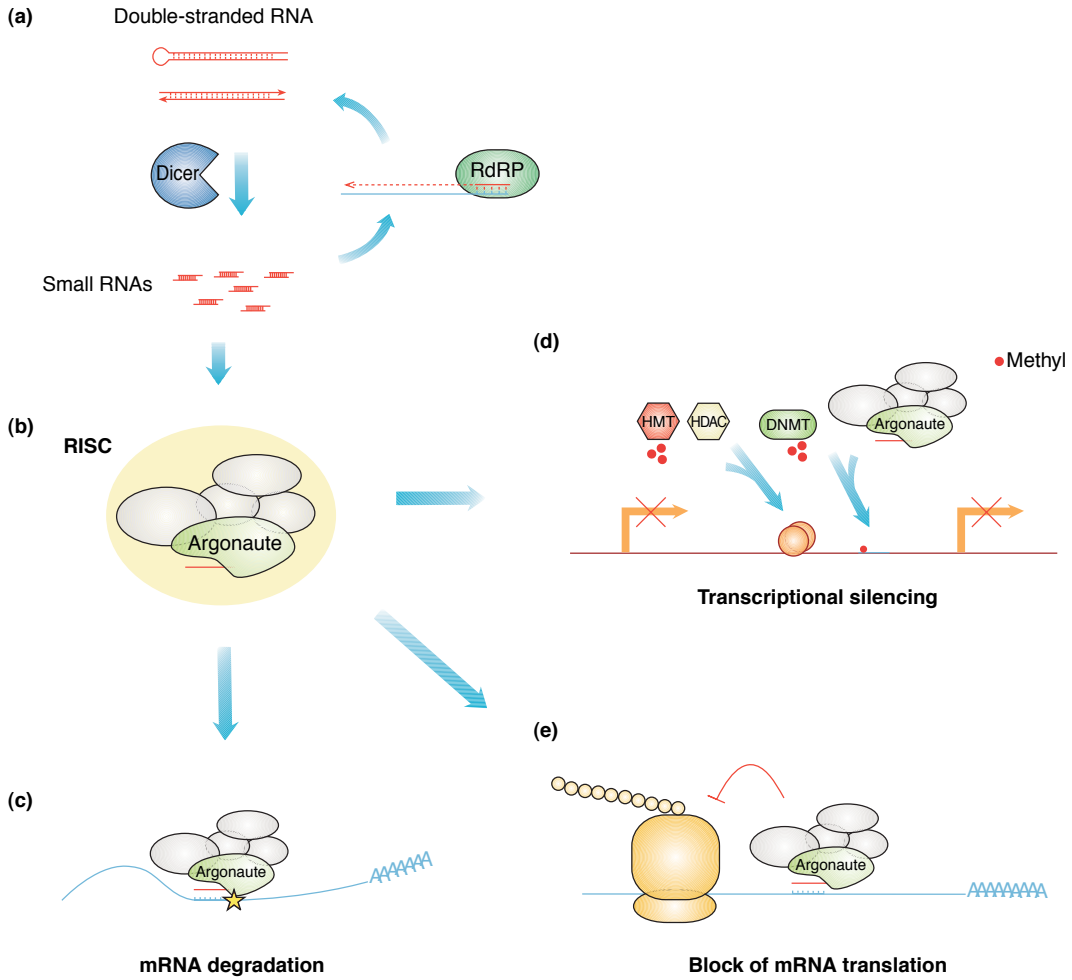

Figure 6. Different forms of RNA silencing according to Almeida and Allshire (2005). (a) Double-stranded RNA molecules are cleaved into small RNAs by Dicer (blue). The RdRP protein (green) acts in a positive-feedback loop for the siRNA signal by producing complementary strands of the target RNA molecule (Makeyev and Bamford, 2002). (b) The RISC complex, primed with a small RNA, can exert silencing in a variety of forms. The small RNA confers target specificity, whereas the protein components effect repression. (c) The conventional RNAi, PTGS or quelling pathway. The RISC complex associates with the target mRNA and employs the RISC 'slicing' activity of Argonaute protein to cleave the transcript (Liu et al., 2004; Song et al., 2004) (d). RISC can also induce transcriptional gene silencing (TGS) by using the siRNA specificity to direct silent chromatin modifications over homologous DNA loci by methylation. (e) A typical microRNA-loaded RISC does not affect mRNA turnover but binds to the 30-UTR of the target transcript (blue line) and effectively blocks its translation by an unknown manner.

RNA silencing approaches have been rapidly developed and employed in plants and animals as a tool for exploring gene function (Nakayashiki et al., 2005). Recently, gene silencing using RNAi was reported in several ascomycetous filamentous fungi, e.g. *Magnaporthe oryzae* (Kadotani et al., 2003), *Colletotrichum lagenarium* (Nakayashiki et al., 2005), *A. fumigatus* (Mouyna et al., 2004), *Cladosporium fulvum* (Lacroix and Spanu, 2009), and *F. graminearum* (McDonald et al., 2005). With the progress of genome analysis, RNAi is also regarded as an efficient approach to the functional analysis of genes in filamentous fungi, especially for those species limited in conventional gene

replacement by the low efficiency of homologous recombination (Lacroix and Spanu, 2009; Matityahu et al., 2008; Nakayashiki et al., 2005; Yamada et al., 2007).

1.6 Transformation of filamentous fungi

1.6.1 Polyethylene glycol-mediated protoplast transformation

The most established method for transformation of filamentous fungi is the use of protoplasts incubated in $CaCl_2$ and polyethylene glycol (de Groot et al., 1998; Hynes, 1996) and was first described by Tilburn et al. (1983). Protoplasts were prepared using germinated asexual spores, young mycelial fragments or basidiospores and various cell wall digesting enzymes. Protoplasts are osmotically stabilised with sodium chloride, magnesium sulphate, mannitol or, most commonly, sorbitol. Uptake of DNA (linear or circular double-stranded) by protoplasts is carried out in the presence of calcium ions followed by addition of high concentrations of polyethylene glycol (Hynes, 1996). Calcium is necessary for sufficient DNA absorption. Isolated DNA co-precipitates with calcium phosphate microcrystals. The insoluble DNA complex is engulfed through endocytosis. Within the cytoplasm of the cell, the microcrystals dissociate from the co-precipitate, allowing the DNA to reach the nucleus freely (Hain et al., 1985; Razdan, 2003). Polyethylene glycol stimulates the DNA uptake by endocytosis without any gross damage to protoplasts. This is followed by cell wall formation and initiation of cell division.

1.6.2 *Agrobacterium tumefaciens*-mediated transformation (ATMT)

Agrobacterium tumefaciens is a Gram-negative soil bacterium that is widely used to transform plants (Bundock et al., 1995; de Groot et al., 1998). It has the natural ability to transfer a segment of DNA from its Ti plasmid, known as the T-DNA, into plant cells by inducing tumours or crown gals at plant wound sites so that the T-DNA integrates at random into the nuclear chromosomes (Bundock et al., 1995; de Groot et al., 1998). The ends of the T-DNA, and thus the length of the transferred DNA, are defined by two short repeated sequences, the left and right borders. *A. tumefaciens* can transfer at least 150 kb of foreign DNA to the plant cell (Hamilton et al., 1996). The process of T-DNA transfer is dependent upon expression of *A. tumefaciens*' *vir* genes, which are induced by compounds secreted by wounded plant cells, such as acetosyringone (Charles et al.,

1992; Winans, 1992). *Agrobacterium* attaches itself on the wall of the wounded plant cell and introduces the T-DNA into the plant cell. Vir proteins bind on the T-DNA and protect the complex against endonucleases. Moreover, specific vir proteins are responsible for the translocation of the T-DNA complex into the nucleus (Winans, 1992). In addition, the virD2 protein offers homologies to the DNA ligase of *Escherichia coli* and hence could be part of the integration process of the single-stranded T-DNA complex into the genomic DNA of the plant (Winans, 1992). Repair systems probably complement the missing strain of the integrated T-DNA. The structural similarities of the infection of *Agrobacterium* with bacterial conjugation suggests a common evolution of both DNA transfers (Zambryski et al., 1989). Expression of *onc* genes on the T-DNA leads to plant cell proliferation and formation of a tumour. vir-mediated plant cell transformation represents the only example of naturally occurring trans-kingdom DNA transfer known to date (Bundock et al., 1995).

ATMT was originally developed for plants and it has subsequently been applied to the transformation of yeasts and several filamentous fungi (Covert et al., 2001; de Groot et al., 1998; Mullins et al., 2001; Talhinhas et al., 2008). With this method, transformation can be achieved using either protoplasts or intact cells (Zwiers and De Waard, 2001). ATMT has a high frequency of gene replacement when a mutated gene from the transformation host was cloned between the T-DNA border sequences (Chen et al., 2000; de Groot et al., 1998; Rho et al., 2001; Zeilinger, 2004; Zwiers and De Waard, 2001). In *Kluyveromyces lactis* the frequency of gene replacement by ATMT was more than ten times higher than that by electroporation with linear double-stranded DNA (Bundock et al., 1999). Furthermore, integration of the transformed DNA into the genome generally occurs as a single copy (Staats et al., 2007; Zwiers and De Waard, 2001). *A. tumefaciens* strains are used for transformations, which carry a hypervirulent Ti helper plasmid that transforms at very high frequencies, but which lacks one border sequence and thus an intact T-DNA region (Hood et al., 1986; Komari et al., 1986; Lazo et al., 1991). If these strains are to function in transformation they must also carry a binary vector (Bevan, 1984), in which the T-DNA is defined by both left and right border repeats.

1.7 Aim of the study

1.7.1 Hydrophobin 5 from *F. culmorum* (FcHyd5p)

Since gushing factors are assumed to be surface-active molecules that stabilise carbon dioxide bubbles (Draeger, 1996; Pellaud, 2002), hydrophobins have been under discussion as gushing activators (Haikara, 2000; Laitila et al., 2007). As mentioned earlier, hydrophobin levels in malts could be related with lost volume due to gushing in beer (Haikara, 2000). However, differences in the gushing inducing capacity were observed with hydrophobins produced by various fungi (Sarlin et al., 2005). Actually, hydrophobins are produced by all mycelial fungi, regardless, whether or not they are gushing inducers. It has been shown that the properties of especially class II hydrophobins are highly variable between species (Askolin et al., 2006). Hydrophobins of gushing inducing filamentous fungi, such as *F. culmorum* and *F. graminearum,* may have special structural properties resulting in a higher activity in gushing induction (Zapf et al., 2006), but have not yet been isolated in order to study their gushing activity. However, recent studies demonstrated that beer fermented with *S. cerevisiae* transformed with the *FcHyd5* gene from *F. culmorum* displayed gushing (Zapf et al., 2006). Admittedly, expression of the gene in the *S. cerevisiae* transformants as well as detection of the protein in beer and brewing raw materials has not been demonstrated yet. Consequently, the role of hydrophobins in beer gushing needs further elucidation.

Therefore, the aim of this study was to produce and purify FcHyd5p, a hydrophobin of *F. culmorum* produced in naturally infected cereals and malt and to investigate its role in gushing induction. FcHyd5p without signal peptide has a predicted molecular weight of 8.84 kDa and a theoretical pI of 4.29. The amino acid sequence exhibits the characteristic pattern of eight cysteine, the second and the third being immediate neighbours as are the sixth and the seventh. This pattern is typical for class II hydrophobins as described by Wessels (1994). FcHyd5p has similarities to other hydrophobins, e.g. 85% amino acid sequence identity with FvHyd5p from *F. verticillioides* (Fuchs et al., 2004) and 100% amino acid identity with FgHyd5p from *F. graminearum* (Zapf et al., 2006). This fact makes FcHyd5p an interesting model substance for beer gushing because the gene is present in both fungal agents, which have been most closely associated to the induction of the phenomenon in breweries (Haikara, 1983; Munar and Sebree, 1997; Schwarz et al., 1996).

1.7.2 Alkaline foam protein A from *F. graminearum* (AfpA)

Its surface activity and similarity of characteristics with hydrophobins may allow speculations about the natural function of AfpA in the life cycle of the producing fungus. According to its structural features, a role in hyphal growth, surface hydrophobicity, conidium development and sporulation can be anticipated. Furthermore, its structural relatedness to cell wall proteins would include an influence on infection mechanisms as was described for *F. oxysporum*, where cell wall proteins were supposed to be responsible for penetration of the root cortex and consequently for the infection of crops (Prados-Rosales et al., 2009). But also hydrophobins are supposed to be important for plant-pathogen interactions (Templeton et al., 1994). In transformants of *Magnaporthe grisea,* in which the hydrophobin MHP1 was inactivated, pleiotropic effects on fungal morphogenesis occurred, including reduction in conidiation and conidial germination, reduced appressorium development and pathogenicity (Kim et al., 2005b). In contrast to hydrophobins, gushing experiments revealed that AfpA could not be connected with the induction of gushing. However, it could be demonstrated that AfpA was produced in contaminated malt and it was able to enhance gushing in its native form and even more after modification by Maillard reaction (Zapf et al., 2007).

Therefore, the aim of the current study was to investigate the influence of AfpA on some characteristics of *F. graminearum* cultures. One possible way to study these parameters is to disrupt the *AfpA* gene in the fungus and to compare the resulting knock out mutants to the wild type parent strain by determining differences in morphology, fitness, surface activity and plant pathogenicity. Furthermore, a goal of the work was to establish RNA silencing in *F. graminearum* in order to inactivate the gene rather than knocking it out. By comparing phenotypical differences between AfpA silencing mutants, knock out mutants and the corresponding wild type strain, differences between silencing and disruption of a gene should be explored. Additionally, the gene coding for *polyketide synthase 12* (*PKS12*), which is responsible for the biosynthesis of the red pigment aurofusarin in *F. graminearum* (Frandsen et al., 2006) should be used together with *AfpA* in a co-silencing construct. This approach would combine rapid analysis of transformants with efficient silencing by comparing colony pigmentation as phenotypical marker.

2 Materials and Methods

2.1 Materials

2.1.1 Strains

E. coli strain TOP10F´ (Invitrogen, USA) with the genotype F´(*lac*Iq Tn*10* (TetR)) *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G was used for cloning procedures. TOP10F´ possessed an additional F´ episome, which carries the tetracycline resistance gene and allows isolation of single-stranded DNA from vectors that have an f1 origin of replication. In addition, the F´ carries the *lac*I^q repressor for inducible expression from *trc*, *tac*, and *lac* promoters using IPTG.

The *Pichia pastoris* wild-type strain X33 with a positive methanol utilisation (Mut^+) was used for transformation and heterologous expression. For the determination of methanol utilisation (Mut) of putative transformants, the *P. pastoris* strain GS115/pPICZ/*lac*Z Mut^+ with a mutation in the histidinol dehydrogenase gene (*his4*) that prevents it from synthesising histidine (His^-) and a positive methanol utilisation (Mut^+) was used as positive control. The *P. pastoris* strain GS115/Mut^S Albumin with a slow methanol utilisation (Mut^S) was used as negative control. The gene for serum albumin was cloned with its native secretion signal into the *AOX1* locus, which leads to a secretion of albumin (67 kDa) and a Mut^S phenotype.

F. graminearum strain TMW 4.0157, an isolate from barley with a high level of sporulation and a growth inhibition by a hygromycin B concentration of 40 µg/ml was used for amplification of genomic DNA as well as knock out and silencing experiments.

The *A. tumefaciens* strain AGL1 was used for homologous integration and disruption of the *AfpA* gene in *F. graminearum*. AGL1 carries the hypervirulent Ti helper plasmid pTiBo542ΔT with a deleted border sequence (Hood et al., 1986; Komari et al., 1986) and an insertion mutation in its *recA* general recombination gene, which stabilises the recombinant plasmids (Lazo et al., 1991).

All strains were stored in the strain collection of the Lehrstuhl für Technische Mikrobiologie, Weihenstephan. The related TMW numbers are listed in Table 1.

Table 1: TMW numbers of strains used in the study

Organism	Strain	TMW number
Pichia pastoris	X33	3.177
	X33 [pPICZαA-FcHyd5]	3.213
	X33 [pPICZαA-FcHyd5-HisTag]	3.293
Fusarium graminearum	wild type	4.0157
	FG [pPK2-ΔAfpA]	4.2254 – 4.2257
	FG [pSilent1-AfpAi:TF1]	4.2258
	FG [pSilent1-AfpAi:TF2]	4.2259
	FG [pSilent1-AfpAi:TF3]	4.2260
	FG [pSilent1-AfpAi+PKS12i:TF1]	4.2261
	FG [pSilent1-AfpAi+PKS12i:TF2]	4.2262
	FG [pSilent1-AfpAi+PKS12i:TF3]	4.2263
	FG [pSilent1-AfpAi+PKS12i:TF4]	4.2264
	FG [pSilent1-AfpAi+PKS12i:TF5]	4.2265

2.1.2 Chemicals

Unless otherwise stated, chemicals were ordered by Merck (Darmstadt, Germany), Carl Roth GmbH & Co. KG (Karlsruhe, Germany), Fluka Serva (Heidelberg, Germany) or Sigma-Aldrich (Steinheim, Germany).

2.1.3 Oligonucleotides

All oligonucleotides were synthesised by Eurofins MWG-Operon (Ebersberg, Germany) and diluted to a concentration of 100 pmol/µl with deionised water. All used oligonucleotides are listed in Table 1.

Table 2: Primers used in the study

Standard primer

Name	Sequence 5' → 3'	Annealing
357f	CCCTACGGGAGGCAGCAG	63°C
Agro1r	GTCTCCAATGCCCATACCC	59°C
Agro2r	GTCTCCACTGCCCAAACCC	61°C

Expression of FcHyd5p

Name	Sequence 5' → 3'	Annealing
EcoRI-FcHyd5al-f	CCGGAATTCCGGTTGCCAGCTAACGAAAAAAGA	68°C
SalI-FcHyd5-r	ACGCGTCGACATCTTGAACACCTGTTGG	68°C
AgeI-FcHyd5-r	ACCGGTTCAATCTTGAACACCTGTTGG	68°C
AOX1-f	GACTGGTTCCAATTGACAAGC	55°C
AOX1-r	GCAAATGGCATTCTGACATCC	55°C
α factor	TACTATTGCCAGCATTGCTGC	55°C

Silencing

Name	Sequence 5' → 3'	Annealing
XhoI-AfpA-sen-f	CCGCTCGAGTTCACTGCTTCTCTCCTCAGC	57°C
SnaBI-AfpA-sen-r	TATATACGTATGCAGCTAACAGGCTTCTTG	56°C
ApaI-AfpA-anti-f	TATAGGGCCCTTCACTGCTTCTCTCCTCAGC	57°C
BglII-AfpA-anti-r	GGAAGATCTTGCAGCTAACAGGCTTCTTG	56°C
MOE-AfpA-f	CCGGCATGGGCCAAGGTGTTCTCCAGT	55°C
MOE-AfpA-r	CACCTTGGCCCATGCCGGATCGGTC	56°C
XbaI-AfpA-sen-f	TGCTCTAGATTCACTGCTTCTCTCCTCAGC	57°C
XbaI-PKS-anti-f	TGCTCTAGATGGAAGTCATTCGGTGTTGA	58°C
XhoI-PKS-anti-r	CCGCTCGAGGCTGTAGATGAAGGGGACCA	61°C
EcoRI-AfpA-anti-f	CCGGAATTCTTCACTGCTTCTCTCCTCAGC	57°C
EcoRI-PKS-sen-f	CCGGAATTCTGGAAGTCATTCGGTGTTGA	58°C
ApaI-PKS-sen-r	TATAGGGCCCGCTGTAGATGAAGGGGACCA	61°C
pSILENT-Intron-f	GCTGGAGGATACAGGTGA	56°C
pSILENT-Intron-r	GCCGTTCCCTGGCTGTGT	60°C

ATMT knock out

Name	Sequence 5' → 3'	Annealing
ATMTafpaUpKpnI-f	GGGGTACCCCTGTCAAATATCGGTAGTTGG	70°C
ATMTafpaUpKpnI-r	GGGGTACCCCGATGAGAGTGAAGTGAATGC	71°C
ATMTafpaDoXbaI-f	GCTCTAGAGCTAACAGCGAGAAGGAGTGC	70°C
ATMTafpaDoHind3r	CCCAAGCTTGGGGCTGCCAGAGTGATGAAAG	72°C
hph1_ppk2_f	TTCGATGTAGGAGGGCGTGGAT	62°C
hph1_ppk2_r	CGCGTCTGCTGCTCCATACAAG	64°C
AfpA-ko-r	AAGTGCACTGGCTTCAGAG	57°C

PEG-mediated knock out

Name	Sequence 5' → 3'	Annealing

AfpA-ko2-EcoRV-f	TATAGATATCTCAAGGATGGGCTTCATTTC	57°C
AfpA-ko2-EcoRV-r	TATAGATATCACAGGCTTCTTGGTCTCAGC	61°C
PKS12-ko-EcoRV-f	TATAGATATCGACGCTAGGTTCTTCAACATG	57°C
PKS12-ko-EcoRV-r	TATAGATATCGTGCCTGTGCCGTGCATCTC	65°C
pSM1-seq_f	TTAGTCAAGCTGCGATGAAG	55°C
pSM1-ko-rev	CGGTCGGCATCTACTCTATT	57°C
AfpA-ko-f	ACCAACAATTCAGAGGCTGT	57°C
PKS12-ko-f	TAAGAACACCAGCAAGGTCA	57°C

Realtime-PCR

Name	Sequence 5' → 3'	Annealing
Fgtub-f	GGTCTCGACAGCAATGGTGTT	61°C
Fgtub-r	GCTTGTGTTTTTCGTGGCAGT	59°C
EF1a-f	GGCTTTCACCGACTACCCTCCTCT	67°C
EF1a-r	ACTTCTCGACGGCCTTGATGACAC	65°C
UBC-f	TCCCCTTACTCTGGCGGTGTC	66°C
UBC-r	TTGGGGTGGTAGATGCGTGTAGT	64°C
RT-AfpA-f	AGGCAGTCCTTTCCACCTCTC	62°C
RT-AfpA-r	AGTGTAGGCAACCTGCTGCTC	62°C

2.1.4 Restriction enzymes

All restriction enzymes used in this work were provided by MBI Fermentas GmbH (St. Leon-Rot, Germany) and applied as recommended in the manufacturer's instructions. If available, FastDigest® enzymes were used.

2.1.5 Buffers

Unless otherwise stated all components for the buffers were dissolved in deionised water and percentage values correspond to volume per volume (v/v) for liquid components and weight per volume (w/v) for solid components.

Table 3: Buffers used in the study

Agarose gel electrophoresis

Name	Composition
TAE buffer	2.0 M Tris, 1.0 M acetic acid, 0.1 M EDTA, pH 8.2

5x Loading dye	50 mM EDTA, 40% sucrose, 0.001% bromophenol blue

DNA extraction

Name	Composition
TES buffer	100 mM Tris, pH 8.0, 10 mM EDTA, 2% SDS
TE buffer	10 mM Tris, 1 mM EDTA, pH 8.0
Lysis buffer	2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

PEG-mediated transformation

Name	Composition
STC buffer	0.8 M sorbitol, 50 mM Tris–HCl, pH 8.0, 50 mM $CaCl_2$
SPTC buffer	STC buffer, 40% polyethylene glycol 4000

SDS-PAGE

Name	Composition
Separating gel	16% acrylamide, 1.0 M Tris, pH 8.45
Stacking gel	4% acrylamide, 0.74 M Tris, pH 8.45
Gel buffer	3.0 M Tris, pH 8.45
5x-Cathode buffer	0.5 M Tris, 0.5 Tricine, 0.5% SDS, pH 8.25
5x-Anode buffer	1 M Tris, pH 8.9
2x incubation buffer	8% SDS (w/v), 24% glycerol (v/v), 100 mM Tris-HCl, 3.1% DTT (w/v), 0.01% bromophenol blue (w/v), pH 6.8

Hot coomassie blue staining

Name	Composition
Staining solution	0.03%(w/v) PlusOne™ Coomassie™ Blue PhastGel™ R-350 (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), 10% acetic acid
Destaining solution	10% acetic acid

Western blot

Name	Composition
Blotting buffer	10 mM CAPS, 10% methanol, pH 11.0
Blocking buffer	20 mM Tris, 150 mM NaCl, pH 7.5, 1% BSA shortly before usage
TBS buffer	20 mM Tris, 50 mM NaCl, pH 7.5
TBS-T buffer	20 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.5

Developer solution	100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 7.5
Stop solution	10 mM EDTA
BCIP solution	50 mg BCIP, 1.0 ml 70% N,N´-dimethyl-formamid, 1.0 ml H_2O
NBT solution	75 mg NBT, 1.0 ml 70% N,N´-dimethyl-formamid

FPLC

Name	Composition
Binding buffer	20 mM NaH_2PO_4, 500 mM NaCl, 50 mM imidazole, pH 7.4
Elution buffer	20 mM NaH_2PO_4, 500 mM NaCl, 500 mM imidazole, pH 7.4

2.1.6 Media

All media were autoclaved at 121°C for 20 min. Unless otherwise stated, percentage values correspond to volume per volume (v/v) for liquid components and weight per volume (w/v) for solid components. Heat-sensitive solutions were filter sterilised (diameter 0.2 µm) and added to the medium after cooling down to 55°C. For solidification 1.5%, Agar (Difco) was added. Sugar components were dissolved in a proportionate volume of deionised water and autoclaved separately to avoid formation of Maillard products.

Table 4: Media used in the study

Escherichia coli

Medium	Composition
LB	1% peptone, 0.5% yeast extract, 0.5% NaCl. pH 7.5
SOC	0.5% yeast extract, 2% peptone, 10 mM NaCl, 2.5 mM KCl, pH 7; After autoclaving: 10 mM $MgCl_2$ x 6 H_2O,10 mM $MgSO_4$ x 7 H_2O, 20 mM glucose

Pichia pastoris

Stock solution	Composition
10x YNB	13.4% YNB with ammonium sulphate without amino acids, filter sterilised
500x Biotin	0.02% biotin, filter sterilised, 4°C
100x Histidine	0.4% histidine, filter sterilised, 4°C
10x Dextrose	20% dextrose, filter sterilised, 4°C
10x Methanol	5% methanol, filter sterilised, 4°C

1 M potassium phosphate buffer	132 ml of 1 M K_2HPO_4, 868 ml of 1 M KH_2PO_4, pH = 6.0, autoclaved

Medium	Composition
YPD	1% yeast extract, 2% peptone; after autoclaving: 2% dextrose
YPDS	YPD, 1 M sorbitol
MDH	After autoclaving: 1.34% YNB, 4 x 10^{-5}% biotin, 2% dextrose, 4 x 10^{-3}% histidine
MMH	After autoclaving: 1.34% YNB, 4 x 10^{-5}% biotin, 0.5% methanol, 4 x 10^{-3}% histidine
BMMY	1% yeast extract, 2% peptone; after autoclaving: 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4 x 10^{-5}% biotin, 0.5% methanol
BMGY	1% yeast extract, 2% peptone; after autoclaving: 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4 x 10^{-5}% biotin, 1% glycerol
BMM	After autoclaving: 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4 x 10^{-5}% biotin, 0.5% methanol

Fusarium graminearum

Medium	Composition
CM	0.6% yeast extract, 0.6% casein hydrolysate; after autoclaving: 1% sucrose
Regeneration	0.1% yeast extract, 0.1% casein hydrolysate; after autoclaving: 0.1 M sucrose
Malt extract	3% malt extract, 0.2% soy peptone, pH 6.5
Mung bean	Green mung beans (4% w/v) were heated in water until boiling. After 5 min of boiling, the broth was filtered off and agar (2% w/v) was added.
Mannitol agar	0.2% mannitol, 1.5% agar

Agrobacterium tumefaciens

Stock solution	Composition
Salts for induction medium (Hooykaas et al., 1979)	3.625 g/l KH_2PO_4, 5.125 g/l K_2HPO_4, 0.375 g/l NaCl, 1.250 g/l $MgSO_4$ x 7 H_2O, 0.165 g/l $CaCl_2$ x 2 H_2O, 0.0062 g/l $FeSO_4$ x 7 H_2O and 1.250 g/l $(NH_4)_2SO_4$. Salts were separately dissolved in water
MES	1 M MES, filter sterilised, pH 5.3
Acetosyringone	10 mM acetosyringone, filter sterilised, pH 8.0
Micronutrients for M-100 salts	60 mg/l H_3BO_3, 140 mg/l $MnCl_2$ x 4 H_2O, 400 mg/l $ZnCl_2$, 40 mg/l Na_2MoO_4 x 2 H_2O, 100 mg/l $FeCl_3$ x 6 H_2O, 400 mg/l $CuSO_4$ x 5 H_2O.
Salts for M-100	1.6% KH_2PO_4, 0.4% Na_2SO_4, 0.8% KCl, 0.2% $MgSO_4$ x H_2O, 0.1% $CaCl_2$,

medium	0.8% micronutrients stock solution

Medium	Composition
Induction	10 mM glucose (5 mM for agar plates), 0.5% glycerol, 40% salt stock solution After autoclaving: 40 mM MES, 200 μM acetosyringone
M-100	1% glucose, 0.3% KNO_3, 6.25% M-100 salt stock solution

2.1.7 Plasmids

The plasmid pPICZαA (Invitrogen, USA) was used for heterologous expression of a gene of interest by integration of the construct into the *alcohol oxygenase 1* (*AOX1*) gene of *P. pastoris*. The vector was part of the EasySelect™ *Pichia* Expression Kit (Invitrogen, USA). The plasmid contains the pUC origin for replication in *E. coli*, the 5´ upstream region of the *AOX1* gene followed by *S. cerevisiae* α factor secretion signal, multiple cloning site, *c-myc* epitope, 6 x His-tag and the *AOX1* terminator. The zeocin resistance cassette for selection in *E. coli* or *P. pastoris* is driven by the promoters P_{EM7} that drives expression of the *Sh ble* gene in *E. coli* or P_{TEF1} that drives expression of the *transcription elongation factor 1* gene from *S. cerevisiae,* the *CYC1* transcription termination region stops transcription in *S. cerevisiae*.

The plasmid pSM1 (Pöggeler et al., 2003) was used for knock out constructs in *F. graminearum.* The vector contains the pUC origin and an ampicillin resistance cassette for replication and selection in *E. coli* and a hygromycin and GFP expression cassette flanked by multiple cloning sites. The GFP expression cassette contains the promoter from *glyceraldehyde-3-phosphate dehydrogenase* (Pgpd) of *A. nidulans*, the gene for the *enhanced green fluorescent protein* (egfp) and the *tryptophane C* terminator (TtrpC) of *A. nidulans*. The hygromycin B resistance cassette contains the *tryptophane C* promoter (PtrpC) of *A. nidulans* and the *hygromycin B phosphotransferase* gene. For the disruption of a gene of interest, parts of the genomic DNA upstream and downstream of the gene were integrated into the multiple cloning sites and the construct linearised by using a single cutter between the upstream and the downstream flank.

The plasmid pPK2 (Covert et al., 2001) was used for the disruption of genes in *F. graminearum* mediated by *A. tumefaciens.* The vector carries the pBR322 origin and a canamycin resistance for replication and selection in *E. coli* and *A. tumefaciens*. The transfer-DNA contains a hygromyin B resistance cassette consisting of the promoter Pgpd of *A. nidulans,* the *hygromycin B phosphotransferase* gene and the terminator TtrpC

of *A. nidulans*. The T-DNA is flanked by cloning sites for integration of flanking genomic DNA of the gene of interest and by T-DNA border regions, which assign T-DNA and are necessary for the transfer into the genome of *F. graminearum* mediated by *A. tumefaciens*.

The plasmid pSilent-1 (Nakayashiki et al., 2005) developed for ascomycetous fungi was used for gene silencing in *F. graminearum*. The plasmid carries an ampicillin resistance gene as selective marker for *E. coli* and a hygromycin resistance gene flanked by the promoter PtrpC and terminator TtrpC of *A. nidulans* for selection in *F. graminearum*. The transcriptional unit for hairpin RNA expression, also flanked by PtrpC and TtrpC, carries two multiple cloning sites, which are separated by intron 2 of the *cutinase* gene from *M. oryzae*.

Maps of all plasmids can be found in the appendix at chapter 8.1, sequences at chapter 8.2.

2.2 Microbiological methods

2.2.1 Production of chemically competent *E. coli*

10 ml of LB broth were inoculated with *E. coli* TOP10F´ and grown overnight at 37°C and shaking (190 rpm) to an OD_{590} of 0.5. Cells were harvested by centrifugation (5,000 x g, 5 min, 4°C) and resuspended in 5 ml of ice-cold 100 mM $CaCl_2$ solution. After incubation on ice for 30 min, cells were again harvested by centrifugation (5,000 x g, 4°C, 5 min) and resuspended in 1 ml of ice-cold 100 mM $CaCl_2$ solution. After addition of 175 µl of ice-cold glycerol (87% v/v), cells were aliquoted in 0.1 ml portions, frozen in liquid nitrogen and finally stored at -80°C.

2.2.2 Production of electrocompetent *P. pastoris*

The *P. pastoris* wild type strain X33 (TMW 3.177) was used for electroporation. *P. pastoris* was incubated overnight at 30°C and shaking (180 rpm) in YPD broth. 200 µl of the overnight culture were used for inoculation of 500 ml fresh YPD medium. After an OD_{600} of 1.5 was reached, cells were washed as follows: centrifugation (1,500 x g, 4°C, 5 min), resuspension of the pellet in 500 ml of ice-cold sterile and deionised water, centrifugation, resuspension of the pellet in 250 ml of ice-cold sterile and deionised

water, centrifugation, resuspension of the pellet in 20 ml of ice-cold 1 M sorbitol, centrifugation, resuspension of the pellet in 1 ml of ice-cold 1 M sorbitol.

2.2.3 Production of electrocompetent *Agrobacterium tumefaciens*

Electrocompetent *A. tumefaciens* were generated according to Shen et al. (1989). A single colony of *A. tumefaciens* AGL1 was picked and inoculated in 10 ml LB medium containing 100 µg/ml ampicillin (25°C, 180 rpm, overnight). 500 µl of the overnight culture were transferred into 100 ml LB medium containing 100 µg/ml ampicillin and grown at 25°C to an OD_{660} of 0.7. The culture was chilled on ice for 10 min and afterwards pelleted by centrifugation (4,000 rpm, 5 min, 4°C). The pellet was resuspended in 15 ml ice-cold 10% glycerol followed by a centrifugation step. After resuspension in 4 ml 10% glycerol and centrifugation, the pellet was finally suspended in 1.5 ml 10% glycerol and aliquoted in portions of 100 µl. Cells were frozen in liquid nitrogen and stored at -80°C.

2.2.4 Transformation of chemically competent *E. coli*

E. coli transformation was performed as described by Hanahan et al. (1983). 100 µl of chemically competent *E. coli* TOP10F´ were thawed on ice and mixed with 10 ng of plasmid or 10 µl of ligation reaction. After incubation for 30 min on ice, cells were subjected to a heat shock at 42°C for 40 sec and chilled on ice for 2 min. Afterwards, cells were incubated in 1 ml SOC medium for at least 1 h at 37°C and 120 rpm shaking. For selection, 100 µl as well as the remaining pellet of the cell suspension were plated on selective agar plates and incubated at 37°C for 1 day.

2.2.5 Transformation of *P. pastoris*

Up to 10 µg of linearised plasmid DNA of pPICZαA-FcHyd5 or pPICZαA-FcHyd5-HisTag (chapter 2.3.7) with a concentration of 1 µg/µl (dissolved in sterile, deionised water) was used for transformation. 80 µl of the ice-cold competent *P. pastoris* X33 cells (chapter 2.2.2) were mixed with the linearised vector and transferred to an ice-cold 0.2 cm electroporation cuvette. After incubation for 5 min, cells were pulsed with the GenePulser II Electroporation System from Bio-Rad (Hercules, USA) with the following conditions: 25 µF, 200 Ω, 2 kV. 1 ml of ice-cold 1 M sorbitol were added to the cuvette

and transferred to a 15 ml centrifugation tube. After incubation for 2 h at 30°C without shaking, 10 to 200 µl were spread on YPDS plates containing 100 µg/ml zeocin. After a maximum of 10 days, colonies were transferred to fresh YPD plates with zeocin.

2.2.6 Transformation of *A. tumefaciens*

Electroporation of *A. tumefaciens* was performed as described by Shen et al. (1989). Electrocompetent *A. tumefaciens* cells (chapter 2.2.3) were thawed on ice. Up to 200 ng plasmid DNA of pPK2-AfpA-ko (chapter 2.3.8) were added to 50 µl cells and transferred to an ice-cold 0.2 cm electroporation cuvette. After incubation for 5 min, cells were pulsed with the GenePulser II Electroporation System from Bio-Rad (Hercules, USA) with following conditions: 25 µF, 400 Ω, 2.5 kV. 1 ml of LB medium were added to the cuvette and transferred to a 15 ml centrifugation tube. After an incubation for 3 h at 26°C without shaking, 100 µl were spread on LB agar plates containing 50 µg/ml canamycin and incubated at 26°C for 3 days.

2.2.7 Production of a *F. graminearum* spore suspension

Mung bean agar plates were inoculated with *F. graminearum* and incubated for 14 days at ambient temperature using UV light (universal white light and black light blue) with a photoperiod of 12 h to ensure efficient induction of *F. graminearum* sporulation. Conidia were harvested from the plates with 2 ml of sterile tap water or CM medium. Conidia were counted using a counting chamber. Conidial concentrations (conidia per ml) were calculated according to the manufacturer of the counting chamber. Average conidial numbers were calculated from at least 13 independent counts for each culture.

2.2.8 Generation of *F. graminearum* protoplasts

Conidia were collected from at least 5 mung bean agar plates with each 2 ml CM medium (chapter 2.2.7). 90 ml CM medium were inoculated with 10 ml conidial suspension and incubated for 10 h at ambient temperature and shaking (150 rpm). Germinated conidia were collected in a Steritop™ filtration unit (0.22 µm, Millipore, Billerica, MA, USA) and washed twice with 50 ml 0.7 M NaCl. Then, 1 g (wet weight) of conidia were resuspended in 50 ml 0.7 M NaCl (pH 5.6) containing 10 ml driselase solution (20 mg/ml driselase in 1 M NH_4Cl, sterile filtrated 0.2 µm, Sigma-Aldrich, Steinheim,

Germany) and digested for 3 h at 28°C, 75 rpm. The protoplasts were pelleted by centrifugation (1,300 x g, 10 min, 4°C) in a swing–out rotor and washed twice by resuspending in 10 ml ice cold STC buffer and mild centrifugation (830 x g, 10 min, 4°C). The protoplasts were suspended at a concentration of 1 x 10^8 protoplasts/ml in four parts STC buffer and one part SPTC buffer. Spores were used immediately or stored at -80°C in 100 µl aliquots supplemented with 1 µl DMSO each.

2.2.9 PEG-mediated transformation of *F. graminearum* protoplasts

For integration of the knock out vectors pSM1-AfpA-ko and pSM1-PKS12-ko (chapter 2.3.8) or the silencing vectors pSilent1-AfpA and pSilent1-AfpA-PKS12 (chapter 2.3.9) into the genome of *F. graminearum*, transformation of fungal protoplasts was performed as described by Maier et al. (2005). 30 µg of linearised plasmid DNA were added to 100 µl of *F. graminearum* protoplast suspension (chapter 2.2.8). Samples were mixed and incubated on ice for 30 min. Afterwards, 1 ml SPTC was mixed with the suspension and incubated at room temperature for 20 min. Protoplasts were mixed gently into 200 ml regeneration medium at 45°C and 20 ml were poured in each 94 mm Petri dish. After incubation at 28°C for 24 h, plates were overlaid with 10 ml selective agar (450 µg/ml hygromycin B) and further incubated. Transformants appeared after 4 days and were transferred to fresh plates of CM medium with 150 µg/ml of hygromycin B.

2.2.10 Transformation of *F. graminearum* using ATMT

The *A. tumefaciens* mediated transformation was performed according to Schmidt-Heydt et al. (2009). *A. tumefaciens* strain AGL1 carrying the knock out plasmid pPK2-AfpA-ko (chapter 2.3.8) was inoculated in 7 ml LB medium containing 50 µg/ml canamycin and streptomycin at 250 rpm and 29°C for 20 h. After cultivation, cells were diluted with induction medium accomplished with 200 µM acetosyringone to an OD_{660} of 0.15 and following incubated at 29°C and 250 rpm until an OD_{660} of 0.6 to 0.7 was reached. A *F. graminearum* TMW 4.0157 spore suspension diluted with induction medium to a final concentration of 10^5 to 10^6 spores/ml was used (chapter 2.2.7). Sterile cellophane membranes were cut to size and put on agar plates containing induction medium with 200 µM acetosyringone. 100 µl spore suspension and 100 µl *A. tumefaciens* culture with an OD_{660} of 0.6 to 0.8 were mixed, plated on the cellophane membranes and co-

cultivated at 27 °C for 48 h. Afterwards, cellophane membranes were put on M-100 plates containing 300 µg/ml mefoxin and 150 µg/ml hygromycin B and incubated at ambient temperature for approximately 6 days. Putative transformants were again transferred on M-100 plates containing 300 µg/ml of mefoxin and 150 µg/ml of hygromycin B in order to avoid *A. tumefaciens* contamination.

2.2.11 Determining the Mut phenotype of *P. pastoris* transformants

Due to the presence of the *AOX1* sequence in pPICZαA, recombination can occur not only in the 5´ region of the native *AOX1* gene, but also in the 3´ region, disrupting the wild-type *AOX1* gene and creating Mut^S (methanol utilisation slow) transformants instead of Mut^+ (methanol utilisation positive). Testing on MDH and MMH plates will allow confirmation of the Mut^+ phenotype. For differentiation, Mut^+ strain GS115/pPICZ/*lac*Z Mut^+ and the Mut^S strain GS115/Mut^S Albumin were used. Control strains and putative recombinants were plated on both an MDH and MMH plate. After incubation for 2 days at 30°C growth rates of the strains were compared. Mut^+ strains will grow normally on both plates.

2.2.12 Single-spore isolation

A spore suspension of putative *F. graminearum* transformants was prepared as described in chapter 2.2.7, but mung bean medium plates with 150 µg/ml hygromycin B as selective were used. 100 µl of a spore solution with 3 x 10^2 spores/ml were plated on selective mung bean plates (150 µg/ml hygromycin B) and incubated for 5 h at 20°C. Afterwards, germination of spores were microscopically analysed and exposed spores were transferred to freshly prepared selective mung bean plates.

2.2.13 Phenotypical analysis of aurofusarin expression

F. graminearum silencing transformants with pSilent1-AfpA-PKS12 hairpin cassette and the wild type strain were inoculated on mannitol agar with 0.1% PCNB (Böhm-Schraml et al., 1993) and incubated in the dark at 30°C for 10 days. Colonies showing an albino phenotype indicate an inhibition of the *PKS12* gene.

2.3 Molecular biological methods

2.3.1 Polymerase chain reaction (PCR)

DNA was amplified with PCR techniques by using Taq-DNA polymerase (Qbiogene, MP Biomedicals, Heidelberg, Germany) or proofreading KOD Hot Start DNA-polymerase from Novagen (Madison, USA). Thermo Cycler Primus96 plus (MWG-Biotech AG, Ebersberg, Germany) and specific oligonucleotides (chapter 2.1.3) were used for amplification. PCR conditions were used as follows: Initial denaturing of DNA at 95°C for 4 min followed by 35 cycles of denaturing at 95°C for 45 sec, primer annealing for 45 sec and elongation at 72°C for 1 min/kb. Final elongation time was 5 min. For annealing temperatures of oligonucleotides, see Table 2 in chapter 2.1.3.

2.3.2 Agarose gel electrophoresis

DNA was separated on 1% agarose gels (Biozym Scientific GmbH, Hess. Oldendorf, Germany) using 1x TAE buffer in an Easy Cast electrophoresis system (Owl Separation Systems, Portsmouth, NH, USA). Samples were mixed with 5x loading dye and separated by application of voltage of 90 to 120 V for 1 to 1.5 h. The Lambda DNA/*Eco*RI+*Hind*III Marker 3 (Fermentas, St. Leon-Rot, Germany) or GeneRuler™ 100 bp DNA Ladder (Fermentas, St. Leon-Rot, Germany) was used for sizing and estimation of DNA concentrations. Gels were stained with dimidium bromide and visualised by UV transillumination (Herolab UVT 28M, Herolab GmbH Laborgeräte, Wiesloch, Germany) and documented with a video camera and Intas® GDS equipment and software.

2.3.3 DNA isolation from *P. pastoris* TMW 3.177

DNA isolation from *P. pastoris* was accomplished as described by Harju et al. (2004). *P. pastoris* was grown for 24 h at 30°C in YPD broth. 1.5 ml of the culture was pelleted (20,000 x g, 5 min, room temperature) and resuspended in 200 µl of lysis buffer. Following steps were repeated twice: incubation in a dry ice-ethanol bath for 2 min and in a water bath at 95°C for 1 min. After vortexing for 30 sec, 200 µl of chloroform were added and vortexed again for 2 min. After centrifugation (15,000 rpm, 3 min, room temperature), the upper aqueous phase was transferred into a fresh microcentrifuge tube containing 40 µl of ice-cold 100% ethanol and gently mixed. After precipitation at

room temperature for 5 min, DNA was pelleted (15,000 rpm, 5 min, room temperature) and the supernatant removed. After a washing step with 0.5 ml of 70% ethanol, DNA was again pelleted (15,000 rpm, 5 min, room temperature) and the supernatant removed. The pellet was air-dried and DNA finally re-suspended in 25 – 50 µl TE buffer.

2.3.4 DNA isolation from *F. graminearum* TMW 4.0157

DNA extraction from *F. graminearum* was accomplished as described by Möller et al. (1992). *F. graminearum* was incubated in 10 ml malt extract medium for 7 days at ambient temperature and shaking (130 rpm). Following cultivation, mycelium was filtrated onto sterile filter paper and washed with sterile deionised water. The mycelium was lyophilised and subsequently pestled to a powder in a mortar using sterile sea sand. 50 mg of the dry ground mycelium were transferred to a fresh 2 ml reaction tube with 500 µl TES buffer and 50 µg Proteinase K (peqlab Biotechnologie GmbH, Erlangen, Germany) and incubated for 1 h at 58°C with occasional gentle mixing. Following incubation, 140 µl 5 M NaCl and 65 µl 10% CTAB were added and incubated for 10 min at 65°C. 750 µl CIA 24:1 were added, gently mixed and incubated on ice for 30 min. After centrifugation (15,000 x g, 10 min, 4°C), the supernatant was transferred to a fresh 1.5 ml reaction tube, 225 µl 5 M ammonium acetate were added and gently mixed. After 30 min incubation on ice and centrifugation (15,000 x g, 10 min, 4°C), the supernatant was again transferred to a 1.5 ml reaction tube and 0.55 volumes isopropanol were added. After incubation at ambient temperature for 1 h, DNA was pelleted (15,000 x g, 30 min, 20°C) and the supernatant removed. The DNA pellet was washed twice with 200 µl of cold 70% ethanol, air-dried and resuspended in 50 µl TE buffer.

2.3.5 Precipitation of plasmid DNA

Plasmid DNA was ethanol precipitated using 1/10 volume 3 M sodium acetate and 2.5 volumes of 100% ethanol. After centrifugation (15,000 rpm, 30 min), the pellet was washed with 80% ethanol, air-dried, and resuspended in sterile, deionised water or TE buffer.

2.3.6 General cloning procedures

Cloning, DNA manipulation and gel electrophoresis followed standard procedures as described by Sambrook et al. (1989). DNA was purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) or isolated from agarose gels using an E.Z.N.A. gel extraction kit (Peqlab, Germany). DNA was sequenced by GATC Biotech (Konstanz, Germany). Plasmid DNA from *E. coli* was isolated with the Plasmid Mini Kit from Qiagen (Hilden, Germany) or the PureYield™ Plasmid Midiprep System from Promega (Madison, WI, USA) for higher DNA amounts. Restriction enzymes and T4-DNA ligase were purchased from Fermentas (MBI Fermentas GmbH, St. Leon-Rot, Germany) and reactions were performed following the recommendations given by the manufacturer. Ligation reaction was carried out using a ratio between plasmid and insert of 1:3 to 1:5. For cloning procedures with blunt-end restriction enzymes or single cutter, the vector was dephosphorylated with the Shrimp alkaline phosphatase (Fermentas, St. Leon-Rot, Germany) to avoid self-ligation. DNA and RNA concentration and purity were determined optically using the NanoDrop™ spectrophotometer (peqlab Biotechnologie GmbH, Erlangen, Germany).

2.3.7 Construction of FcHyd5p expression vectors

The signal sequence detection within the *FcHyd5* gene sequence was performed with SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). Plasmid 042849pPCR-Script (Zapf et al., 2006) containing the intron-free gene sequence of FcHyd5 as a synthetic gene was used for amplification (chapter 8.2.2). Nucleotide sequence of the gene was been optimised by GENEART GmbH (Regensburg, Germany) for *S. cerevisiae* codon usage.

The vector pPICZαA (chapter 2.1.7) with the *S. cerevisiae* α factor secretion signal and without *c-myc* epitope was used to ensure secretion of FcHyd5p in *P. pastoris,* Two different PCR fragments of the *FcHyd5* without native signal peptide were amplified by using the primer pairs EcoRI-FcHyd5al-f / AgeI-FcHyd5-r and EcoRI-FcHyd5al-f / SalI-FcHyd5-r. The stop codon TGA instead of the native stop codon TAA was used to ensure termination of translation in *P. pastoris*. Nucleotide sequences of primers are shown in Table 2 in chapter 2.1.3. Proofreading KOD Hot Start DNA-polymerase was used for amplification. The PCR products were digested with the restriction enzymes mentioned in the name of the primers. Each PCR fragment was ligated with the vector pPICZαA

resulting in pPICZαA-FcHyd5 without His-tag and pPICZαA-FcHyd5-HisTag with an additional C-terminal 6 x His-tag and transformed into *E. coli*. The correctness of all constructs was confirmed by DNA sequencing. The AOX primer pair was used for amplification. For transformation into *P. pastoris*, the constructs were linearised with *Sac*I.

2.3.8 Construction of disruption vectors

For the disruption of the *AfpA* gene in *F. graminearum* by using the PEG-mediated transformation of protoplasts, a fragment of 795 bp containing a part of the upstream region and a part of the coding sequence of the *AfpA* gene was amplified from *F. graminearum* TMW 4.0157 using the primer pair AfpA-ko2-EcoRV-f / AfpA-ko2-EcoRV-r. The fragment was ligated into the *EcoR*V site of the knock out vector pSM1 (chapter 2.1.7) generating the construct pSM1-AfpA-ko. The *AfpA* fragment contains a single cutting *EcoN*I site, which was used for linearisation of pSM1-AfpA-ko. For the disruption of the *PKS12* gene as phenotypical control for efficient transformation according to Maier et al. (2005), a fragment of 833 bp of *PKS12* was amplified from *F. graminearum* TMW 4.0157 using the primer pair PKS12-ko-EcoRV-f / PKS12-ko-EcoRV-r and ligated into the *EcoR*V site of the knock out vector pSM1. The single cutting *Eco47*III (*Afe*I) within the *PKS12* fragment was used for linearisation of the *PKS12* knock out construct pSM1-PKS12-ko.

For the knock out of the *AfpA* gene in *F. graminearum* with ATMT, the vector pPK2 was used (chapter 2.1.7). A part of the *AfpA* gene from *F. graminearum* TMW 4.0157 followed by a downstream flank with a total length of 439 bp was amplified with the primer pair ATMTafpaDoXbaI-f / ATMTafpaDoHind3r. The template was ligated upstream of the right border of the T-DNA within the binary vector pPK2 using the restriction sites *Xba*I and *Hind*III. Furthermore, an upstream flank of the AfpA gene with a length of 803 bp was amplified using the primer pair ATMTafpaUpKpnI-f / ATMTafpaUpKpnI-r. The *Kpn*I restriction site was used to ligate the flank downstream of the left border of the T-DNA within the binary vector pPK2 resulting in pPK2-AfpA-ko. Primers hph1_ppk2_f or hph1_ppk2_r, which bind on the hygromycin B resistance cassette of the pPK2 vector, were combined with ATMTafpaDoHind3r or ATMTafpaUpKpnI-f in order to check the properness of the construction by DNA sequencing.

2.3.9 Construction of silencing vectors

The vector pSilent-1 was used for gene silencing constructs (chapter 2.1.7). Two different silencing vectors were constructed: pSilent1-AfpA, which silences the gene of *AfpA* exclusively, and pSilent1-AfpA-PKS12, which silences both *AfpA* and *PKS12* genes as phenotypical marker.

For the pSilent1-AfpA vector, a 535 bp fragment of the *AfpA* gene was integrated in sense and antisense direction into the pSilent1 vector generating a hairpin RNA cassette. Site-directed mutagenesis by overlap extension-PCR according to Ho et al. (1989) was applied in order to exclude the intron of the *AfpA* gene. For the construction of the $AfpA_{sense}$ part of the silencing construct, two fragments were amplified using primer pairs XhoI-AfpA-sen-f / MOE-AfpA-r and MOE-AfpA-f / SnaBI-AfpA-sen-r. Both templates were then connected in a subsequent PCR using primers XhoI-AfpA-sen-f / SnaBI-AfpA-sen-r resulting in the $AfpA_{sense}$ fragment without intron. $AfpA_{anti}$ was generated similarly using primer pairs ApaI-AfpA-anti-f / MOE-AfpA-r and MOE-AfpA-f / BglII-AfpA-anti-r for amplification, followed by a subsequent PCR with primer pair ApaI-AfpA-anti-f / BglII-AfpA-anti-r resulting in the intron-free $AfpA_{anti}$ fragment. $AfpA_{sense}$ and $AfpA_{anti}$ were inserted into the *Xho*I / *SnaB*I and *Apa*I / *Bgl*II restriction sites of plasmid pSilent-1, generating the vector construct pSilent1-AfpA (Figure 7).

For the pSilent1-AfpA-PKS12 vector, $AfpA_{sense}$ and $AfpA_{anti}$ were generated as described above to exclude introns, but forward primers XbaI-AfpA-sen-f and EcoRI-AfpA-anti-f were used instead of XhoI-AfpA-sen-f and ApaI-AfpA-anti-f. For co-silencing, an intron-free 505 bp fragment of the *PKS12* gene was amplified using primer pairs EcoRI-PKS-sen-f / ApaI-PKS-sen-r for PKS_{sense} and XbaI-PKS-anti-f / XhoI-PKS-anti-r for PKS_{anti}. Fragments PKS_{anti} and $AfpA_{sense}$ as well as $AfpA_{anti}$ and PKS_{sense} were ligated using the restriction sites *Xba*I and *EcoR*I, respectively. Following ligation, PKS_{anti}-$AfpA_{sense}$ and $AfpA_{anti}$-PKS_{sense} were inserted into the *Xho*I / *SnaB*I and *Bgl*II / *Apa*I restriction sites of plasmid pSilent-1 generating the vector construct pSilent1-AfpA-PKS12 (Figure 7). Both cassettes were flanked by a trpC promoter (upstream) and a trpC terminator sequence (downstream). The sequence of the RNA hairpin cassettes was checked by DNA sequencing using primers pSILENT-Intron-f and pSILENT-Intron-r in combination with the primers used for the amplification of the respective fragments.

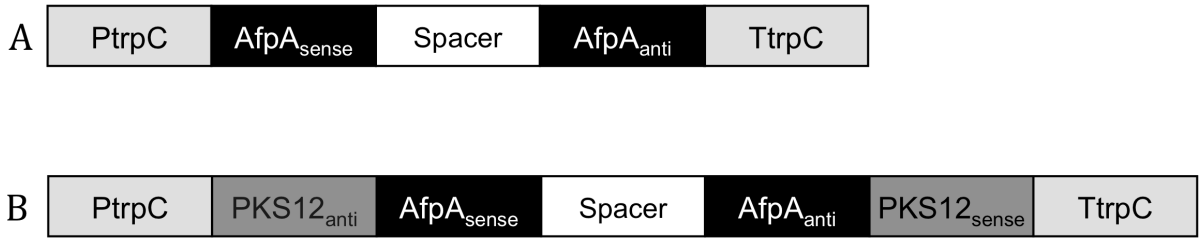

Figure 7. RNA hairpin cassettes for silencing of AfpA (A) or AfpA combined with PKS12 (B). Both cassettes are flanked by a trpC promoter and a trpC terminator sequence. The intron 2 of the *cutinase* gene from *M. oryzae* acts as spacer between the gene fragments of AfpA and PKS12. The hairpin cassettes are part of the silencing constructs in pSilent-1.

2.3.10 RNA isolation from *P. pastoris*

Yeast cells were grown in BMMY broth for 4 days and samples collection was started after 24 h. Cells were disrupted enzymatically using lyticase from *Arthrobacter luteus* (Sigma, St. Louis, USA) and RNA was isolated as described for yeast in the RNeasy Mini Kit manual (Qiagen, Hilden, Germany). Samples were immediately immersed in RNA*later*® Stabilization Reagent (AMBION, Inc., Austin, Texas, USA) and stored at -80°C.

2.3.11 RNA isolation from *F. graminearum*

RNA from *F. graminearum* was isolated using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). *Fusarium* was grown in malt extract medium for 7 days at ambient temperature and shaking (130 rpm). Mycelium was collected by filtration and the remaining liquid was removed by extrusion. Dried mycelium was frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. Liquid nitrogen was frequently added to avoid thawing of mycelium powder. RNA was isolated as described in the manufacturer's manual and stored at –80°C.

2.3.12 Reverse transcription

DNA was removed by treating the samples with RQ1 RNase-free DNase (Promega, Freiburg, Germany) at 37°C for 1 h before the reaction was stopped with 4 µl of Stop solution (Promega) and incubation at 65°C for 10 min. Transcription into cDNA was performed as described by Vermeulen et al. (2005). 10 µl of mRNA was rewritten into cDNA using 2 µg oligo $(dT)_{15}$ primer (Promega), 40 µM dNTPs (Qbiogene), 4 µl RT Buffer (Promega), 0.5 U M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega) and 1.5 µl water. mRNA samples were also prepared without reverse

transcriptase as a control for DNA contamination and included in a PCR to ensure that all residual DNA was fully digested with DNase.

2.3.13 Quantitative real-time PCR analysis

The level of gene expression was determined fluorometrically using SYBR Green I with the LightCycler® system (Roche Diagnostics GmbH, Mannheim, Germany) and the Absolute QPCR SYBR Green Capillary Mix (Thermo Scientific, Wilmington, DE, USA). Real-time PCR was performed according to the instructions of the manufacturer. Relative quantification changes in gene expression were calculated according to Pfaffl (2001). Crossing points were determined by using the Fit Point Method of the Roche LightCycler® software version 3.5. In order to determine the real-time PCR efficiency, standard curves using diluted chromosomal DNA were included for each primer pair. The efficiency was calculated from the slopes given by the LightCycler software according to Pfaffl (2001). Three different housekeeping genes were used as internal controls to which gene expression was normalised: *β-tubulin* using the primer pair Fgtub-f / Fgtub-r, *translation elongation factor 1* using the primer pair EF1a-f / EF1a-r and *ubiquitin conjugating enzyme* using UBC-f / UBC-r (Lysoe et al., 2006). *AfpA* was amplified using the primer pair RT-AfpA-f / RT-AfpA-r. Real-time PCR amplifications were initiated by a 15 min incubation at 95°C, followed by 44 cycles of 15 s at 95°C, 10 s at 59°C, and 34 s at 72°C. A melting curve analysis was conducted to ensure that the detected amplified products were from the target sequence and not from primer dimers, which have lower melting temperatures.

2.3.14 Verification of *P. pastoris* and *F. graminearum* transformants

The integration of the constructs pPICZαA-FcHyd5 or pPICZαA-FcHyd5-HisTag into the *P. pastoris* genome was checked by sequencing. The AOX primer pair was used for amplification. Sequences were compared by using the alignment tool ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Transcription of *FcHyd5* was verified by RT-PCR using cDNA and the primer pair α factor / AgeI-FcHyd5-r. Verified transformants were annotated as X33 [pPICZαA-FcHyd5] or X33 [pPICZαA-FcHyd5-HisTag].

Integration of the constructs pSM1-AfpA-ko and pSM1-PKS12-ko into the genome of *F. graminearum* was verified by sequencing with the primer pSM1-seq_f. Disruption of the *AfpA* gene was checked by sequencing with the primer AfpA-ko2-EcoRV-f and the disruption of the *PKS12* gene with the primer PKS12-ko-EcoRV-f.

For identification of successfully transformed *F. graminearum* AfpA knock out transformants carrying the T-DNA of the construct pPK2-AfpA-ko inside of the native *AfpA* gene, isolated DNA of the fungal strains were subjected to PCR with the primer pair hph1_ppk2_f and AfpA-ko-r. The oligonucleotides binds to the hygromycin B resistance cassette of the T-DNA and downstream of the *AfpA* gene fragment used for integration of the construct. The correct integration and disruption of the *AfpA* gene was verified by sequencing. Absence of *A. tumefaciens* was verified by PCR with the oligonucleotides 357f combined with the reverse primers Agro1r and Agro2r, which amplify specifically a part of the 16S rRNA gene of *A. tumefaciens*. Stability and integrity of the disruption was verified by qRT-PCR using the primer pair RT-AfpA-f and RT-AfpA-r for the *AfpA* gene (chapter 2.3.13). Housekeeping genes encoding *β-tubulin*, *translation elongation factor 1*, and *ubiquitin conjugating enzyme* were used as positive controls (Lysoe et al., 2006). Verified transformants were annotated as FG [pPK2-ΔAfpA].

Integration of the RNA hairpin cassette of the silencing constructs pSilent1-AfpA and pSilent1-AfpA-PKS12 was checked using the primer pair pSILENT-Intron-f / pSILENT-Intron-r. Verified silencing transformants were annotated as FG [pSilent1-AfpAi] or FG [pSilent1-AfpAi+PKS12i]

2.4 Protein chemical methods

2.4.1 Expression and preparation of recombinant FcHyd5p

Recombinant *P. pastoris* strain were inoculated in 25 ml BMGY broth at 30°C in a shaking incubator (250 rpm) until the culture reached an OD_{600} of 2-6. Cells were harvested by centrifugation (3,000 x g, 5 min, room temperature) and resuspended to an OD_{600} of 1 in BMMY or BMM broth to induce expression. BMMY medium was used to achieve a high amount of expressed FcHyd5p; BMM medium was used to keep the fraction of other proteins low. Cultures were placed in Fernbach flasks and covered with sterile gauze to ensure efficient aeration. Cultures were incubated at 30°C and shaking, 100% methanol was added every 24 hours to a final concentration of 0.5% to maintain

induction. Cells were harvested after 5 days by centrifugation at 10,000 x g for 30 min. Proteins in the supernatant of the BMMY medium were precipitated with 90% ammonium sulphate (4°C, 12 h), pelleted (17,000 x g, 40 min) and diluted in deionised water. The solution was dialysed for 24 h at 4°C with Membra-Cel dialysis tubing MWCO 3500 with a 3.5 kDa cut off (Serva Electrophoresis, Heidelberg, Germany) in deionised water. The supernatant of the BMM medium was freeze-dried after dialysis to concentrate proteins.

2.4.2 Protein quantification

Proteins were quantified as described by Bradford (1976) using the Bio-Rad Protein Assay from Bio-Rad (Hercules, USA). One part of the Dye Reagent Concentrate was diluted with four parts deionised water and filtered to remove particles. Triplicates of a dilution series of bovine serum albumin (BSA) with concentrations of 0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml, 1.0 mg/ml and 1.2 mg/ml were prepared for calibration. For measurement 10 µl of sample or standard were pipetted into separate micro plate wells and mixed with 200 µl of diluted dye reagent to each well. After incubation of at least 5 min at room temperature, absorbance was measured at 595 nm. Linear regression line was determined and protein concentration of the samples was calculated.

2.4.3 SDS-Polyacrylamide gel electrophoresis

Protein size and pureness were checked by one dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The Tricine electrophoresis system was used as described by Schägger and von Jagow (1987) due to the separation of small proteins in the range of 5 to 20 kDa and the high salt tolerance. SDS-PAGE was set up in a MiniProtean III chamber (Bio-Rad).

For assembling of two SDS-Polyacrylamide gels, 40 µl of 25% SDS solution, 7 µl TEMED, and 50 µl of freshly prepared 10% APS solution were added to 10 ml of separating gel solution and immediately mixed thoroughly. Gels were casted and overlaid with isopropanol. When the gel was polymerised after 30 min isopropanol was removed and 16 µl of 25% SDS solution, 7 µl TEMED and 33 µl of 10% APS solution were added to

5 ml of stacking gel solution and mixed. Combs were inserted and the stacking gel was casted on top of the solidified separating gel and polymerised for another 30 min.
Gel sandwiches were mounted in an electrophoresis apparatus. 1x cathode buffer and 1x anode buffer were added into the respective electrode chamber. Protein samples were mixed 1:1 with incubation buffer and incubated at 75°C for 10 min. After centrifugation (13,000 x g, 1 min), 15 µl of samples were applied to the gel cavities. For protein size determination the PageRuler™ unstained protein ladder (Fermentas, St. Leon-Rot, Germany) was used. Separation was started at 10 mA per gel for 15 min, followed by increasing the current to 18 mA for 90 to 120 min. Electrophoresis was stopped 15 min after the bromophenol blue front had left the gel.

2.4.4 Hot Coomassie blue staining

The Hot Coomassie blue staining method was used for visualisation of protein bands on SDS-polyacrylamide gels. Gels were covered with staining solution and heated in a water bath at 60°C for at least 30 min. Afterwards, gels were covered with destaining solution and incubated by gentle shaking until staining background was removed. Finally, gels were washed with deionised water and documented by scanning (Epson Expression 1600 Pro, Meerbusch, Germany).

2.4.5 Western blot

Western blot analysis was performed as described by Zapf et al. (2007). A Hybond ECL nitrocellulose membrane from Amersham Pharmacia Biotech (Uppsala, Sweden) was used. SDS-PAGE was made as described above but the PageRuler™ prestained protein ladder plus (Fermentas, St. Leon-Rot, Germany) was used as size marker and gels were not stained. After electrophoresis, gels were incubated in precooled blotting buffer for 20 min. A nitrocellulose and four slices of filter paper were prepared and equilibrated in blotting buffer. A blotting sandwich was assembled in a HEP-1 Panther™ semi-dry electroblotting system (Owl Separation Systems, Portsmouth, NH, USA) according to the manufacturer's directions and proteins were blotted onto the membrane at 0.8 mA/cm^2 for 1 h. Membrane was incubated in blocking buffer accomplished with 1% BSA for 1 h at 37°C and gentle shaking. Afterwards, membrane was incubated in 20 ml TBS buffer containing 5 µl (dilution 1:2000) primary antibodies (immunoglobulin from rabbit)

against the 6 x His epitope from Rockland (Philadelphia, USA) or anti-AfpA antibodies (Zapf et al., 2007) for 1.5 h at 37°C and gentle shaking, followed by three washing steps with TBS-T at room temperature for 5 min each. Secondary antibody (goat-antirabbit-IgG, coupled with alkaline phosphatase, from Rockland, Philadelphia, USA) was added as 1:2000 dilution in TBS for 1.5 h, followed by three washes with TBS-T and one with TBS at room temperature for 5 min each. Staining was carried out using 75 µl BCIP solution and 100 µl NBT solution and stopped by addition of stop solution.

2.4.6 Fast protein liquid chromatography (FPLC)

For FPLC all buffers and solutions were degassed and filtrated (0.2 µm). One 1 ml HisTrap HP affinity column (Amersham Biosciences) was washed with 5 ml deionised water followed by loading 0.5 ml of 0.1 M nickel salt solution ($NiSO_4$) on the column. Loading was followed by a washing step with 5 ml deionised water. The column was equilibrated with at least 10 column volumes of buffer A (binding buffer). Dialysed and freeze-dried FcHyd5p was dissolved in buffer A and applied on the column. While target protein was bound to column particles due to the His-Tag, residual proteins were flushed out by washing the column with 10 volumes of buffer A (2 ml/min). A concentration gradient ranging from 100% buffer A and 0% buffer B to 0% buffer A to 100% buffer B (elution buffer, four column volumes, 2 ml/min) released the target protein from the His-Trap column. Washing the column with ten volumes of buffer B (2 ml/min) regenerated the column by removing of any residual protein. A steep gradient from 0% buffer A, 100% buffer B to 100% buffer A, 0% buffer B with 2 ml at 2 ml/min terminated the elution. Finally, the column was equilibrated again by washing with six column volumes of buffer A. To store the His-Trap columns, buffer was replaced by 20% ethanol. During the FPLC process, eluate was collected in 1 ml fractions and the process was monitored by UV detection at 280 nm.

2.4.7 Purification of recombinant FcHyd5p by preparative isoelectric focussing

FcHyd5p produced extracellularly by *P. pastoris* transformants in BMMY medium was separated from other proteins by using a preparative isoelectric focussing system (Rotofor Cell, Bio-Rad, Hercules, USA). Ampholyte Biolyte 3-10 (Bio-Rad, Hercules, USA) with a pH range from 3 to 10 was used and the focussing was performed according to

the Rotofor Cell manual. Voltage stabilised after 4.5 h, the total run took approximately 6 h. Further purification was accomplished using Amicon Ultra 30K and 5K Centrifugal Filter Devices (Millipore, Tullagreen, Ireland).

2.5 Determination of surface activity of FcHyd5p

2.5.1 Foam stability

Wild type strain of *P. pastoris* X33 and FcHyd5p producing clones were grown in 250 ml BMMY medium and FcHyd5p was overexpressed. Cells were harvested by centrifugation (10,000 x g, 30 min) and the supernatant was mixed with an ultra turrax homogeniser (Art-Labortechnik, Müllheim, Germany) at 10,500 rpm for 1 min in a 500 ml Erlenmeyer flask to determine the stability of the foam. Parafilm from Pechiney Plastic Packaging (Menasha, USA) with a hydrophobic surface was used to screen for surface activity of culture supernatants (Martin et al., 1999).

2.5.2 Gushing experiments

Beverages were cooled to 1°C in an ice bath and hydrophobins were added at different concentrations. Each amount was applied at least twice, respectively. Two untreated bottles in each experiment were used in order to determine the gushing tendency without adding proteins. Proteins secreted by the wild type strain *P. pastoris* X33 were treated and added as negative control. Bottles were tightly closed with a sterilised crown seal and rotated overhead at 28 rpm at ambient temperature for 24 h upon addition of proteins, followed by a rest of 2 h. Bottles were weighed before and after opening. Bonaqa™ sparkling water from Coca Cola (carbonated, 7.3 g/l) in 330 ml bottles and Export type beer from a German brewery in 500 ml bottles were used for gushing experiments.

2.6 Comparison of *F. graminearum* wild type and transformants

2.6.1 Comparison of growth rate

Mung bean medium plates were used to determine the growth rate of the *F. graminearum* TMW 4.0157 wild type strain and transformants because of a distinct and consistent growth and sporulation. The same batch of medium was used for each

experiment in order to avoid variation caused by the medium. Wild type strains and transformants were inoculated on a mung bean medium plate and incubated for 6 days at ambient temperature. Following incubation, an inverted sterile 1 ml pipet tip was used to cut uniform discs from the cultures as inoculum. Inoculum discs were cut in an equidistant circle around the colony centre to avoid differences in age and density of inoculum. Inoculi were placed in the centre of fresh mung bean medium plates and incubated at 19°C. The overgrown area of 18 plates for each strain was measured after 41, 67, 96 and 119 hours, respectively.

2.6.2 Comparison of the development of aerial and submerged mycelium

F. graminearum TMW 4.0157 wild type strain and transformants were inoculated in 50 ml malt extract broth in 200 ml Erlenmeyer flasks for the analysis of submerged mycelia or on malt extract agar plates for analysis of aerial mycelia. Inoculation of plates was done as described in chapter 2.6.1. Liquid cultures were inoculated by addition of one inoculum disc per culture. Substrate mycelia were collected after 5 days of incubation at 19°C with horizontal shaking at 110 rpm. Aerial hyphae were harvested by scraping the complete areal mycelia from the surface of agar plates after 7 days of incubation at 19°C. Following harvest, the collected mycelia were dried for 30 h at 60°C and weighed.

2.6.3 Comparison of number, length and germination ratio of conidia

Inoculation of *F. graminearum* was accomplished as described in chapter 2.6.1 in order to avoid variation caused by the medium. Induction of sporulation and harvesting of conidia was performed as described in chapter 2.2.7. The ratio between conidial counts for the wild type strain and transformants was calculated. Furthermore, the length of conidia was determined using a video imaging software package (AxioVision 4.7 Imaging Solution Software, Zeiss, Germany) by measuring the distance between the tips of foot and head cells of at least 500 conidia per isolate. Moreover, germination potential of conidia was measured by diluting conidia of the wild type strain and transformants to 6×10^4, 6×10^5, 3×10^6 and 6×10^6 conidia/ml, respectively, in malt extract broth and incubated for 5 h at 19°C. Subsequently, the proportion of ungerminated conidia was determined microscopically.

2.6.4 Surface hydrophobicity assay

The strains to be assayed were plated onto malt extract agar and incubated at 22°C until sporulation started. Sterile distilled water (10 µl) was placed on the surface of each culture. In addition, wettability of aerial, conidiating hyphae by application of a drop of an aqueous solution containing 0.2% SDS and 50 mM EDTA was also assessed as described by Stringer and Timberlake (1995). Additionally, 25% SDS solution in water was applied to investigate differences in surface hydrophobicity of mycelia when applying different surfactants.

2.6.5 Foam stability testing

F. graminearum wild type strain and transformants were grown in 50 ml malt extract broth for 11 days at 19°C on a rotary shaker at 110 rpm. Following cultivation, mycelia were removed by filtration and 15 ml of the cell free culture supernatant were transferred into a sterile 50 ml centrifuge tube (Sarstedt, Nümbrecht, Germany). The supernatant was vigorously mixed with a homogeniser (Art-Labortechnik, Müllheim, Germany) for 1 min at 10,500 rpm and the height of the foam layer above the liquid fraction was measured after a rest of 20 min at ambient temperature.

2.6.6 Pathogenicity testing

In order to determine differences in plant pathogenicity between the *F. graminearum* wild type strain and transformants, performing a root rot infection test with wheat assessed virulence and barley as described by Babaeizad et al. (2009). The wild type strain and transformants were inoculated and sporulation was induced as described at chapter 2.6.1 and 2.2.7. Wheat and barley seeds were surface sterilised in sodium hypochlorite (10% active chlorine) for 15 min and washed three times with sterile tap water for 5 min. Seeds were laid on sterile water-soaked filter paper for germination at ambient temperature. After 3 days, seedlings with a shoot length of approximately 1 cm were inoculated for 2 h in a spore suspension with 5 x 10^4 spores/ml in 0.02% tween 20 (v/v) and 0.5% gelatine (w/v) aqueous solution. An inoculation without spores was accomplished as negative control. After inoculation, seedlings grew in open 50 ml centrifugation tubes filled with 17 ml sterile expanded clay (Seramis, Mars,

Verden/Aller, Germany) soaked with 0.5% liquid fertiliser ("Vitalnahrung für Grünpflanzen", N/P/K: 1.8/1.0/2.3, Seramis, Verden/Aller, Germany) at a photoperiod of 16 h (full spectrum light with up to 5% of UVB and up to 30% UVA). Seedlings were fertilised every 3 days. After 9 days, differences in length of plantlets were measured.

2.7 Statistic

Significance of results was determined by using the Students t test. A statistical tendency was defined at $P < 0.1$, a significant difference at $P < 0.05$. All results are based upon three independent experiments with at least three measurements unless otherwise stated.

2.8 GenBank accession number

Nucleotide sequence data of FcHyd5p are available in the GenBank databases under the accession number DQ449530. Nucleotide sequence data of AfpA from *F. graminearum* are available under HM185071 and AfpA from *F. culmorum* under DQ336148.

3 Results

3.1 Cloning and verification of *FcHyd5* expression in *Pichia pastoris*

The intron-free sequence of the *FcHyd5* gene, which was codon optimised for the *Saccharomyces cerevisiae* codon usage, was used for amplification. The SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) was used for the prediction of a putative signal peptide in FcHyd5p. The analysis of the amino acid sequence using the hidden Markov model (hmm) resulted in a presence of a signal peptide with a probability of 1.000. A putative restriction site between the amino acids 17 (A) and 18 (L) was indicated with a probability of 0.821 (Figure 8). The restriction site is placed between the following nucleotides: TCT GCT ^ TTG CCA. The likelihood for a signal anchor sequence instead of a signal peptide sequence is 0.000.

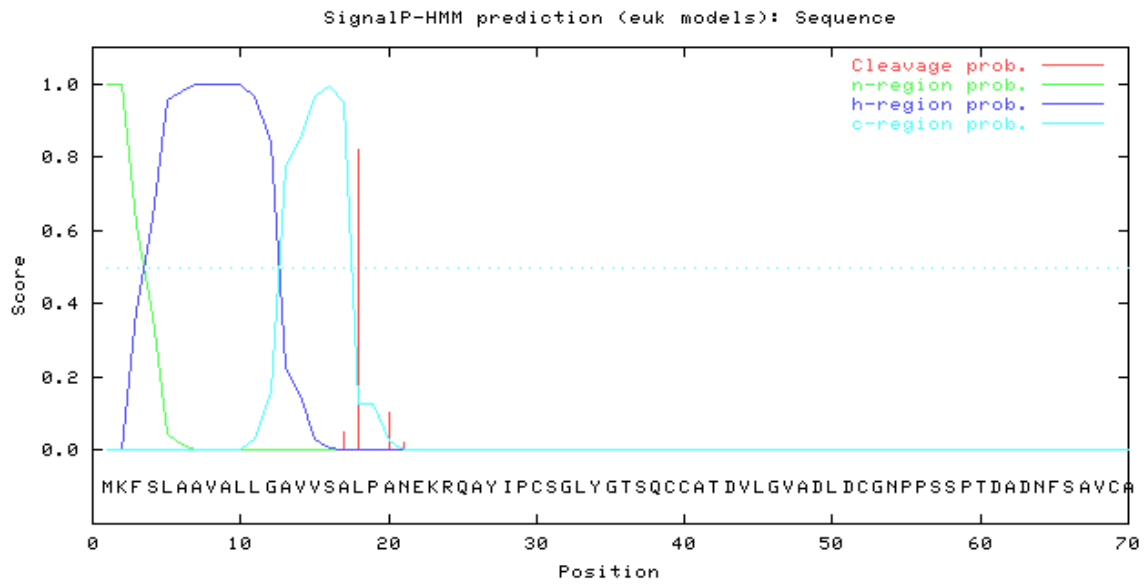

Figure 8. Prediction of a signal peptide in the *FcHyd5* gene using the SignalIP 3.0 server and the hidden Markov model (hmm). A restriction site with a probability of 0.821 was predicted between amino acids 17 (A) and 18 (L) of the protein.

The *FcHyd5* native signal peptide was excluded from the gene sequence by PCR using the primer pairs EcoRI-FcHyd5al-f / AgeI-FcHyd5-r and EcoRI-FcHyd5al-f / SalI-FcHyd5-r, which binds to the sequence region directly adjacent to the 3´-end of the leader signal. The PCR product was restricted with the respective endonucleases and ligated into the corresponding restriction sites of vector pPICZαA resulting in pPICZαA-FcHyd5 without His-tag and pPICZαA-FcHyd5-HisTag with an additional C-terminal 6 x His-tag. The constructs were transformed by electroporation into the genome of the

P. pastoris X33 wild type strain. The resulting *P. pastoris* transformants X33 [pPICZαA-FcHyd5] and X33 [pPICZαA-FcHyd5-HisTag] were verified by sequencing with the AOX primer pair. Below, the sequence of X33 [pPICZαA-FcHyd5] and X33 [pPICZαA-FcHyd5-HisTag] is shown. Underlined letters indicate the *FcHyd5* gene, letters in grey mark the *S. cerevisiae* α factor secretion signal, letters marked in black indicate the start / stop codon, the 6 x His-tag letters are shown in italic and bold letters indicate the restriction sites used for cloning procedures:

X33 [pPICZαA-FcHyd5]:

GAAGATCAAAAAACAACTAATTATTCGAAACGATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCAT
CCTCCGCATTAGCTGCTCCAGTCAACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCG
GTTACTCAGATTTAGAAGGGGATTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGT
TTATAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCT**G**
AATTCCGGTTGCCAGCTAACGAAAAAAGACAAGCTTATATTCCATGTTCTGGATTATACGGTACTTCTCAATGTT
GTGCTACAGATGTTTTGGGAGTTGCAGATTTGGATTGTGGTAATCCACCATCTTCTCCAACAGATGCTGATAACT
TTTCTGCAGTTTGTGCTGAAATTGGTCAAAGAGCAAGATGTTGCGTTTTGCCAATTTTGGATCAAGGAATTTTGT
GTAATACTCCAACAGGTGTTCAAGATTGA**ACCGGT**CTTTTTTCGTTAGCTGGCAACCGGAATTCCGGTTGCCA

X33 [pPICZαA-FcHyd5-HisTag]:

NTNCNNNGACAAGCTTTTTATNTTAACGACTTTTAACGACAACTTGAGAAGATCAAAAAACAACTAATTATTCGA
AACGATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACAC
TACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGA
TGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGC
TGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCT**GAATTC**CGGTTGCCAGCTAACGAAAAAAG
ACAAGCTTATATTCCATGTTCTGGATTATACGGTACTTCTCAATGTTGTGCTACAGATGTTTTGGGAGTTGCAGA
TTTGGATTGTGGTAATCCACCATCTTCTCCAACAGATGCTGATAACTTTTCTGCAGTTTGTGCTGAAATTGGTCA
AAGAGCAAGATGTTGCGTTTTGCCAATTTTGGATCAAGGAATTTTGTGTAATACTCCAACAGGTGTTCAAGAT**GT**
CGAC*CATCATCATCATCATCATCAT*TGAGTTTGTAGCCTTAGACATGACTGTTCCTCAGTTCAAGTTGGGCACTTACG
AGAAGACCGGTCTGNNNNNNNNNNN

The correctness of the transformants was confirmed by sequence alignment. Additionally, RT-PCR analysis confirmed transcription of the recombinant *FcHyd5* gene in clone X33 [pPICZαA-FcHyd5] after 24 h.

3.2 Determination of the Mut phenotype of *P. pastoris* transformants

Screening for the phenotype of methanol utilisation of transformants X33 [pPICZαA-FcHyd5] and X33 [pPICZαA-FcHyd5-HisTag] resulted in a positive methanol utilisation

(Mut$^+$) for all transformants compared to the Mut$^+$ strain GS115/pPICZ/*lac*Z Mut$^+$ and the Mut^S strain GS115/Mut^S Albumin. Consequently, recombination occurred in the 5´ region of the native *AOX1* gene and not in the 3´ region, which would disrupt the wild-type *AOX1* gene and would create Mut^S (methanol utilisation slow) transformants instead of Mut$^+$ (methanol utilisation positive).

3.3 Production and purification of heterologously expressed FcHyd5p

Proteins of the culture medium of clone X33 [pPICZαA-FcHyd5], X33 [pPICZαA-FcHyd5-HisTag], and wild type strain X33 were subjected to SDS-PAGE. A band of 12 kDa was detected in the recombinant strain X33 [pPICZαA-FcHyd5], whereas X33 [pPICZαA-FcHyd5-HisTag], which expressed FcHyd5p with a 6 x His-tag, produced a 13 kDa band. These bands were absent in the wild type strain (Figure 9). The SDS-PAGE gel was blotted on a nitrocellulose membrane and treated with anti 6 x His-tag antibodies conjugated to alkaline phosphatase. Following staining, samples from clone X33 [pPICZαA-FcHyd5] and wild type strain X33 revealed no visible bands. A signal was visible with proteins extracted from clone X33 [pPICZαA-FcHyd5-HisTag], indicating the presence of a 6 x His-tag at the proteins separated by SDS-PAGE (see lane 4 in Figure 9).

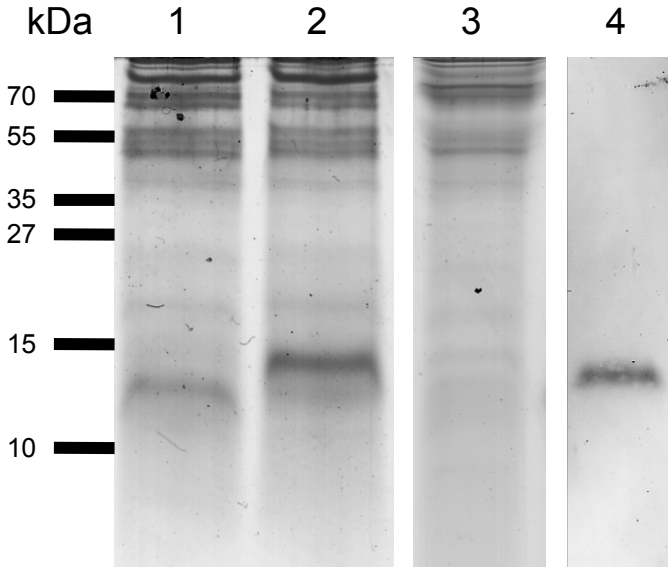

Figure 9. SDS-PAGE of proteins secreted into the culture medium by clones X33 [pPICZαA-FcHyd5] (lane 1), X33 [pPICZαA-FcHyd5-HisTag] (lane 2), and the wild type strain X33 of *P. pastoris* (lane 3). Western blot analysis of the purified proteins from clone X33 [pPICZαA-FcHyd5-HisTag] using anti 6 x His-tag antibodies (lane 4).

Purification and concentration of FcHyd5p with 6 x His-tag using a His trap column was applied but failed during the current study. No apparent protein peak could be detected. A lack of UV-detection at 280 nm could be excluded by analysing the fractions with a Bradford protein assay kit. However, analysing the collected fractions of the FPLC run with a Western blot by using anti 6 x His-tag antibodies revealed that the protein was present in various fractions coming from the column (Figure 10), whereas no FcHyd5p was visible on SDS-PAGE.

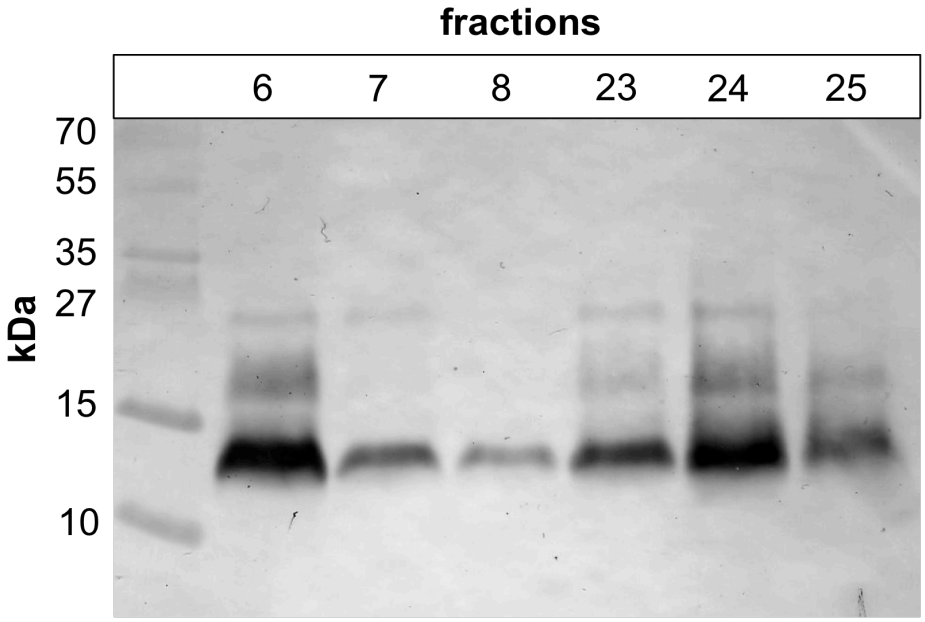

Figure 10. Western blot with fractions of a FPLC run using a His trap column and proteins separated from *P. pastoris* clone X33 [pPICZαA-FcHyd5-HisTag]. A total of 40 fractions were collected at each FPLC run. FcHyd5p with His-tag is detectable at the beginning of the run (fractions 6, 7, 8), when proteins without affinity to the His trap column are separated as well as at the end of the run (fractions 23, 24, 25), when proteins with His-tag should be collected. No apparent protein peak could be monitored by UV detection.

The application of a hydrophobic interaction chromatography was also not successful. As an alternative, preparative isoelectric focussing (IEF) was successfully applied for protein purification in order to avoid self-aggregation and especially aggregation on surfaces. IEF in a pH range from 3.0 to 10.0 and subsequent ultrafiltration of the fractions was used to purify the heterologously expressed hydrophobin from spent culture media of clones X33 [pPICZαA-FcHyd5] and X33 [pPICZαA-FcHyd5-HisTag] as well as from the wild type strain X33. SDS-PAGE (Figure 11) and immuno-staining of the IEF fractions revealed that the protein migrated to a pH between 4.0 and 5.0 in IEF as expected with a maximum between pH 4.3 and 4.6. No such protein was visible in wild type culture preparations at any of the pH tested.

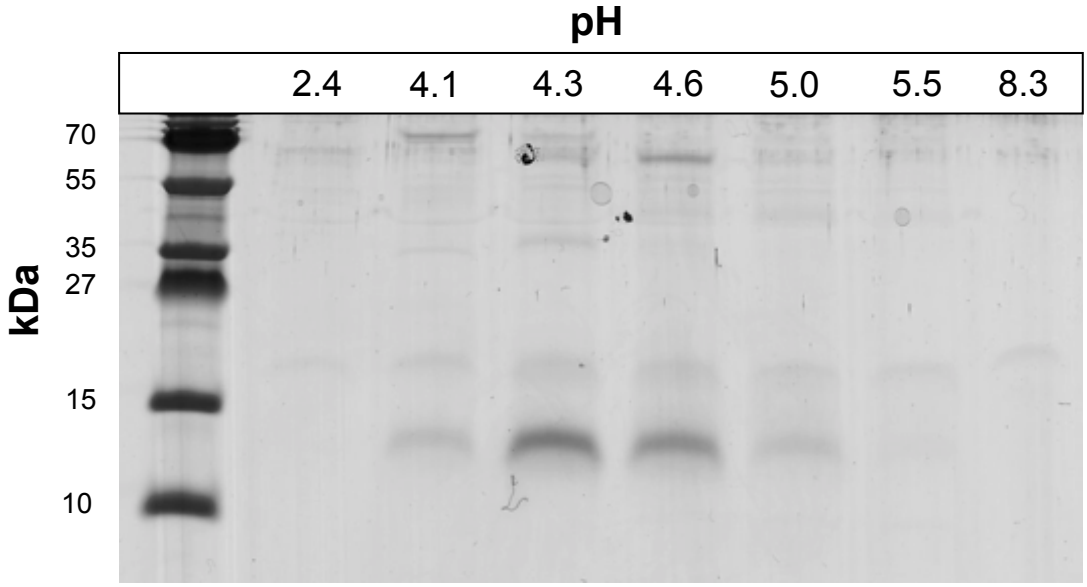

Figure 11. SDS-PAGE with fractions of isoelectric focussing using an ampholyte with a pH range from 3 to 10. Staining revealed that FcHyd5p migrated to a pH between 4.0 and 5.0 in IEF as expected whereas no such protein was visible in wild type culture preparations.

Purified FcHyd5p with C-terminal His-tag has been used to raise polyclonal antiserum in rabbits against epitopes of FcHyd5p, but failed in the current study. The Western blot with proteins from X33 [pPICZαA-FcHyd5], X33 [pPICZαA-FcHyd5-HisTag], and *P. pastoris* wild type strain X33 displayed only bands with proteins from the transformant X33 [pPICZαA-FcHyd5-HisTag] (data not shown).

The ability of hydrophobins to form aggregates (Torkkeli et al., 2002) resulting in dimers, trimers etc. was detectable on SDS-PAGE and verified by Western blot as shown in Figure 12. Using a higher amount of freeze-dried culture supernatant from clone X33 [pPICZαA-FcHyd5-HisTag] for gel electrophoresis and immuno-staining, bands between 13 and 55 kDa were visible with the smallest band having the highest intensity and the bigger ones being more faint with size increments of about 13 kDa.

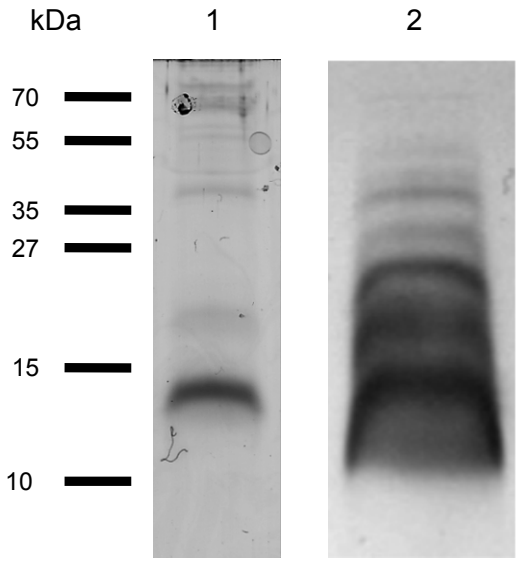

Figure 12. SDS-PAGE (lane 1) and Western blot (lane 2) of the freeze-dried culture supernatant from clone X33 [pPICZαA-FcHyd5-HisTag] using 6 x His-tag antibodies. 375 µg of supernatant per lane were applied to ensure distinct immuno-staining.

3.4 Determination of surface activity and gushing potential of FcHyd5p

3.4.1 Foam stability and surface-activity

In order to prove the ability of FcHyd5p to stabilise air bubbles in fluids by formation of a layer at the gas/water interface, cell-free supernatants were mixed with a ultra turrax homogeniser. The homogenised supernatant of liquid cultures of wild type strain X33 clarified after 10 min and the foam disintegrated after 2 h. On the contrary, the cell-free spent culture supernatant of the FcHyd5p producing clones X33 [pPICZαA-FcHyd5] and X33 [pPICZαA-FcHyd5-HisTag] remained turbid for nearly 24 h. The foam above cell-free culture supernatants of both FcHyd5p producing clones was stable for more than 72 h with a height of approximately 7 cm above the culture liquid (Figure 13).

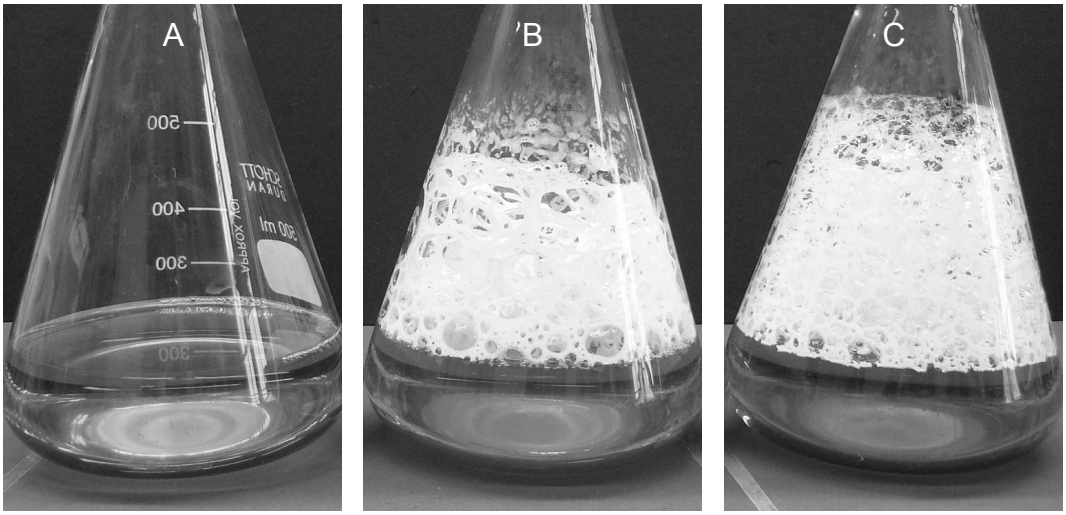

Figure 13. Foam stability of cell-free culture supernatants from *P. pastoris* wild type strain X33 (A), clone X33 [pPICZαA-FcHyd5] (B) and clone X33 [pPICZαA-FcHyd5-HisTag] (C) grown in BMMY medium. The supernatants were mixed with a ultra turrax homogeniser at 10,500 rpm for 1 min. Photographs were taken after 69 h.

In order to test the ability to change hydrophobic properties, Parafilm™ with a highly hydrophobic surface was coated with cell-free culture supernatants of the clones and the wild type strain. The surface of the Parafilm™ became wettable after the treatment with supernatants of clones X33 [pPICZαA-FcHyd5] and X33 [pPICZαA-FcHyd5-HisTag]. No change in hydrophobicity was detectable with the supernatant of wild type strain *P. pastoris* X33.

3.4.2 Gushing potential of FcHyd5p

Recombinant FcHyd5p with 6 x His-tag, which was purified by preparative isoelectric focussing and subsequent ultrafiltration from a spent culture media of X33 [pPICZαA-FcHyd5-HisTag], was used for gushing experiments in beer. Addition of 86, 129 and 215 µg protein resulted in a gushing volume of 7 ml ± 12, 24 ml ± 20 and 77 ml ± 40 per bottle, respectively. No gushing was detectable after addition of 21.5 and 43 µg. Also no gushing was observed with total protein extracted from cell-free culture supernatant of the wild type strain *P. pastoris* X33 added in the same amounts. Untreated beer used for the gushing experiment showed a volume loss of 4 ml ± 0.4 upon opening of bottles (Figure 14A).

Furthermore, culture supernatants from clones X33 [pPICZαA-FcHyd5], X33 [pPICZαA-FcHyd5-HisTag] and the wild type strain *P. pastoris* X33 were dialysed and freeze-dried in order to achieve higher protein amounts. BMM medium was used for protein

production to keep the fraction of proteins from the growth medium low. Untreated beer used for the gushing experiments yielded no gushing volume. Also no gushing was detectable with the freeze-dried culture supernatant from the wild type strain *P. pastoris* X33. Addition of 0.1, 0.5, 1.0 and 2.0 mg of freeze-dried supernatant from X33 [pPICZαA-FcHyd5] resulted in a gushing volume of 0 ml, 88 ml ± 7, 211 ml ± 18 and 252 ml ± 20 per bottle, respectively. Adding freeze-dried supernatant of clone X33 [pPICZαA-FcHyd5-HisTag] in the same amounts resulted in 7 ml ± 9, 66 ml ± 4, 77 ml ± 27 and 122 ml ± 66 lost volume due to gushing, respectively (Figure 14B).

Dialysed and freeze-dried culture supernatants from clones X33 [pPICZαA-FcHyd5] and X33 [pPICZαA-FcHyd5-HisTag] and the wild type strain *P. pastoris* X33 were added to Bonaqa™ sparkling water in 330 ml bottles. Untreated water used for the gushing experiments yielded no gushing volume. Also no gushing was detected with the supernatant from the wild type strain *P. pastoris* X33. Addition of 0.1, 0.5, 1.0 and 2.0 mg freeze-dried supernatant from clone X33 [pPICZαA-FcHyd5] caused a lost volume of 162 ml ± 9, 165 ml ± 0.3, 172 ml ± 7 and 179 ml ± 7, respectively. Addition of supernatant from clone X33 [pPICZαA-FcHyd5-HisTag] in the same amounts caused a gushing volume of 147 ml ± 1, 156 ml ± 10, 166 ml ± 2 and 172 ml ± 1, respectively (Figure 14C).

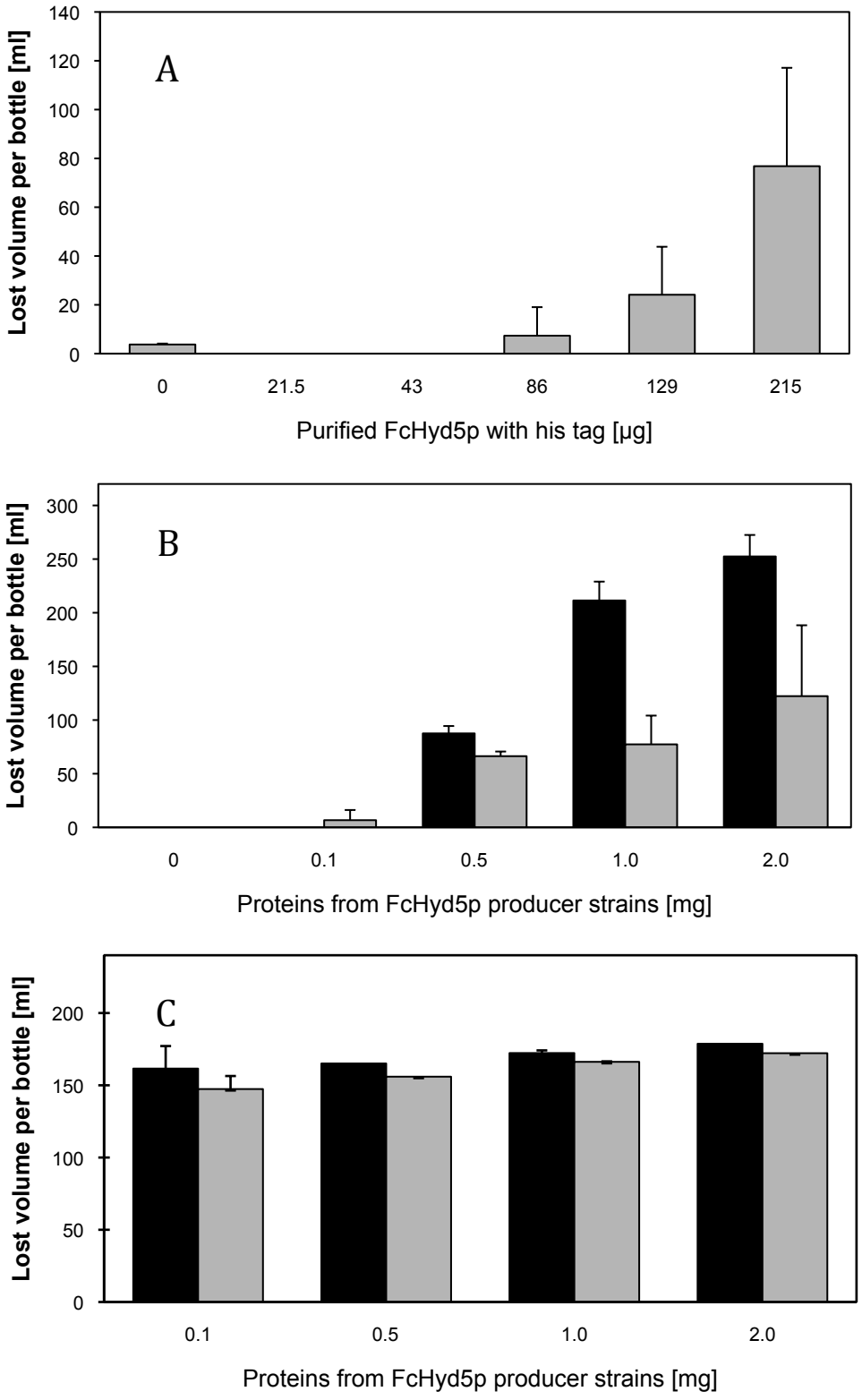

Figure 14. Gushing experiments with FcHyd5p producing clones X33 [pPICZαA-FcHyd5] (black), X33 [pPICZαA-FcHyd5-HisTag] (grey) and the wild type strain *P. pastoris* X33. No gushing was detectible in the experiments using the wild type strain. (A) Purified FcHyd5p with 6 x His-tag was added to beer (500 ml) in different amounts (0 to 215 µg) and the gushing volume per bottle was determined. (B) Freeze-dried culture supernatants from clones X33 [pPICZαA-FcHyd5] and X33 [pPICZαA-FcHyd5-HisTag] were added to beer (500 ml) in different amounts (0 to 2 mg). (C) Freeze-dried culture supernatants from clones X33 [pPICZαA-FcHyd5] and X33 [pPICZαA-FcHyd5-HisTag] were added to carbonated water (330 ml) in amounts from 0 to 2 mg.

3.5 Knock out of AfpA via PEG-transformation

F. graminearum wild type strain TMW 4.0157 was transformed with the *AfpA* disruption vector pSM1-AfpA-ko and the *PKS12* disruption vector pSM1-PKS12-ko as phenotypical control for transformation efficiency. Transformation was performed using protoplasts stabilised by PEG. Two transformant cultures of each construct were selected by single-spore isolation from a hygromycin resistant colony obtained after cultivation of a transformation experiment. Sequencing of all transformants using the primer pSM1-seq_f confirmed integration of the constructs into the genome of the transformants. In addition, expression of GFP in the transformant cultures could be detected by fluorescence using UV $_{366\ nm}$ light (Figure 15).

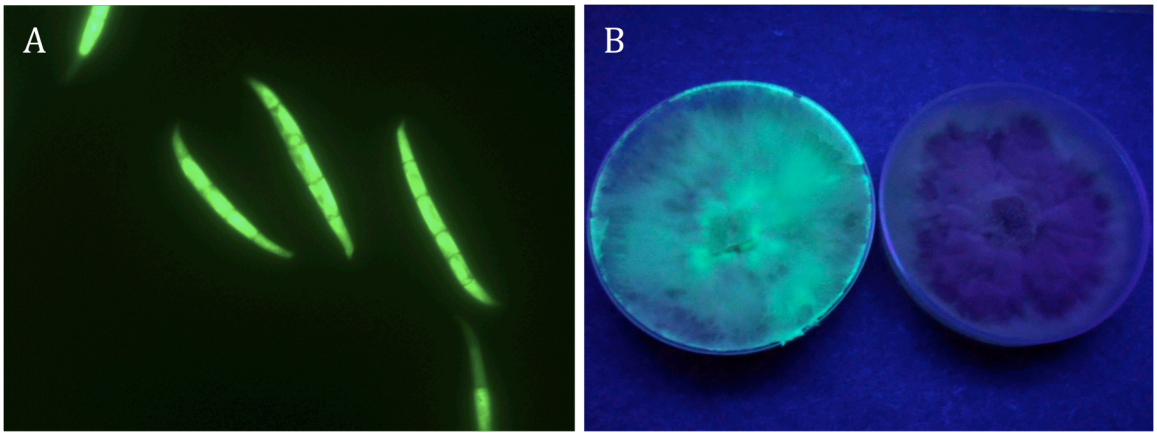

Figure 15. GFP fluorescence in a transformant of *F. graminearum* strain TMW 4.0157. The plasmid pSM1 containing a GFP expression cassette was integrated into the genome of the fungus. (A) Fluorescent spores. (B) Fluorescent mycelium of transformant (left Petri dish) and non-fluorescent wild type strain (right Petri dish) under UV $_{366\ nm}$ light.

However, following verification of *AfpA* knock out by sequencing with the primer AfpA-ko2-EcoRV-f revealed that the GFP-containing construct had not integrated into the *AfpA* gene, which was therefore still intact in genome of the transformants. Checking the putative *PKS12* knock out transformants by sequencing with the primer PKS12-ko-EcoRV-f showed that the knock out construct pSM1-PKS12-ko had also not integrated into the *PKS12* gene. In both cases, the position of integration into the genome could not be assessed.

3.6 Knock out of AfpA from *F. graminearum* by ATMT

F. graminearum wild type strain TMW 4.0157 was transformed with the *AfpA* disruption vector pPK2-AfpA mediated by *A. tumefaciens*. Four transformant cultures were selected

by single-spore isolation from a hygromycin resistant colony obtained after cultivation of a transformation experiment.

Integration of the construct was verified by sequencing with the primer pair hph1_ppk2_f / hph1_ppk2_r binding on the hygromycin B cassette of the vector pPK2. Disruption of the *AfpA* gene was verified in all four FG [pPK2-ΔAfpA] transformant cultures by sequencing the 3´ intersections of the integrated vector construct and the following part of the genome of *F. graminearum* downstream of the native *AfpA* gene. The sequence of FG [pPK2-ΔAfpA] is shown below. Underlined letters indicate the *trpC* termination of the plasmid pPK2 (chapter 2.1.7), letters in black mark the primer pair ATMTafpaDoXbaI-f / ATMTafpaDoHind3r used for the amplification of the AfpA downstream flank (in grey), and italic letters show a portion of the genomic region downstream of the AfpA downstream flank used in the construct pPK2-AfpA:

TCTCAAGCCTACAGGACACACATTCATCGTAGGTATAAACCTCGAAAATCATTCCTACTAAGATGGGTATACAAT
AGTAACCATGCATGGTTGCCTAGTGAATGCTCCGTAACACCCAATACGCCGGCCGAAACTTTTTTACAACTCTCC
TATGAGTCGTTTACCCAGAATGCACAGGTACACTTGTTTAGAGGTAATCCTTCTTTCTAGAGCTAACAGCGAGAA
GGAGTGCTACAGCTTCAGCGCCCGCGTCGCTGAGACCAAGAAGCCTGTTAGCTGCATCTACTCCCAGTACAGCAA
CTAAGCGATAGAGTTTGCACTGTACATAAGACTTGTTTTAGACATGATTTATTATTTTAGATTTTAGTTCTCCTG
TATATACATTGATTAATTACTTCTCTCATTTCTTTATACTTTGTTACTGTGATTGTCTTATGCATGCTAGGCTTA
ATCTGGCCGAGTCAAGCAGGGGATAAGATCAGATCAATGATTGGTTCTATAAAACACGTGACTGTCTGACTAACT
GATCCTTATCTGATCATCTGATAAAAATCTCTCACAAGACCCGACCTCGACCCAGACCGACCCAACCCCTGGGCC
CTGGCTCTCCGCCTGCGACCTGACGAGTTCACTCTTTCATCACGCTGGCAGC*GCCACCATGAAGCTTGCATCGTT*
GAGCTCTCTGCTCACAATACTCTCATTATGCGCCCCATCCGCCCAAGCCGAAGACAG

Moreover, quantitative reverse transcription PCR using three housekeeping genes as internal standards showed that *AfpA* mRNA was absent in the transformed cells thus confirming non-expression of the gene (see Figure 26, page 73). Each of the four verified knock out transformant cultures were used for further experiments to evaluate the role of AfpA for *F. graminearum.* Data given represent averages of all four transformants to exclude an impact of potentially non-homologous integrated T-DNA.

3.7 Comparison of *F. graminearum* wild type and ΔAfpA mutants

AfpA as a surface active protein has some properties similar to hydrophobins (Zapf et al., 2007) and a high degree of sequence homology to the PhiA protein in *Aspergillus nidulans* (Melin et al., 2003). Since the proteins were reported to influence growth rate,

development of mycelium and conidia, surface hydrophobicity of mycelia, foam stability, and pathogenicity, these properties were investigated to characterise knock out transformants obtained during the current study as compared with the wild type strain.

3.7.1 Growth rate

In order to evaluate the role of AfpA for *F. graminearum*, differences in the fitness between wild type strain and FG [pPK2-ΔAfpA] were analysed by comparing the growth rate of each strain. A reduced growth rate was also described as a result of the disruption of the *PhiA* gene in *Aspergillus nidulans* (Melin et al., 2003) as another representative of the fungispumins with a high degree of homology to *AfpA*. The findings were confirmed during the current study for the disruption of the *AfpA* gene. Knock out mutants showed only 90.84% ± 4.07 of the growth rate of the *F. graminearum* wild type strain (Figure 16). Therefore, a statistically significant reduction of the growth rate was determined because of *AfpA* gene disruption.

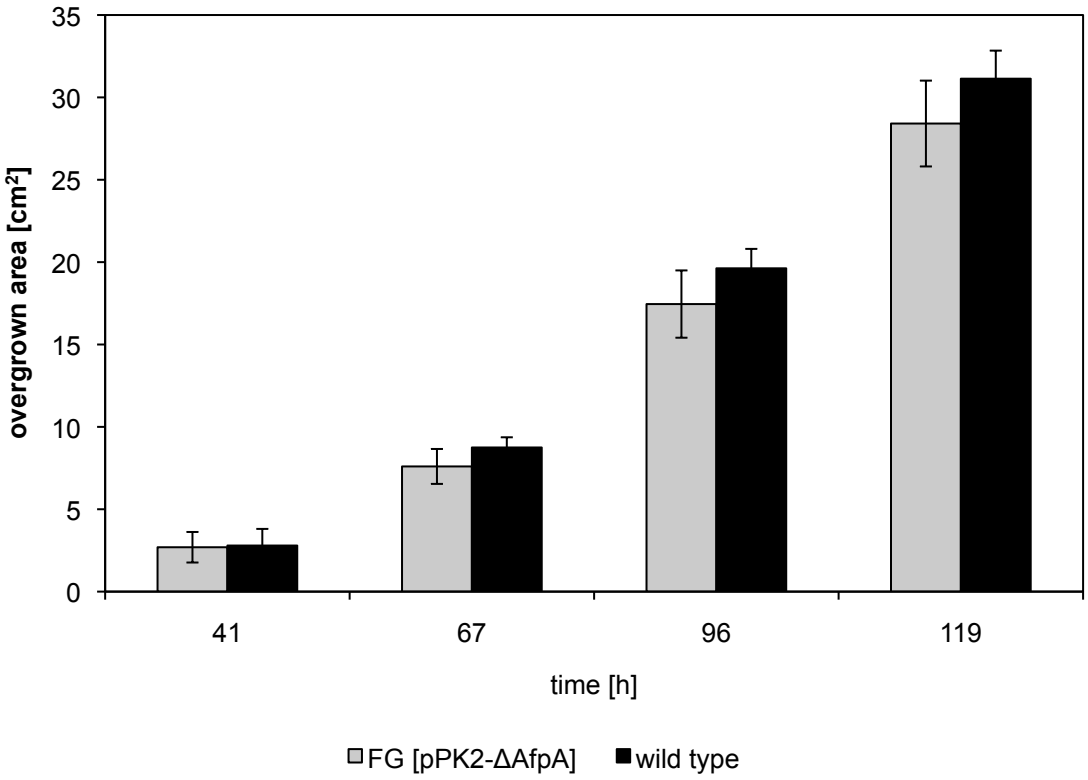

Figure 16. Comparison of the growth rate of *AfpA* knock out mutants FG [pPK2-ΔAfpA] and the *F. graminearum* TMW 4.0157 wild type strain. The overgrown area of Petri dishes was measured after of 41, 67, 96 and 119 hours of incubation.

3.7.2 Development of aerial and substrate mycelium

In addition, the biomass of aerial and submerged mycelium was determined after five days of growth in order to analyse possible impacts of AfpA on the development of both types of hyphae, as was reported for hydrophobins (Wessels et al., 1991a; Wessels et al., 1991b). Similar to the growth rate, development of submerged mycelium was significantly reduced. The wild type strain produced a biomass of 0.28 g ± 0.01 compared to 0.26 g ± 0.02 of the knock out mutants. Thus a reduction of approximately 8% was found. In contrast to submerged mycelia, no significant reduction of the biomass of aerial mycelium was detected in the experiments (Figure 17). The FG [pPK2-ΔAfpA] mutants produced 0.07 g ± 0.01 of aerial hyphal biomass in seven days as compared to 0.08 g ± 0.01 of the wild type strain.

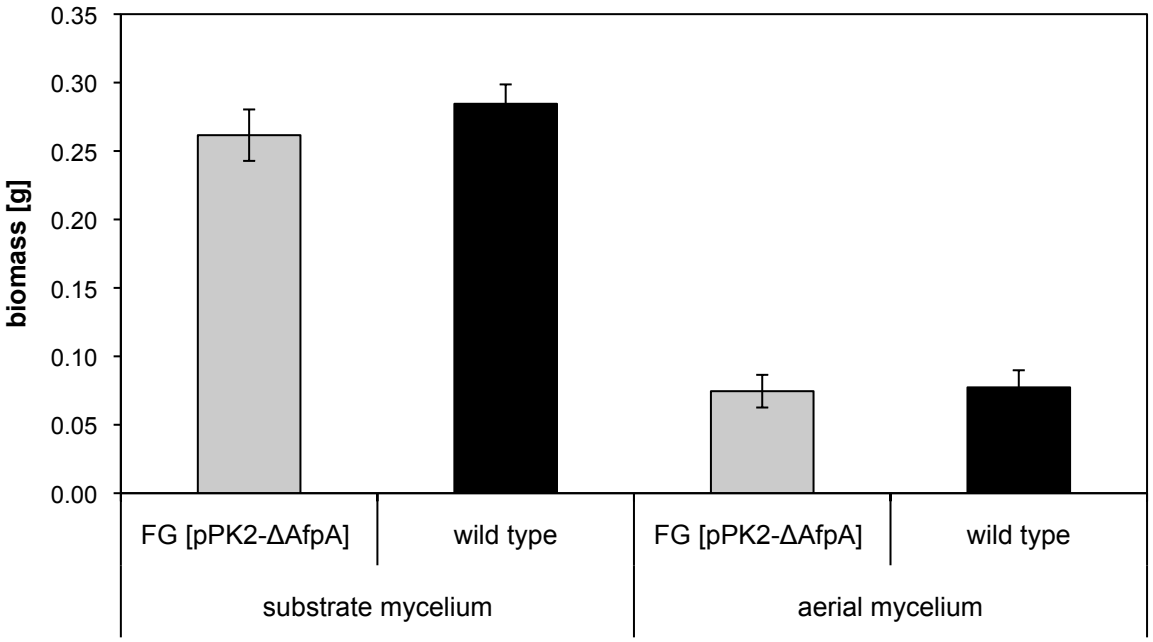

Figure 17. Development of submerged and aerial mycelia from *AfpA* knock out mutants FG [pPK2-ΔAfpA] and the *F. graminearum* TMW 4.0157 wild type. The cultures were incubated in malt extract broth for 5 days for the development of submerged mycelia and for 7 days on malt extract plates for the development of aerial mycelia. Following incubation, mycelia were harvested, dried and the biomass was determined.

Differences occurred in the appearance of submerged mycelium of FG [pPK2-ΔAfpA] mutants growing on mung bean agar with addition of hygromycin B. Whereas substrate mycelium of *F. graminearum* wild type strain grows homogeneously on non-selective mung bean agar plates (see Figure 18A), substrate mycelium of FG [pPK2-ΔAfpA] mutants appears strongly branched and inhomogeneous on selective agar (see Figure 18B). In addition, a high amount of compartments within the hyphae of FG [pPK2-ΔAfpA] mutants could be observed microscopically (see Figure 18C), whereas hyphae of

the wild type strain displayed only few compartments (see Figure 18D). These effects do not result when FG [pPK2-ΔAfpA] mutants grow on mung bean agar without hygromycin B.

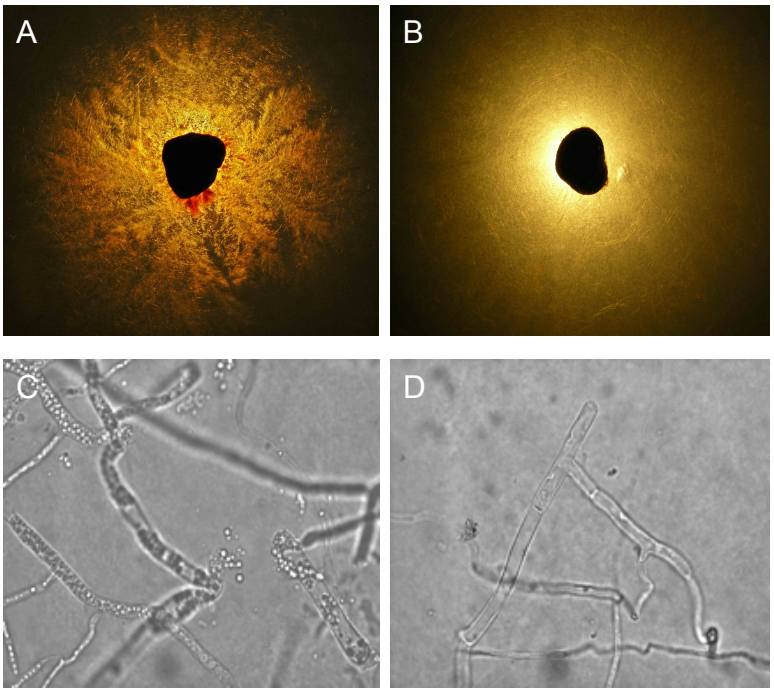

Figure 18. Growth of *AfpA* knock out mutant FG [pPK2-ΔAfpA] and *F. graminearum* wild type strain on mung bean agar. Substrate mycelium of FG [pPK2-ΔAfpA] mutants appears branched and inhomogeneous when hygromycin B was used for selection (A), whereas substrate mycelium of *F. graminearum* wild type strain grows homogeneously on mung medium plates (B). Microscopical analysis revealed a high amount of compartments in the hyphae of the FG [pPK2-ΔAfpA] mutant growing on agar with hygromycin B (C), whereas only few compartments are visible in hyphae of the wild type strain growing without antibiotic (D).

3.7.3 Development of phialides

The protein PhiA as a structurally related protein to AfpA and member of the fungispumin class of proteins has been described to have an impact on conidium development resulting in reduced sporulation rates (Melin et al., 2003). Especially the development of the phialides has been demonstrated to be affected in PhiA knock out mutants. However, during the current study no influence of AfpA on the development of phialides could be observed. Comparing morphology of the phialides and the mycelium of the *F. graminearum* wild type strain with the corresponding features in the knock out mutants, no obvious differences were detected (Figure 19).

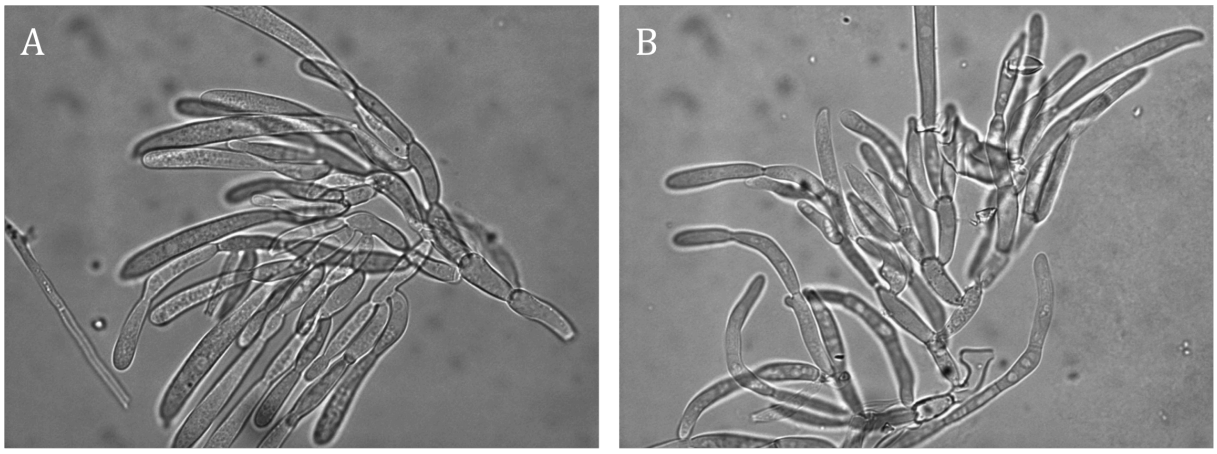

Figure 19. Comparison of phialides from *AfpA* knock out transformant FG [pPK2-ΔAfpA] (A) and *F. graminearum* wild type strain (B) (1000x magnification).

3.7.4 Number, length and germination ratio of conidia

In order to investigate the impact of AfpA on the development of conidia, their length, number, and germination capacity were evaluated to find morphological or physiological differences between the wild type strain and FG [pPK2-ΔAfpA] mutants. A significant difference in conidial length was observed. Conidia of the wild type strain had an average length of 46.09 µm ± 6.89 whereas conidia of the knock out mutants had a length of only 38.33 µm ± 6.14. Thus a length reduction of 17% was found as a result of the mutants failure to produce the AfpA protein. No significant difference was found by comparing the numbers of conidia produced. Knock out mutants showed 97% ± 22 of the sporulation rate as compared to the wild type strain. Furthermore, the disruption of the *AfpA* gene had no influence on the germination capacity of conidia when a concentration of 6 x 10^4 conidia/ml was analysed. Both in the wild type strain and in the knock out mutants 10% of the conidia remained ungerminated after an incubation period of 5 h (Figure 20).

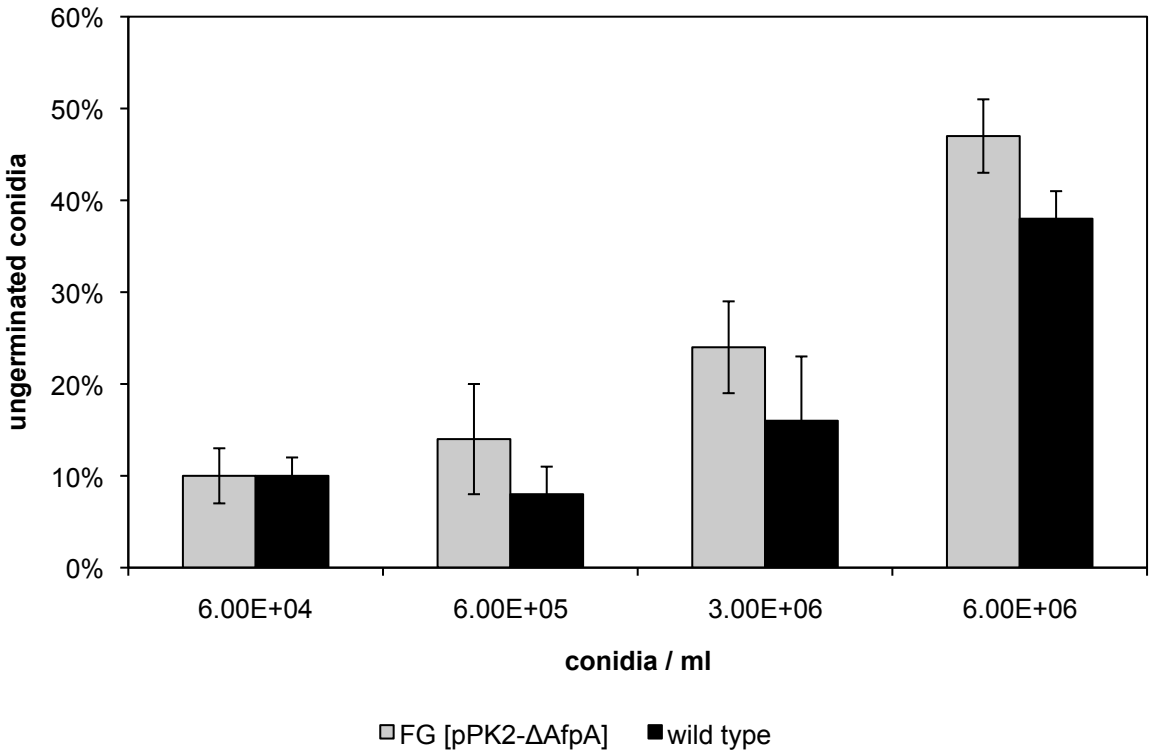

Figure 20. Determination of the germination capacity of conidia from *AfpA* knock out mutants FG [pPK2-ΔAfpA] and the *F. graminearum* TMW 4.0157 wild type strain. Conidia were diluted to different concentrations and incubated in malt extract broth for 5 hours. Following incubation, the percentage of ungerminated conidia was investigated.

However, an increase of the conidial concentration up to 6 x 10^6 conidia/ml resulted in a decrease in germination capacity with a significantly lower capacity in FG [pPK2-ΔAfpA] mutant conidia as compared to the wild type conidia. Consequently, results show that absence of AfpA production has an influence on conidial length as well as on germination capacity of conidia in *F. graminearum* at higher conidial concentrations.

3.7.5 Surface hydrophobicity

AfpA as a surface-active protein may possibly have an impact on surface hydrophobicity of mycelia since disruption of several hydrophobin genes resulted in an easily wettable phenotype in other fungi (Spanu, 1998; Talbot et al., 1993; van Wetter et al., 1996). In order to investigate the possible influence of AfpA on the surface hydrophobicity of *F. graminearum* aerial mycelium, 10 µl of water or of a detergent solution containing both 0.2% SDS and 50 mM EDTA were dripped on aerial mycelia and incubated for 24 h as described by Stringer and Timberlake (1995). No differences in wettability of aerial mycelia were detected between FG [pPK2-ΔAfpA] mutants and the wild type strain (Figure 21). In contrast, application of a 25% SDS solution resulted in wettability of

aerial mycelia in both the wild type strain and the mutants. Results of the wettability tests performed during the current study suggest that there is no influence of AfpA on the surface hydrophobicity of aerial hyphae in *F. graminearum*.

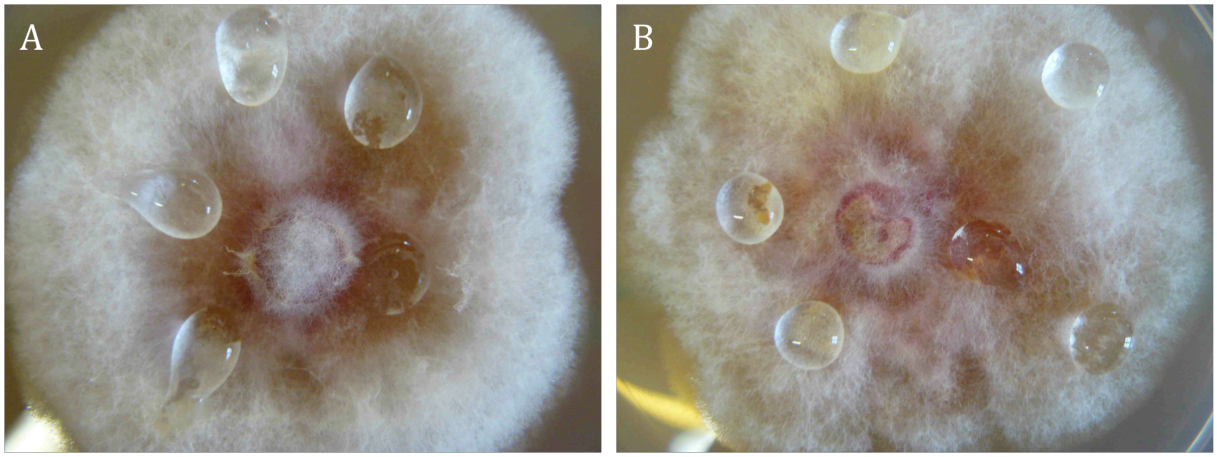

Figure 21. Comparison of the surface hydrophobicity between mycelia of an *AfpA* knock out mutant FG [pPK2-ΔAfpA] (A) and wild type (B). Detergent solutions containing both 0.2% SDS and 50 mM EDTA were dripped on aerial mycelium and incubated for 24 h.

3.7.6 Foam stability

AfpA has been described as a foam stabilising protein (Zapf et al., 2007), which contains a signal peptide. Thus it could be part of the cell membrane, the membrane of an intracellular compartment or it could be excreted outside the cell. In order to investigate the impact of AfpA as surface-active protein on the stability of foam bubbles, the cell free supernatants of wild type and transformant spent liquid cultures were vigorously agitated using an ultra turrax type homogeniser and the stability of the resulting foam was assessed after a 20 min rest. A significant difference between foam stability of wild type strain and the FG [pPK2-ΔAfpA] mutants was detected. The foam layer on top of the homogenised cell free culture liquid of the wild type strain had a height of 33.33 mm ± 5 after a rest of 20 min whereas the foam layer of the FG [pPK2-ΔAfpA] mutant cultures was less stable with a height of 19.83 mm ± 3.34 (Figure 22). As a result, experiments showed a significant difference in foam stability between wild type strain and FG [pPK2-ΔAfpA] mutants demonstrating that the surface active AfpA protein is secreted into the culture liquid in the *F. graminearum* wild type strain.

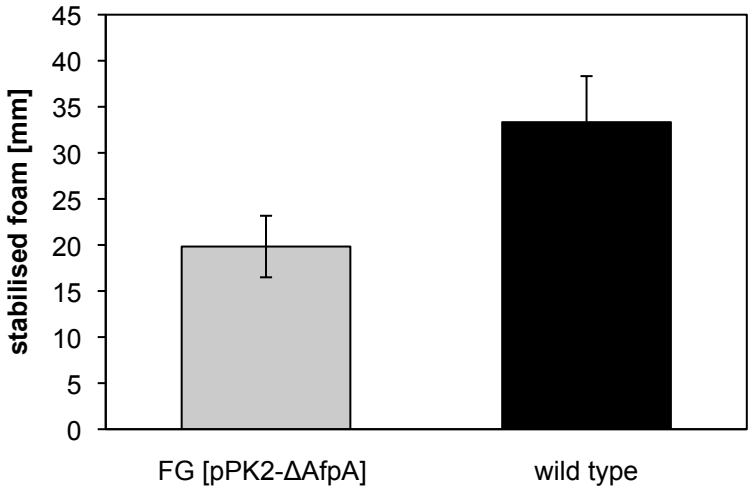

Figure 22. Comparison of foam stability of cell-free spent culture supernatant between *AfpA* knock out mutants FG [pPK2-ΔAfpA] and wild type grown in malt extract broth. The supernatant was mixed with a homogeniser for 1 min and the foam height was measured after a rest of 20 min.

3.7.7 Pathogenicity

The role of AfpA in the pathogenicity of *F. graminearum* was investigated. The fungus has a high pathogenic potential against cereals including wheat and barley (Bottalico, 1998; Brunner et al., 2009; Han et al., 2001). Hydrophobins as well as other cell wall associated fungal proteins were reported to be involved in mechanisms of plant pathogenicity (Kim et al., 2005b; Prados-Rosales et al., 2009). During the current study, it was demonstrated that disruption of the *AfpA* gene had an effect on the virulence of *F. graminearum* TMW 4.0157 against host plants. Barley and wheat seedlings were infected with conidial suspensions of the *F. graminearum* wild type strain and the FG [pPK2-ΔAfpA] mutants and the shoot and root length was compared with uninfected seedlings after a growth period of 9 days.

Uninfected control barley seedlings had an average shoot length of 21.05 cm ± 1.7, whereas seedlings infected with conidia of the *F. graminearum* wild type strain showed a shoot length of 15.89 cm ± 1.77. A shoot length of 18.53 cm ± 2.44 resulted after infection with conidia of the knock out mutants (Figure 23). A significant difference in shoot length was determined between uninfected barley seedlings and seedlings infected with either the wild type strain or FG [pPK2-ΔAfpA] mutants. However, a significant difference in shoot length between seedlings treated with the wild type strain and with the knock out mutants was also observed. In wheat, negative control seedlings had an average shoot length of 23.44 cm ± 2.05. After infection with conidia of the wild type strain, the resultant shoot length was 14.78 cm ± 3.76 whereas shoots of wheat

seedlings treated with FG [pPK2-ΔAfpA] mutant strain conidia had an average length of 17.71 cm ± 3.96 (Figure 23). As observed in barley seedlings, a significant difference in shoot length could be determined between the negative control and seedlings treated either with wild type strain conidia or knock out mutant conidia. Also in wheat, the difference in shoot length between wild type strain and FG [pPK2-ΔAfpA] mutants was significant.

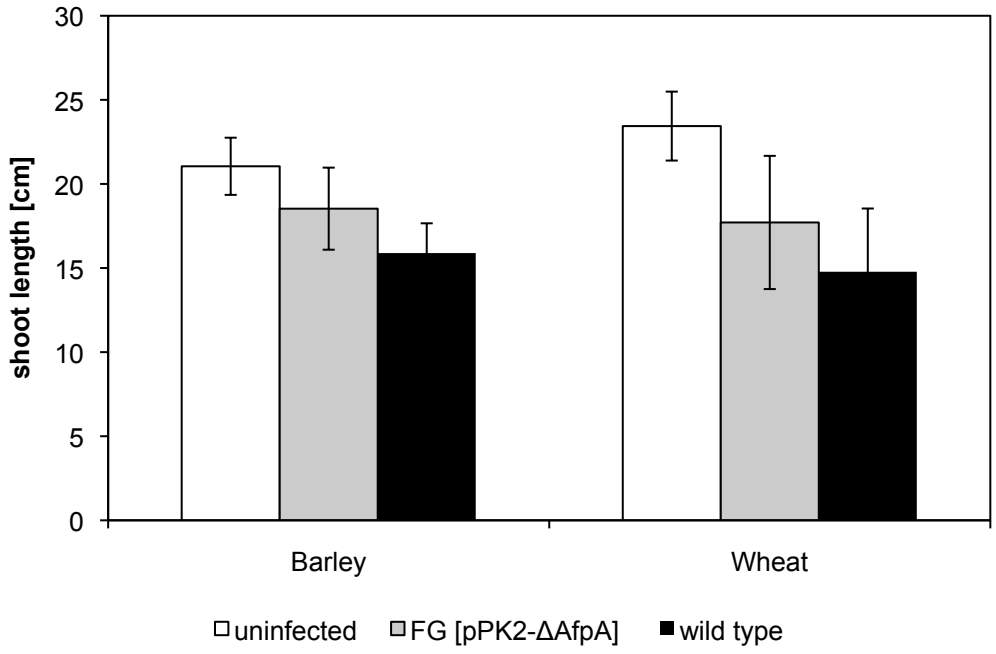

Figure 23. Comparison of the pathogenicity of *AfpA* knock out mutants FG [pPK2-ΔAfpA] with *F. graminearum* TMW 4.0157 wild type strain. Wheat and barley seedling were infected with FG [pPK2-ΔAfpA] mutants and wild type strain conidia. Uninfected seedlings were used as negative control. Shoot length was determined after a growth period of 9 days.

Determining the length of roots, uninfected control barley seedlings had an average root length of 10.43 cm ± 2.57, whereas seedlings infected with conidia of the *F. graminearum* wild type strain showed a root length of 7.61 cm ± 1.51. A root length of 9.37 cm ± 2.07 resulted after infection with conidia of the knock out mutants (Figure 24). A significant difference in root length was determined between uninfected barley seedlings and seedlings infected with the wild type strain. No significant difference could be determined between uninfected barley seedlings and seedlings treated with FG [pPK2-ΔAfpA] mutants, whereas a significant difference in root length between seedlings treated with the wild type strain and with the knock out mutants was observed. In wheat, negative control seedlings had an average root length of 9.91 cm ± 2.04. After infection with conidia of the wild type strain, the resultant root

length was 6.04 cm ± 2.12 whereas roots of wheat seedlings treated with FG [pPK2-ΔAfpA] mutant strain had an average length of 6.86 cm ± 1.81 (Figure 24). A significant difference in root length could be determined between the negative control and seedlings treated either with wild type strain conidia or knock out mutant conidia, but no significant difference could be observed between wild type strain and FG [pPK2-ΔAfpA] mutants.

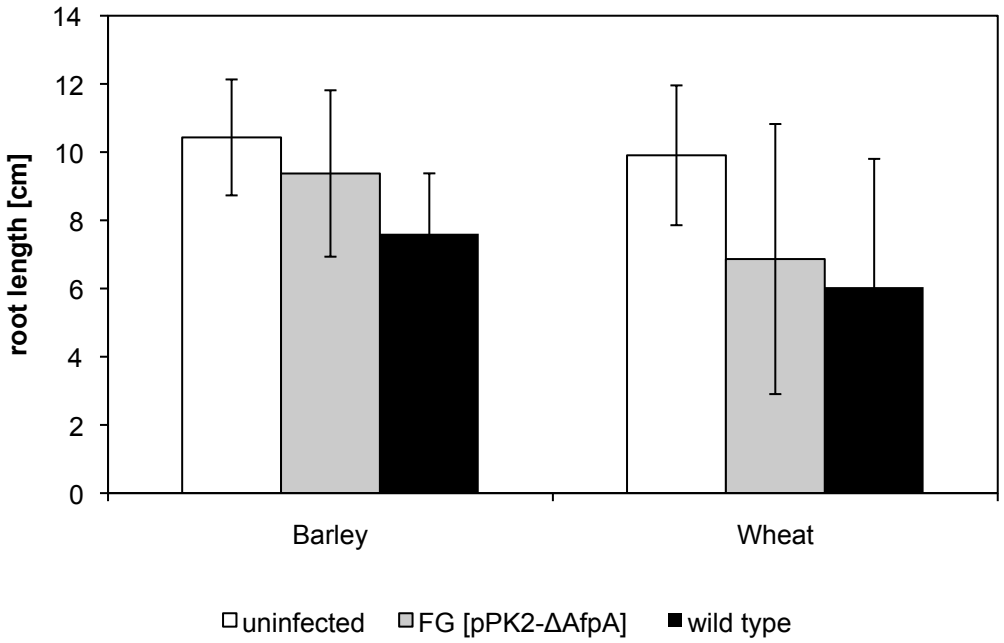

Figure 24. Comparison of the pathogenicity of *AfpA* knock out mutants FG [pPK2-ΔAfpA] with *F. graminearum* TMW 4.0157 wild type strain. Wheat and barley seedling were infected with FG [pPK2-ΔAfpA] mutants and wild type strain conidia. Uninfected seedlings were used as negative control. Root length was determined after a growth period of 9 days.

3.8 Silencing of AfpA in *F. graminearum*

3.8.1 Characterisation of transformants

Protoplasts of the *F. graminearum* wild type strain were transformed with the silencing vector pSilent1-AfpA to achieve a knock down of *AfpA* or the co-silencing vector pSilent1-AfpA-PKS12 for a parallel knock down of *AfpA* as gene of interest and *PKS12* as gene coding for the phenotypical marker. The polyketide synthase 12 is necessary for biosynthesis of the red pigment aurofusarin in *Fusarium* spp. (Frandsen et al., 2006). Transformation with the pSilent1-AfpA vector resulted in three hygromycin B resistant transformants (FG [pSilent1-AfpAi:TF1], FG [pSilent1-AfpAi:TF2], FG [pSilent1-AfpAi:TF3]). Transformants carrying the hairpin cassette of the pSilent1-AfpA-PKS12

vector offered red colonies with a reduced pigmentation as compared to the wild type strain as well as albino colonies without any pigmentation. The three transformants FG [pSilent1-AfpAi:TF1], FG [pSilent1-AfpAi:TF2], FG [pSilent1-AfpAi:TF3] with pSilent1-AfpA and two red pigmented (FG [pSilent1-AfpAi+PKS12i:TF4], FG [pSilent1-AfpAi+PKS12i:TF5]) as well as three albino colonies (FG [pSilent1-AfpAi+PKS12i:TF1], FG [pSilent1-AfpAi+PKS12i:TF2], FG [pSilent1-AfpAi+PKS12i:TF3]) with pSilent1-AfpA-PKS12 were purified by single-spore isolation and the presence of the transcriptional unit for hairpin RNA expression was checked by PCR. The stability of the phenotype of all transformants could be confirmed by repeating the incubation on mannitol agar with PCNB for three inoculation cycles (Figure 25). Transformants carrying the pSilent1-AfpA RNA hairpin cassette exclusively exhibited only red-pigmented transformant colonies thus verifying that there was no influence of *AfpA* silencing on pigmentation.

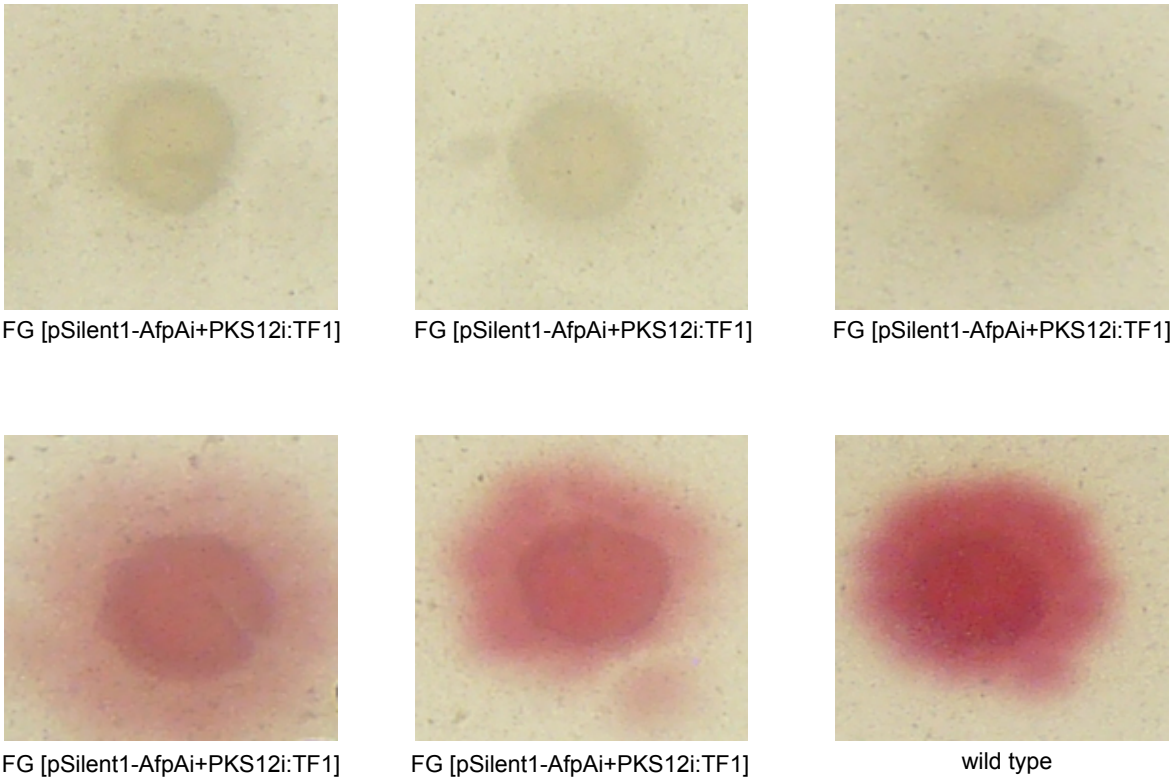

Figure 25. Phenotypes of the *AfpA* + *PKS12* co-silencing transformants FG [pSilent1-AfpAi+PKS12i:TF1] to FG [pSilent1-AfpAi+PKS12i:TF5] as compared to the wild type strain. The strains were grown on mannitol agar with PCNB at 30°C for 10 days in the dark. The strains show different colours due to silencing of the *PKS12* gene with different amounts of aurofusarin produced.

3.8.2 Quantification of AfpA expression

The wild type strain *F. graminearum* TMW 4.0157, the *AfpA* knock out mutants FG [pPK2-ΔAfpA] as well as *AfpA* silencing (FG [pSilent1-AfpAi]) and co-silencing transformants (FG [pSilent1-AfpAi+PKS12i]) were incubated for 5 days in malt extract broth. Following growth, expression intensity of *AfpA* mRNA as relative to the wild type strain was quantified and normalised against three different housekeeping genes (Figure 26). No *AfpA* expression was found in the four knock out mutants FG [pPK2-ΔAfpA]. *AfpA* silencing transformants showed a significant decrease in *AfpA* expression, i.e. 56% ± 15 in FG [pSilent1-AfpAi:TF1], 51% ± 9 in FG [pSilent1-AfpAi:TF2] and 58% ± 22 in FG [pSilent1-AfpAi:TF3], respectively. Highly efficient *AfpA* silencing was found in the co-silencing transformants, which were screened by their albino phenotype. As compared to the wild type strain, their *AfpA* expression was significantly reduced to 37% ± 5 (FG [pSilent1-AfpAi+PKS12i:TF1]), 21% ± 3 (FG [pSilent1-AfpAi+PKS12i:TF2]), and 10% ± 4 (FG [pSilent1-AfpAi+PKS12i:TF3]). On the other hand, co-silencing transformants, which still showed red pigmentation displayed a very low decrease in *AfpA* expression as compared to the wild type, 81% ± 15 in FG [pSilent1-AfpAi+PKS12i:TF4] and 74% ± 29 in FG [pSilent1-AfpAi+PKS12i:TF5]. Silencing of *AfpA* correlated very well with the silencing of *PKS12* and the resulting lack of pigmentation of the albino type transformants.

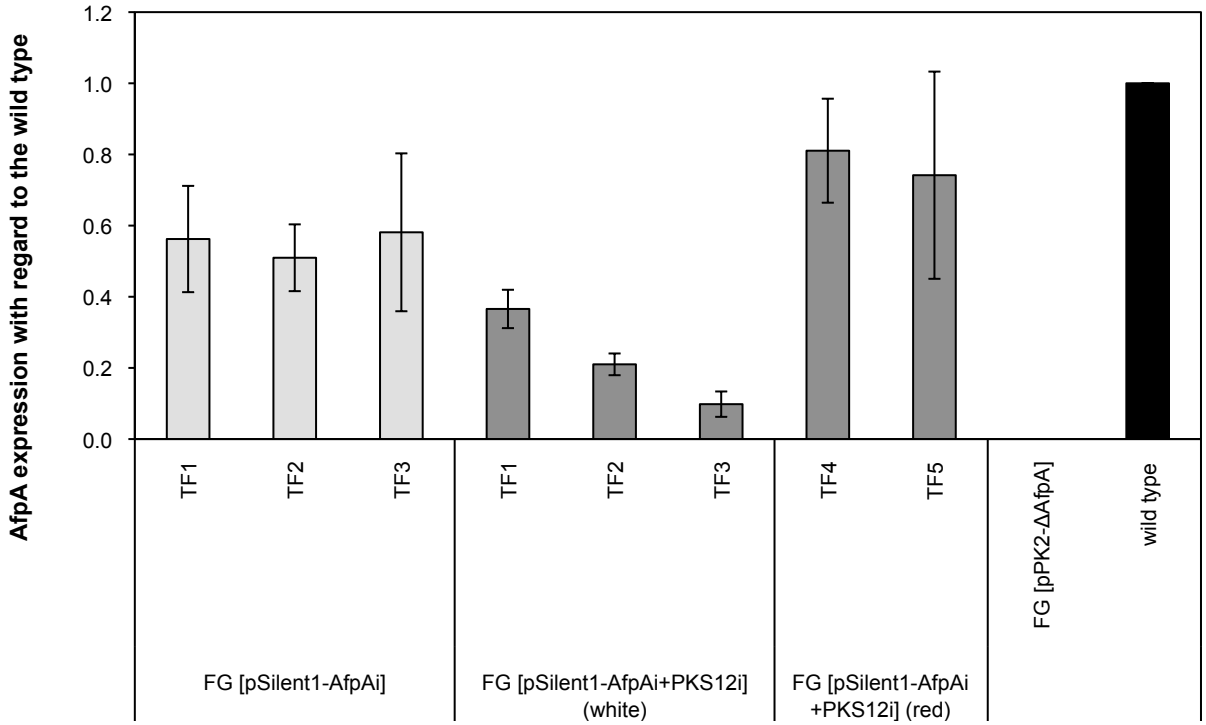

Figure 26. Quantification of the *AfpA* expression levels in *AfpA* silencing transformants, *AfpA* + *PKS12* co-silencing transformants, *AfpA* knock out transformants as compared to the wild type strain. The co-silencing transformants were subdivided into red and white pigmentation types. Three different housekeeping genes were used as internal controls to which gene expression was normalised.

3.9 Comparison of silencing transformants with *AfpA* knock out mutants

In order to confirm the impact of AfpA in phenotypical differences, which could be observed between *AfpA* knock out mutants and *F. graminearum* wild type strain (see chapter 3.7), silencing transformants were analysed in the same way and compared to the results of the *AfpA* knock out mutants FG [pPK2-ΔAfpA] and wild type strain. In addition, potential differences between disruption and silencing should be investigated as it has been described in other studies (Bai et al., 2003; Nakayashiki, 2005). The transformants FG [pSilent1-AfpAi:TF2] and FG [pSilent1-AfpAi+PKS12i:TF3] with the lowest *AfpA* expression level were used in further experiments

3.9.1 Growth rate

Determination of the growth rate of transformants and the wild type strain revealed correlations between silencing and knock out transformants. *AfpA* knock out mutants FG [pPK2-ΔAfpA] showed a growth rate of 91% ± 8 (see also chapter 3.7.1) and the

silencing transformants FG [pSilent1-AfpAi:TF2] and FG [pSilent1-AfpAi+PKS12i:TF3] had a reduction of growth rate to 92% ± 13 as compared to the wild type (Figure 27).

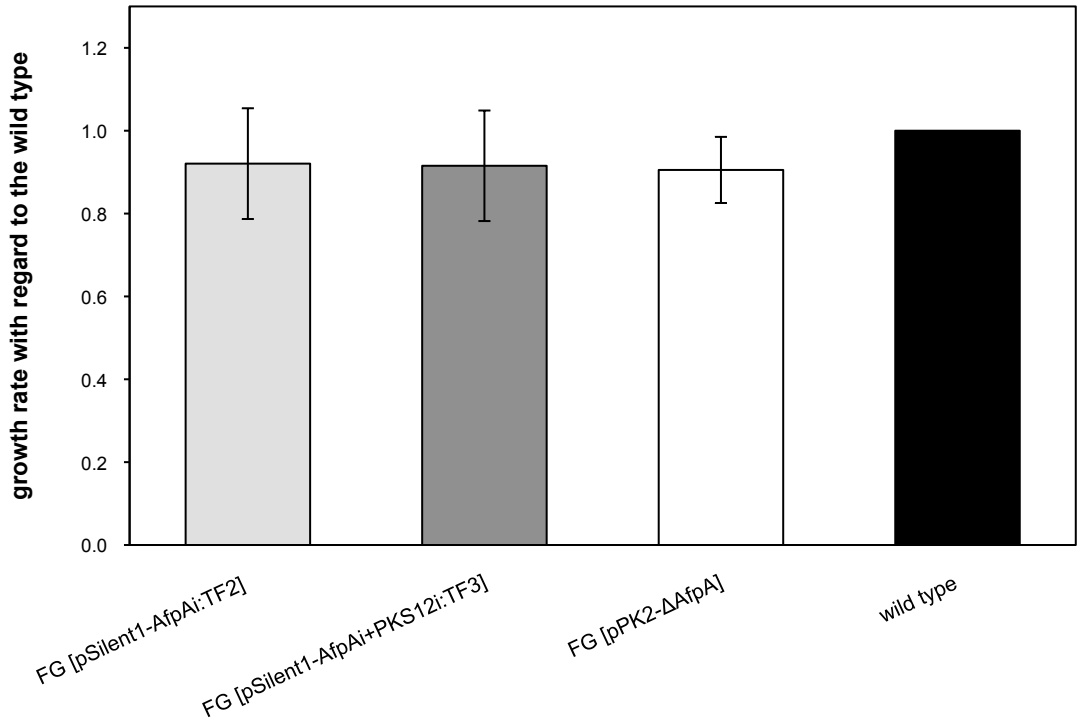

Figure 27. Comparison of the growth rate between *AfpA* silencing transformant FG [pSilent1-AfpAi:TF2], *AfpA* + *PKS12* co-silencing transformant FG [pSilent1-AfpAi+PKS12i:TF3] and *AfpA* knock out mutants FG [pPK2-ΔAfpA] compared to the wild type. The overgrown area of a petri dish was measured and the ratio with regard to the wild type strain was calculated.

Results showed that there is a significant influence of AfpA on the growth rate of the *F. graminearum* strain used during the current study. A similar reduction in growth rate could be assigned for silencing and knock out mutants.

3.9.2 Development of aerial and substrate mycelium

Similar to growth rates, determination of the biomass of submerged mycelia revealed correlations between silencing and knock out transformants. The wild type strain produced a submerged mycelial biomass of 0.28 g ± 0.01 as compared to 0.26 g ± 0.02 in the knock out mutants FG [pPK2-ΔAfpA] (see also chapter 3.7.2). A reduced development because of an inhibited AfpA expression could also be confirmed by the silencing transformants. The *AfpA* silencing transformant FG [pSilent1-AfpAi:TF2] produced 0.27 g ± 0.02 submerged mycelia and the *AfpA* and *PKS12* co-silencing transformant FG [pSilent1-AfpAi+PKS12i:TF3] 0.25 g ± 0.05 submerged mycelia (Figure

28). Thus a significant reduction was found for the knock out transformants, whereas the difference in submerged mycelial biomass production between wild type strain and FG [pSilent1-AfpAi:TF2] or FG [pSilent1-AfpAi+PKS12i:TF3] transformant was not statistically significant.

In contrast to the submerged mycelia, no correlation between silencing and knock out transformants could be detected at the production of aerial mycelial biomass. The knock out mutants FG [pPK2-ΔAfpA] offered a slightly decreased aerial hyphal biomass of 0.07 g ± 0.01 (see also chapter 3.7.2), whereas the silencing transformants FG [pSilent1-AfpAi:TF2] and FG [pSilent1-AfpAi+PKS12i:TF3] showed an increased biomass of 0.09 g ± 0.02 and 0.13 g ± 0.02 as compared to the wild type strain with 0.08 g ± 0.01 (Figure 28).

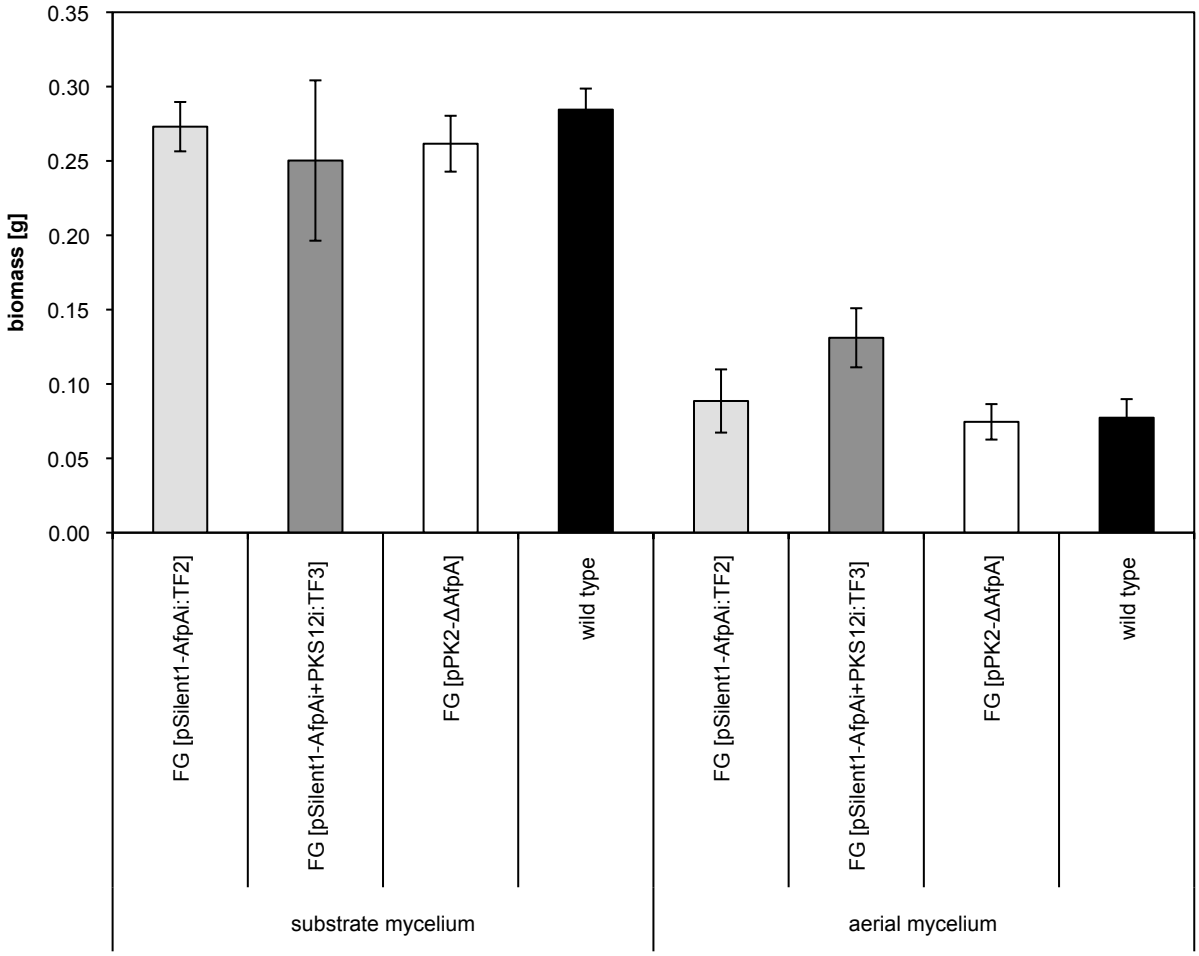

Figure 28. Development of submerged and aerial mycelial biomass in *AfpA* knock out mutants FG [pPK2-ΔAfpA], *AfpA* silencing transformant FG [pSilent1-AfpAi:TF2], AfpA and PKS12 co-silencing transformant FG [pSilent1-AfpAi+PKS12i:TF3], and the *F. graminearum* TMW 4.0157 wild type strain. The cultures were incubated in malt extract broth for 5 days for the development of submerged mycelia and for 7 days on malt extract plates for the development of aerial mycelia. Following incubation, mycelia were harvested, dried and the biomass was determined.

3.9.3 Number and length of conidia

Furthermore, number and length of conidia produced was determined to distinguish the differences in the development of conidia. No significant differences in the number of conidia could be detected for knock out transformants FG [pPK2-ΔAfpA] (see also chapter 3.7.4) as well as for silencing transformants FG [pSilent1-AfpAi:TF2] and FG [pSilent1-AfpAi+PKS12i:TF3] when compared to the wild type strain. However, correlations between knock out and silencing transformants were investigated by measuring conidial length. As it is also described in chapter 3.7.4, conidia of FG [pPK2-ΔAfpA] mutants had an average length of 38.33 µm ± 6.14 and were significantly shorter as compared to the wild type conidia, which had an average length of 46.09 µm ± 6.89 (Figure 29). The silencing transformants FG [pSilent1-AfpAi:TF2] and FG [pSilent1-AfpAi+PKS12i:TF3] also showed conidia with significantly reduced length averaging 40.71 µm ± 6.23 and 36.19 µm ± 4.92, respectively (Figure 29).

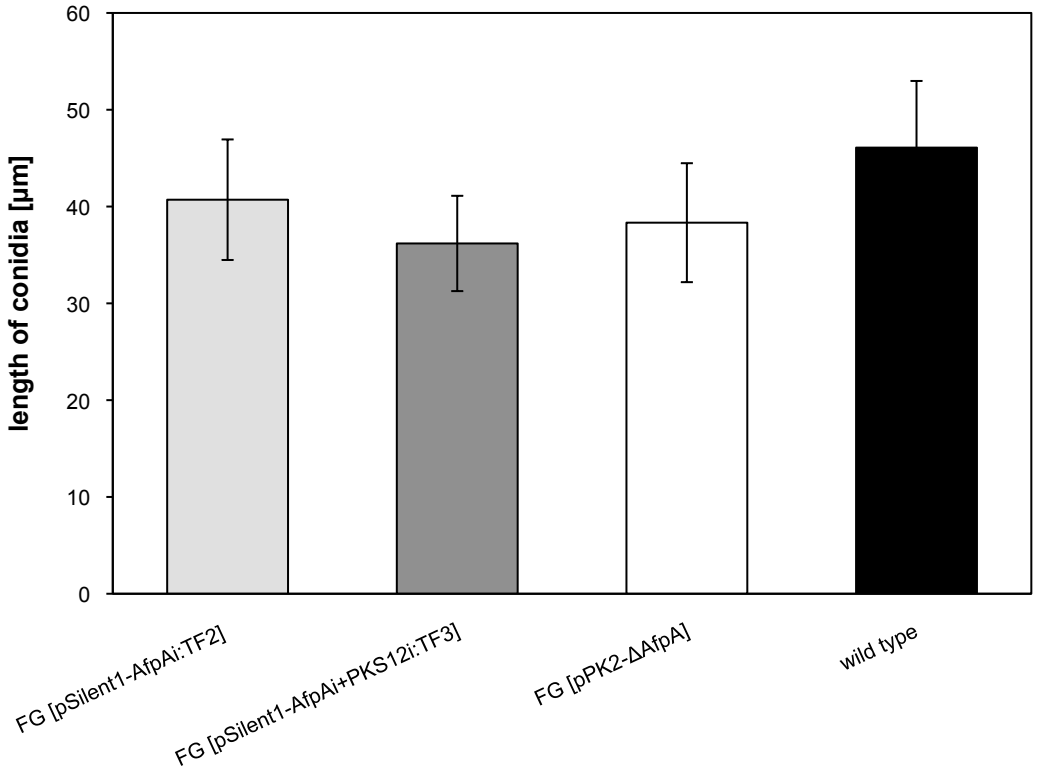

Figure 29. Determination of the length of conidia between *AfpA* silencing transformant FG [pSilent1-AfpAi:TF2], *AfpA* + *PKS12* co-silencing transformant FG [pSilent1-AfpAi+PKS12i:TF3] and *AfpA* knock out mutants FG [pPK2-ΔAfpA] compared to the wild type.

3.9.4 Surface hydrophobicity

Testing the surface hydrophobicity of mycelia by placing a detergent solution on aerial hyphae, no differences in phenotype could be distinguished between *AfpA* knock out mutants FG [pPK2-ΔAfpA] and wild type strain (see also chapter 3.7.5). This result could also be confirmed with the silencing transformants FG [pSilent1-AfpAi:TF2] and FG [pSilent1-AfpAi+PKS12i:TF3], a phenotypical difference to the wild type strain was not observed.

3.9.5 Foam stability

A significant difference between foam stability of the wild type strain supernatant and the supernatant of *AfpA* knock out mutant cultures could been shown in chapter 3.7.6. Following a 20 min rest, an average 19.8 cm ± 3.3 of the foam remained in the homogenised cell free culture liquid of the knock out mutant whereas 33.3 cm ± 5 of the foam height remained in the wild type strain culture liquid. Reduced foam stability of the culture supernatant as a result from a lack of AfpA was also observed for the silencing transformants. FG [pSilent1-AfpAi:TF2] and FG [pSilent1-AfpAi+PKS12i:TF3] displayed foam heights of 19.0 cm ± 3.5 and 6.7 cm ± 2.5, respectively (Figure 30).
Differences in foam stability between silencing clone FG [pSilent1-AfpAi:TF2] and the knock out mutant FG [pPK2-ΔAfpA] were not statistically significant. However, differences in foam stability between the co-silencing clone FG [pSilent1-AfpAi+PKS12i:TF3] and the knock out mutant were highly significant.

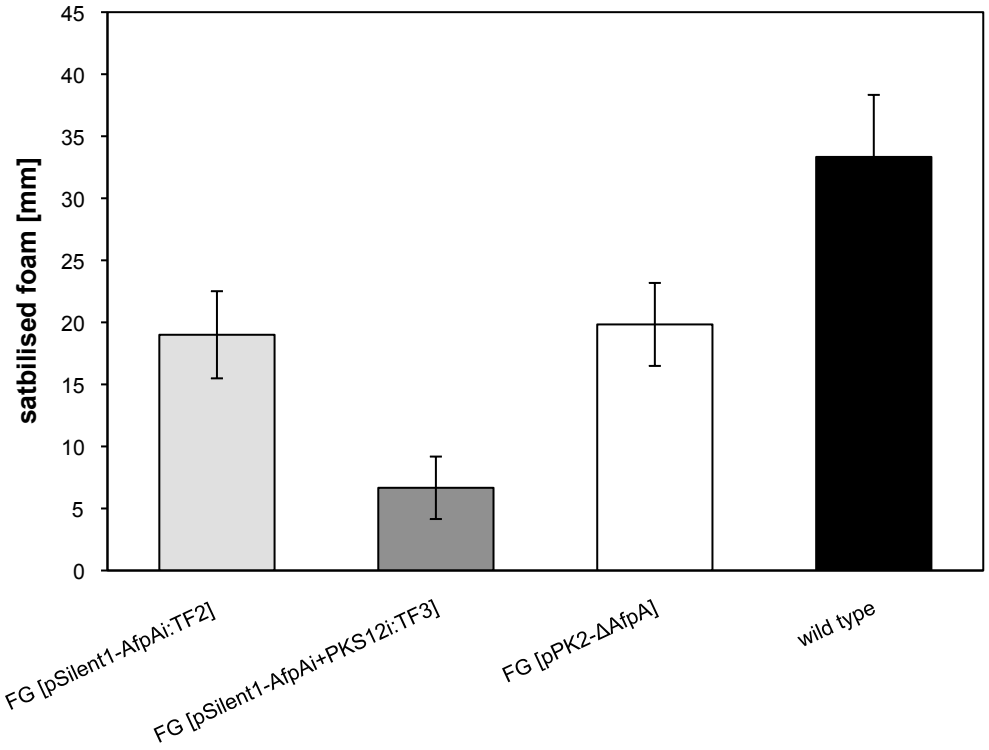

Figure 30. Comparison of foam stability of cell-free culture supernatant grown in malt extract broth between *AfpA* silencing transformant FG [pSilent1-AfpAi:TF2], *AfpA* + *PKS12* co-silencing transformant FG [pSilent1-AfpAi+PKS12i:TF3], and *AfpA* knock out mutants FG [pPK2-ΔAfpA] compared to the wild type. The supernatant was mixed with a homogeniser for 1 min and the foam height was measured after a rest of 20 min.

3.9.6 Pathogenicity

Finally, it was demonstrated that *AfpA* knock out mutants as well as silencing transformants showed a significantly reduced pathogenicity towards barley seedlings as compared to the wild type strain. After a growth period of 9 days, uninfected barley seedlings had an average shoot length of 21.05 cm ± 1.7. Seedlings infected with spores of the *F. graminearum* wild type strain showed a shoot length of 15.89 cm ± 1.77 whereas shoots treated with spores of the knock out mutants had an average length of 18.53 cm ± 2.44 (see also chapter 3.7.7). Barley seedlings treated with spores of the silencing transformants FG [pSilent1-AfpAi:TF2] and the co-silencing transformant FG [pSilent1-AfpAi+PKS12i:TF3] showed a shoot length of 17.82 cm ± 2.07 and 17.80 cm ± 2.61, respectively (Figure 31).

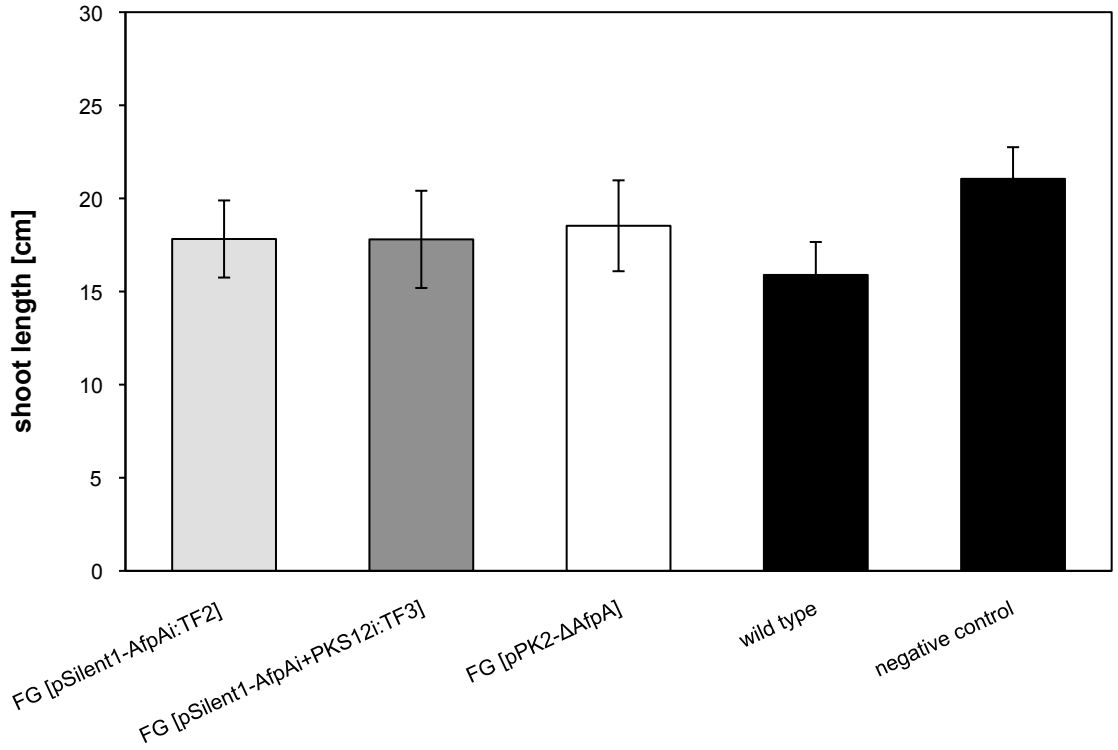

Figure 31. Comparison of pathogenicity against barley seedlings between *AfpA* silencing transformant FG [pSilent1-AfpAi:TF2], *AfpA* + *PKS12* co-silencing transformant FG [pSilent1-AfpAi+PKS12i:TF3], and *AfpA* knock out mutants FG [pPK2-ΔAfpA] and the wild type. Barley seedlings were infected with conidia for 2 h. Seedlings treated with 0.02% tween 20 (v/v) and 0.5% gelatine (w/v) aqueous solution without spores was used as negative control. Shoot length was determined after a 9 d growth period.

Results suggest that a correlation between the presence of the *AfpA* gene in *F. graminearum* and its aggressiveness against barley seedlings exists. This could be verified by the disruption as well as the silencing of the *AfpA* gene.

4 Discussion

Several *Fusarium* species are widespread pathogens on cereals and can infect various economically important crops such as wheat, barley, rice, and maize. In addition, certain strains are also capable of producing mycotoxins, some of which of notable impact to human and animal health. But not only the production of mycotoxins is problematic, the accumulation of surface-active proteins in malt causes severe economic losses to breweries because of gushing. However, the mechanisms and inducing agents of the gushing phenomenon have not been fully identified. Furthermore, the role of surface-active proteins for the fungus is often not completely understood. For this reason, two classes of surface-active fungal proteins, the hydrophobins and the fungispumins, were characterised more closely in this study. It could be shown that the hydrophobin FcHyd5p, a class II hydrophobin from *Fusarium culmorum*, is a highly surface-active protein with the ability to change hydrophobic into hydrophilic surfaces, to stabilise air bubbles in aqueous solutions, and finally to induce gushing of carbonated liquids. These characteristics are very interesting since it was found that especially *Fusarium* species (Haikara, 1983; Munar and Sebree, 1997; Schwarz et al., 1996) and class II hydrophobins are important gushing activators (Zapf et al., 2006). Furthermore, the function of the fungispumin AfpA for *F. graminearum* was investigated more closely by gene disruption. AfpA knock out mutants showed a significant decrease in the rates of radial growth and submerged mycelial biomass, conidial length, and pathogenicity against wheat and barley seedlings compared to the *F. graminearum* wild type strain. Despite structural similarities of AfpA to hydrophobins (Zapf et al., 2007), no significant differences occurred in the biomass, morphology and wettability of aerial hyphae produced. However, stability of foam produced by vigorous agitation of the cell free spent culture supernatants was significantly decreased. In addition, gene silencing by RNA interference was successfully applied in *F. graminearum*. All significant differences, which occurred between knock out mutants and wild type strain, could be confirmed with the *AfpA* silencing transformants, whereas significant differences between the silencing and the disruption of *AfpA* could not be found.

4.1 Cloning of the *FcHyd5* gene into *Pichia pastoris*

Recent studies demonstrated that beer fermented with *S. cerevisiae* cells previously transformed with the *FcHyd5* gene from *F. culmorum* showed gushing (Zapf et al., 2006). However, the expression of the gene in the *S. cerevisiae* transformants as well as detection of the protein in beer and brewing raw materials has not been demonstrated yet. One reason for this is that isolation of the protein from natural sources, e.g. cultures of *F. culmorum*, failed. During the current study, hydrophobin 5 from *F. culmorum* was heterologously expressed by transformant cultures of *P. pastoris* in order to produce high amounts of FcHyd5p for further experiments, e.g. investigation of the protein properties such as surface activity and its effect on gushing (Askolin et al., 2005; Zapf et al., 2006). *P. pastoris* was chosen as the expression system because as eukaryote it is able to produce and secrete correctly folded recombinant proteins that have undergone all post-translational modifications required for functionality with an inducible system (Daly and Hearn, 2005). In contrast, no such effective expression system for secreted proteins is available for *S. cerevisiae*. For our experiments, we applied codon usage optimisation and removed the intron from the *FcHyd5* gene to ensure efficient expression. To facilitate purification of this highly amphiphilic protein, a version with a 6 x His-tag was cloned in addition to the native hydrophobin 5.

4.2 Heterologous expression of FcHyd5p in *Pichia pastoris*

FcHyd5p has a predicted size of 8.84 kDa. With an additional 6 x His–tag, the protein is predicted to have a molecular weight of 9.87 kDa. Transgenic FcHyd5p produced by clones during the current study had a size of approximately 10 to 15 kDa according to SDS-PAGE and Western blot analysis. In spite of these findings, identity of the cloned protein with FcHyd5p could be verified by immuno-staining of the His-tag labelled protein with 6 x His-tag specific antibodies. In addition, the pI as well as the weight difference between transgenic FcHyd5p with and without 6 x His-tag was characteristic of the protein (Figure 9). Possible explanations for the difference in molecular weight observed between the native and the cloned protein are e.g. residual amino acids from restriction sites, incomplete cleavage of the α signal peptide or partial renaturation of the protein in SDS-PAGE. An N-glycosylation, acetylation and two phosphorylation sites were found in the primary sequence of FcHyd5p. Such post-translational modifications may also increase the theoretical molecular weight of proteins (Daly and Hearn, 2005).

Furthermore, a variation in staining intensity of proteins was detected between the two transgenic proteins (Figure 9), which can be explained by the fact that the histidine tag has been reported to intensify staining with Coomassie blue (Batoni et al., 2002).

4.3 Purification of heterologously expressed FcHyd5p

High surface activity is a characteristic feature of hydrophobins as was demonstrated with the cloned protein, which turned a hydrophobic surface like parafilm™ from hydrophobic to wettable (Wösten et al., 1994a). However, this feature often complicates handling of hydrophobins. Therefore, isolation of FcHyd5p with 6 x His-tag using a His trap column failed during the current study. The application of a hydrophobic interaction chromatography was also not successful. As an alternative, solution-phase isoelectric focussing was successfully applied for protein purification in order to avoid self-aggregation and especially aggregation on surfaces.

Furthermore, also the production of a specific polyclonal antiserum against epitopes of FcHyd5p failed in this study. This result may be appearing due to a low immunogenicity of FcHyd5p. Reasons for a minor immune response could be referred to self-aggregation and conformational changes in the molecule, which are reported for hydrophobins by Wösten et al. (2001). But differences in the post-transcriptional folding between FcHyd5p with and without His-tag may also be responsible for changes in the surface of the protein and thus for specificity of the antibodies. Differences in gushing potential of FcHyd5p with and without His-tag confirm the assumption that significant modifications in the structure of the protein are present due to the His-tag. However, the 6 x His-tag is frequently used in many studies because of its small size and low immunogenicity (Wang et al., 2003). Finally, a low concentration of FcHyd5p because of its adhesion on surfaces during the immunisation procedure may be responsible for insufficient antibody production.

4.4 Determination of surface activity of FcHyd5p

The theory, according to which hydrophobins stabilise air bubbles in fluids by forming a layer around air bubbles (Draeger, 1996; Pellaud, 2002) was supported by experiments in which cell-free culture supernatants of FcHyd5p producing *P. pastoris* clones were homogenised with a blender, thus bringing air into the liquid phase. The hydrophobin

strongly aggregated on the air/water interface surrounding the bubbles and leading to stable foam on top of the supernatant as well as strong turbidity formed by aggregated protein. Aggregated FcHyd5p was insoluble in hot 2% SDS, 8 M urea, and organic solvents (ethanol, methanol, acetone). Only addition of a strong denaturing agent like trifluoroacetic acid was able to clarify the turbid liquid (data not shown). This is interesting because this property has previously only been described for class I hydrophobins (Wösten and de Vocht, 2000). Actually, the use of organic solvents should solubilise the aggregated class II hydrophobins (Linder et al., 2005; Wessels, 1994). However, the strong aggregation of FcHyd5p confirmed the high variability in properties of especially class II hydrophobins as described by Askolin et al. (2006).

4.5 Gushing potential of FcHyd5p

A homologous sequence of the *FcHyd5* gene was detected in the genome *F. graminearum* (Zapf et al., 2006). This fact makes FcHyd5p an interesting model substance for beer gushing because the gene is present in both fungal agents, which have been most closely associated to the induction of the phenomenon in brewing experiments (Haikara, 1983; Munar and Sebree, 1997; Schwarz et al., 1996). Similar to the generation of very stable foams and microbubbles in homogenised culture supernatants, also in beer a stable layer of amphiphilic proteins at the gas/water interface of carbon dioxide bubbles can be assumed to play an important role. Such layer may prevent bubbles from completely disappearing after solubilisation of carbon dioxide under pressure in bottled beer. The unique solubilisation properties of FcHyd5p (see above) may further contribute to the stability of such aggregates. Pressure release at opening of a bottle may lead to re-entering of carbon dioxide into the gas phase using the hydrophobic inner part of the empty bubbles as condensation nuclei. In non gushing beer, the bubble layer is made up from nsLTP1, an amphiphilic protein produced by cereals and known as the major foam protein in beer (Sorensen et al., 1993). This layer stabilises microbubbles and prevents them from growing to a size at which bubbles would rise to the surface of the liquid. Results of the gushing experiments conducted with FcHyd5p in this study confirm the theory according to which nsLTP1 bubble layers are impurified by hydrophobins resulting in unstable bubble skins in gushing beer.

In the current study, we also used carbonated water instead of beer to exclude other proteinaceous factors reported to affect the gushing behaviour. We demonstrated that

as few as 86 µg of highly purified FcHyd5p with 6 x His-tag were able to induce gushing when added to a 500 ml bottle of beer (Figure 14A, page 59). Similarly, addition of 2 mg freeze-dried culture supernatant from the FcHyd5p producing clone X33 [pPICZαA-FcHyd5] into beer induced a volume loss of 50% per bottle (Figure 14B). Using carbonated water, 0.1 mg freeze-dried culture supernatant from the same clone was sufficient to induce gushing with a volume loss of 49% per bottle (Figure 14C). Addition of more culture supernatant did not significantly increase gushing since a 20-fold (2 mg) higher amount of freeze dried supernatant increased gushing in water to only 54% volume loss per bottle (Figure 14C). From the results, it is concluded that FcHyd5p could be an essential factor for gushing induction in carbonated aqueous liquids and thus could be an inducer of gushing in the brewing industry. This result is consistent with other studies, in which hydrophobins have been discussed as gushing inducing factors (Haikara, 2000; Laitila et al., 2007) and in which gushing was caused by addition of small amounts of hydrophobins isolated from *Fusarium* cultures (Sarlin et al., 2005). Also brewing experiments were reported in which a culture of *S. cerevisiae* transformed with the FcHyd5p gene yielded a gushing volume of 26.2 ml in the beer produced (Zapf et al., 2006). In the same study, brewing yeast transformed with the class I hydrophobin FcHyd3p and the fungispumin AfpA had no influence on the gushing potential of the beer produced. Differences in gushing tendencies between beer and carbonated water were demonstrated during the current study (Figure 14B/C) where higher amounts (at least 20x) of the same culture supernatant were necessary to induce a similar gushing volume in beer as compared to carbonated water. This fact may refer to the presence of gushing inhibiting components in beer such like certain hop compounds (Hough et al., 1982; Lutterschmid et al., 2010; van Cleemput et al., 2009). Also differences occurred between supernatants from clones X33 [pPICZαA-FcHyd5] and X33 [pPICZαA-FcHyd5-HisTag] because FcHyd5p with 6 x His-tag appeared to provoke lower gushing volumes (Figure 14B/C). The histidine tag can influence protein procession and accordingly may change protein properties as described in other studies (Klose et al., 2004; Ledent et al., 1997).

4.6 Discovering the function of the AfpA protein using targeted gene disruption

As a member of the ascomycetes *F. graminearum* forms haploid mycelia, with regularly uninucleate cells (Dörfelt, 1994; Schlegel, 2006). Hence, disruption of a single-copy gene should lead to complete inhibition of its expression. Restriction analysis revealed that *AfpA* is a single copy gene (Zapf et al., 2007). Therefore, targeted gene disruption of *AfpA* should be a simple method for analysing the role of the protein for the fungus.

Usually, transformation is achieved by polyethylene glycol treatment of protoplasts (Hynes, 1996). This method is often described as being time-consuming, poorly reproducible and inefficient in transformation and homologous recombination (Covert et al., 2001; Talhinhas et al., 2008; Zeilinger, 2004; Zwiers and De Waard, 2001). *A. tumefaciens*, a pathogen of dicotyledonous plants (Hooykaas and Beijersbergen, 1994; Kado, 1991) transfers T-DNA into plant cells during tumorigenesis and represents the only example of naturally occurring trans-kingdom transfer of genetic material (Bundock et al., 1995). Therefore, it is used as tool for transferring genes into plant cells (Hiei et al., 1994; Ishida et al., 1996) yeasts (Bundock et al., 1995) and even filamentous fungi (de Groot et al., 1998). The *A. tumefaciens*-mediated transformation is described as simpler and less labour-intensive as compared to protoplast-requiring transformation protocols (Covert et al., 2001; Kemppainen et al., 2005). In filamentous fungi a high number of ectopic vector integrations occur by illegitimate recombination (Ruiz-Diez, 2002). Using ATMT as alternative, integration of the T-DNA into a genome generally occurs as a single copy event (Staats et al., 2007; Zwiers and De Waard, 2001).

During the current study, conventional protoplast-requiring transformation was applied besides ATMT for targeted gene disruption in *F. graminearum*. Two different knock out constructs were used to achieve a targeted disruption of *AfpA* or *PKS12*, but transformation resulted exclusively in non-homologous integration of both disruption constructs. On the contrary, ATMT turned out to be less labour-intensive. Furthermore, ATMT is more efficient in transformation and homologous recombination as it was described in other studies and for other filamentous fungi (Chen et al., 2000; de Groot et al., 1998; Rho et al., 2001; Zeilinger, 2004; Zwiers and De Waard, 2001), since *A. tumefaciens*-mediated targeted gene disruption of AfpA was very efficient in *F. graminearum*.

Successful disruption of AfpA in *F. graminearum* could be verified by sequencing, but using the anti-AfpA antiserum described by Zapf et al. (2007), bands with the expected size revealed in both samples. As mentioned by Zapf et al. (2007), Western blotting of proteins of *F. culmorum* strains displayed only one band per strain, but with proteins of different *F. graminearum* strains consistently displayed two bands of similar size separated only by a narrow space. Zapf et al. (2007) suggested the presence of a post-translationally modified form of AfpA or a co-reacting protein of slightly higher molecular mass. However, current results suggest a cross-reactivity of the AfpA antiserum with another protein in *F. graminearum* of similar size.

Several knock out mutants were used in the current study to investigate the impact of the AfpA protein. Since no significant differences among the knock out mutants could be determined, an impact of potentially non-homologous integrated T-DNA on the results obtained can be excluded. Furthermore, the ability to disrupt AfpA in *F. graminearum* showed that the fungus does not essentially need the protein. It could be shown that targeted gene disruption of *AfpA* has pleiotropic effects on *F. graminearum.* Similar to *PhiA*, also *AfpA* was shown to have an impact on the development of conidia. *AfpA* knock out mutants exhibited significantly shorter conidia as compared to the wild type strain. In contrast to *PhiA* and *MHP1*, mutants, who do not produce AfpA, displayed only a slight decrease in the number of spores produced, which was statistically insignificant. Furthermore, the AfpA protein seems to affect the germination capacity of conidia. At concentrations above 6×10^4 spores/ml, the *AfpA* knock out mutants had significantly more ungerminated conidia as compared to the wild type strain. A similar effect was reported for the hydrophobin MHP1 in *M. grisea* (Kim et al., 2005b). *AfpA* knock out mutants featured a significantly reduced rate of radial growth as compared to the wild type strain. A similar behaviour was also observed in *PhiA* knock out mutants of *A. nidulans* (Melin et al., 2003). Results suggest that there is either a direct influence of the AfpA protein on the development of mycelia with a reduction of growth rate or an indirect influence, which might be caused by a general lack of fitness of the knock out mutants as compared to the wild type strain. Comparing biomass of submerged mycelia with the rates of radial growth, a similar decrease was observed in both parameters since both depend on the development of substrate mycelium. Surprisingly, no significant difference in the development of aerial mycelia was observed between *AfpA* knock out mutants and the wild type strain since such type of influence has been described for hydrophobins in other fungi. A lower surface hydrophobicity of aerial

hyphae due to disruption of the *AfpA* gene could also not be found with the surface hydrophobicity assays performed during the current study. The phenotype of *AfpA* knock out mutants was unaffected regarding wettability of aerial mycelia with water or detergent solution as has been reported for mutants with a disrupted genes encoding hydrophobins, e.g. MHP1 (Kim et al., 2005b) and dewA (Stringer and Timberlake, 1995). Also staining of hyphae with lipophilic dyes such as nile-red or Hoechst 33342 revealed no apparent differences between the wild type and *AfpA* knock out mutants, which could display differences in the hydrophobicity of the outside of the mycelium cell wall. This would be likely, if AfpA as surface-active protein was exposed on the cell wall of the fungus. Moreover, no apparent differences regarding the morphology of phialides of *F. graminearum* was found during the studies. However, differences occurred in the appearance of submerged mycelium of *AfpA* knock out mutants growing on mung bean agar with addition of hygromycin B. Substrate mycelium appears strongly branched and inhomogeneous with a high amount of compartments within the hyphae (see Figure 18, page 64). These effects do not result when *AfpA* knock out mutants grow on mung bean agar without hygromycin B and consequently may appear as a response to additional stress.

The surface activity and secretion of AfpA was confirmed during the current study. Foam produced from cell-free culture supernatants of knock out mutants was significantly less stable then the foam of the wild type strain after treatment with a homogeniser. Results confirmed that the AfpA protein shows behaviour similar to hydrophobins since it was found to be secreted into the medium by the *F. graminearum* wild type strain and displays a high degree of surface activity with the ability to form stable layers at water air interfaces. This is in agreement with results reported by Zapf et al. (2007) who precipitated AfpA from foam fractions of cell-free *F. graminearum* liquid cultures and showed that AfpA is able to enhance gushing. However, Lutterschmid et al. (2011) tested transgenic AfpA and FcHyd5p for their ability to stabilise foam and found significant differences between hydrophobins and non-hydrophobin proteins. After 1 h, foam with AfpA had completely disintegrated and the initially turbid liquid turned clear, whereas foam with FcHyd5p remained stable as it was also shown in chapter 3.4.1 (page 56). However, AfpA seems to have an impact on stability of foam as it is described to have gushing enhancing (Zapf et al., 2007) as well as gushing reducing properties (Lutterschmid et al., 2011). In *AfpA* knock out mutants, the absence of AfpA may also influence production or secretion of other surface-active proteins such as

hydrophobins, which would also affect foam stability. Surface-active proteins such as hydrophobins and other cell wall associated fungal proteins are often involved in the mechanisms of host-pathogen interaction governing plant infection. Cell wall associated proteins in *F. oxysporum* for example were supposed to be responsible for penetration of the root cortex and consequently for the infection of carnations (Prados-Rosales et al., 2009). Also hydrophobins such as cryparin in *Cryphonectria parasitica* (Kazmierczak et al., 2005) and MPG1 in *Magnaporthe grisea* (Soanes et al., 2002) have been found to be involved in the pathogenicity of the producing organisms. During the current study, a significant difference in pathogenicity between the *F. graminearum* wild type strain used and the knock out mutants was detected by comparing the shoot length of wheat and barley seedlings after infection with conidia produced by the respective cultures. Comparing differences in root length of barley plantlets, no significant difference could be determined between uninfected barley seedlings and seedlings treated with *AfpA* knock out mutants, whereas a significant difference occurred between wild type strain and knock out mutants. In wheat, a significant difference in root length could be determined between the negative control and seedlings treated either with wild type strain conidia or knock out mutant conidia, but no significant difference could be observed between wild type strain and knock out mutants. According to the results obtained, the absence of the AfpA protein in the mutants seemed to have a significant negative impact on the pathogenicity of *F. graminearum*.

Comparing infection of wheat and barley with *F. graminearum*, the growth of wheat seedlings is more inhibited. Shoot length of barley seedlings infected with wild type spores displayed only 75% of the length of uninfected seedlings, whereas infected wheat seedlings offered a shoot length of 63%. A similar result was observed for the length of the roots. However, comparing the differences between wild type strain and knock out mutants, the disruption of AfpA seems to have a smaller impact on infection of wheat than barley.

4.7 Validating phenotypical differences of ΔAfpA mutants by RNA silencing

The efficiency of gene targeting strongly depends on the target species because in filamentous fungi, in contrast to *S. cerevisiae*, a high number of ectopic vector integrations occurs by illegitimate recombination (Ruiz-Diez, 2002). However, the characterisation of multicopy genes as well as the use of diploid cells makes the analysis

difficult. Moreover, most fungi exhibit multicellular hyphae consisting of multinuclear cells, and some of them even have two or more genetically different nuclei in a common cytoplasm. These characteristics make gene targeting even more complicated (Nakayashiki, 2005). The transformation process itself can cause alterations at other loci in addition to the targeted gene (Keller et al., 1990; Oliver and Osbourn, 1995). Therefore, it is necessary to confirm whether observed genotypic or phenotypic changes are caused by disruption of the target gene or by non-target effects. This can be accomplished e.g. by analysis of transformants with an ectopically integrated disruption vector or by complementation of the disruption mutant (Desjardins et al., 1992; Herrmann et al., 1996; Talbot et al., 1996). Finally, the analysis of genes, which are essential to cell viability, is not possible.

An advantage of the RNA interference mechanism is its ability to suppress gene expression relatively easily and quickly. Due to its locus independence, RNA silencing can be used in fungi having multinuclear hyphae or a low targeting efficiency (de Jong et al., 2006; Janus et al., 2007; Nakayashiki et al., 2005). Furthermore, RNA silencing can be successfully applied in down-regulation of multicopy genes as well as genes in cells with a diploid genome. Secondly, suppression of gene expression by RNAi results not necessarily in a null phenotype, which allows the analysis of genes coding for essential proteins in contrast to targeted gene disruption (Janus et al., 2007; Nakayashiki et al., 2005). Further advantages are a controlled expression by using inducible promoters and the co-silencing of several genes (Janus et al., 2007).

Recently, McDonald et al. (2005) reported the presence of Dicer and other RNA silencing enzymes in *Fusarium* spp.. In their study, an inverted repeat transgene construct was used to silence mycotoxin production. Previous results strongly suggested that *Fusarium* could be added to the growing list of RNA silencing–capable fungi. This suggestion was confirmed by the results obtained in the current study since it was demonstrated that gene silencing by RNA interference is a useful and efficient method in *F. graminearum*. A very efficient silencing could be determined with a reduction in *AfpA* expression being as low as 10% ± 4 as compared to the corresponding wild type. Because of the short gene sequence of *AfpA*, the primer pair used for the quantification is located within the sequence used for the RNA haripin cassette. This could possibly lead to an amplification of the hairpin cDNA and thus to a falsely increased expression level. In order to avoid this, primers should ideally be designed so that they do not hybridise within the gene regions corresponding to inhibitory hairpins (Vermout et al., 2007).

Varying degrees of silencing can make experimental results difficult to interpret. Phenotypic differences between knock out and knock down mutants may appear (Bai et al., 2003). Differences in pathogenicity occurred between *Magnaporthe grisea* mutants with a disrupted or silenced *mpg1* gene (Nakayashiki, 2005). In the current study, growth rate, length of conidia, surface hydrophobicity, foam stability and pathogenicity against barley seedlings were determined for *AfpA* knock out mutants and for the *AfpA* silencing transformant. Results were compared to the results of the corresponding wild type strain. It was shown that the significant differences existing between wild type strain and knock out mutants could be confirmed by the silencing transformant. Significant phenotypic differences did not appear between *AfpA* knock out and knock down mutants as was reported in other studies (Bai et al., 2003; Nakayashiki, 2005). Alterations at other loci in addition to the targeted gene, which may cause phenotypical changes in knock out transformants (Keller et al., 1990; Oliver and Osbourn, 1995), could be consequently excluded as reason for the determined differences. However, despite correlations between *AfpA* knock out and knock down mutants, a decreased pathogenicity due to indirect effects such as morphologically aberrant conidia or a reduced growth rate could not be completely excluded in this study. Since *F. graminearum* is more widely known as head blight pathogen, head inoculations should follow in order to confirm the role of AfpA in pathogenicity.

However, differences between *AfpA* knock out and knock down mutants could be found in the morphology of substrate mycelium. Branched and inhomogeneous mycelium could not be observed at silencing transformants growing on mung bean agar with hygromycin B, as it is described in chapter 3.7.2 (page 64) for *AfpA* knock out mutants. The amount of compartments within the hyphae of the silencing transformant was also lower as compared to the *AfpA* knock out mutants. However, an increased compartmentation in comparison to the wild type strain was detectable. Alterations at other loci in addition to the targeted gene may be a reason for these differences. Another possibility refers to the incomplete inhibition of *AfpA* expression in silencing transformants, which may cause less stress and thus normal formation of substrate mycelium on selective agar. Accordingly, a major disadvantage of RNA silencing is that incomplete knock down of a gene sometimes makes experimental results difficult to interpret (Nakayashiki, 2005). Small differences, which occur between gene disrupted and wild type strain, are often phenotypically unrecognised with silencing. Consequently, several transformants with different silencing levels should be examined

to ensure the influence of the gene of interest, but time-consuming expression studies are necessary for the analysis of gene silencing experiments. Consequently, the use of RNA silencing in combination with the gene knock out approach greatly facilitates exploring the function of newly discovered genes in filamentous fungi (Nakayashiki et al., 2005).

4.8 Screening for efficient AfpA silencing with PKS12 as phenotypical marker

Moreover, silencing of *AfpA* was combined with silencing of the *PKS12* gene coding for *polyketide synthase 12* (*PKS12*). The enzyme is responsible for the biosynthesis of the red pigment aurofusarin (Frandsen et al., 2006). Both genes were knocked down together using one co-silencing vector construct. This method turned out to be very useful to screen for transformants displaying very efficient *AfpA* silencing. Without screening by co-silencing the average *AfpA* expression level of the investigated transformants was reduced to 55% whereas co-silencing transformants selected according to their albino phenotype showed a reduction by 77%.

An experiment similar to the current study was described by Janus et al. (2007). The authors integrated the red fluorescent protein (DsRed) from *Discosoma* spp. into the genome of *Acremonium chrysogenum* and used its fluorescence as reporter for gene silencing. The use of DsRed as reporter gene offers a favourable alternative that allows effortless detection of silenced transformants by establishing an easily recognisable red fluorescent phenotype (Janus et al., 2007). However, DsRed is not useful in *Fusarium* spp. because of the natural occurring red pigmentation in many species and especially in *F. graminearum*. For this reason, *PKS12* was used during the current study as an alternative reporter gene in *Fusarium* spp.. An advantage of that approach is that the reporter gene must not be supplemented into the genome of the target fungus. This fact makes the method less time-consuming. Furthermore, integration of additional genes into a genome may cause alterations at loci in addition to the targeted gene (Keller et al., 1990; Oliver and Osbourn, 1995). This may cause additional phenotypical changes, which are attributed to the integration and not to the silencing of the gene of interest. On the other hand, if the role of a native reporter gene in the target organism is not completely understood or an influence on the gene of interest cannot be fully excluded, the observed phenotypical changes could also derive from the silenced reporter gene.

Apart from conidial length, development of aerial mycelia, and foam stability of the homogenised cell-free culture supernatant, no significant differences were observed between the *AfpA* silencing transformant FG [pSilent1-AfpAi:TF2] and the *AfpA* + *PKS12* co-silencing transformant FG [pSilent1-AfpAi+PKS12i:TF3]. A possible reason for the increased reduction in conidial length and foam stability of *AfpA* + *PKS12* co-silencing transformants may be the more efficient silencing of *AfpA* in these clones. In addition, the co-silencing transformants had smaller conidia and lower foam stability as compared to the clones with a knock out of the *AfpA* gene. Thus, the silencing of *PKS12* and consequently the biosynthesis of aurofusarin may also influence conidial length in *F. graminearum*. Aurofusarin has antibiotic properties against both mycelial fungi and yeasts (Medentsev et al., 1993), but the biological role of aurofusarin in *F. graminearum* has not been determined yet (Frandsen et al., 2006; Kim et al., 2005a). The phenotype of the aurofusarin-deficient mutant suggests that aurofusarin biosynthesis is not required for most cultural characteristics of *F. graminearum* or for the virulence of this organism in barley (Kim et al., 2005a). But Malz et al. (2005) reported that *PKS12* mutants have higher growth rate as compared to the wild type, whereas mutants are fully pathogenic on wheat and barley. However, Malz et al. (2005) reported that growth rates were similar on pigmentation non-inducing media, whereas under pigmentation-inducing conditions the pigmented wild type exhibited a clearly reduced growth rate as compared to the albino *PKS12* mutants. During the current study, we observed a similar behaviour by determining the biomass of aerial hyphae of *AfpA* + *PKS12* co-silencing transformants grown on a pigmentation inducing medium. The co-silencing transformant FG [pSilent1-AfpAi+PKS12i:TF3] with an albino phenotype offered an increased development of aerial hyphae (0.13 g ± 0.02) as compared to the wild type strain (0.08 g ± 0.01) (Figure 28, page 75). In the current study, mung bean medium as a pigmentation non-inducing media was used for determination of growth rate, thus an influence of the *PKS12* silencing should be excluded. This could be also confirmed by comparing the growth rates of silencing and co-silencing transformants.

5 Summary

Surface-active proteins have a wide range of different functions for filamentous fungi and thus are very important to this group of organisms. The group of hydrophobins is the largest and most important group. Hydrophobins are involved in formation of hydrophobic aerial structures like aerial hyphae, spores and fruiting bodies and mediate the escape of the hyphae from the liquid growth medium into the air or attachment of hyphae to hydrophobic surfaces and signalling thereof. The latter is important in initial steps of fungal pathogenesis. Furthermore, by self-assembly, hydrophobins stabilise air bubbles in water and change the property of surfaces by forming amphipathic membranes. Due to these characteristics, hydrophobins have therefore been under discussion as inducing factors of gushing, a condition, in which bubbles of carbon dioxide are released unexpectedly and vigorously upon opening of a bottle of carbonated beverages. However, the mechanisms of gushing and the role of hydrophobins are still not completely understood. Therefore, the hydrophobin 5 from *Fusarium culmorum* (FcHyd5p) was heterologously expressed by *Pichia pastoris* in order to produce high amounts of FcHyd5p for further experiments and to examine the role of the hydrophobin for the gushing phenomenon.

Another group of surface-active fungal proteins, the fungispumins, are described to have also an effect on gushing. This new class of proteins was purified after foam fractionation of a cell free culture supernatant of *F. culmorum.* The alkaline foam protein A (AfpA) as representative of this group has similar properties to hydrophobins such as surface activity, incorporation into gas-liquid interphases, heat stability, and the ability to form aggregates. Since the natural function of AfpA still needs to be elucidated, the aim of the study was to examine the role of AfpA by gene disruption.

The class II hydrophobin FcHyd5p from *F. culmorum* was heterologously expressed in *P. pastoris*. Transcription of the recombinant gene was confirmed by RT-PCR and expression of FcHyd5p was demonstrated using SDS-PAGE and immuno-staining with anti-6 x His-tag antibodies. FcHyd5p was purified and concentrated by dialysis, isoelectric focussing and the use of ultra filtration. It was demonstrated that FcHyd5p is able to change the hydrophobic properties of hydrophobic surfaces rendering them wettable after coating with the supernatant of recombinant *P. pastoris* cultures. Furthermore, due to its surface activity, FcHyd5p was able to stabilise air bubbles in aqueous solutions. The supernatant of a culture medium containing a FcHyd5p

producing *P. pastoris* clone remained turbid for 24 h and the foam stable for more than 72 h after the treatment with a homogeniser, whereas the liquid of the wild type strain clarified after 10 min and the foam disintegrated after 2 h. Finally it was demonstrated, that FcHyd5p can induce spontaneous over-foaming of carbonated liquids, referred to as gushing. Addition of 2 mg freeze-dried culture supernatant from an FcHyd5p producing *P. pastoris* clone resulted in a lost volume of 252 ml ± 20 per 500 ml of beer (50%) and 179 ml ± 7 per 330 ml of carbonated water (54%), respectively. Neither untreated beer/water, nor beer/water treated with freeze-dried culture supernatant from the wild type strain showed any gushing. Furthermore, addition of 215 µg highly purified FcHyd5p resulted in a lost volume of 77 ml ± 40 per 500 ml beer (15%).

Furthermore, the gene of AfpA was disrupted in *F. graminearum* in order to investigate its function for the fungus. The *F. graminearum* wild type strain was compared with *AfpA* knock out transformants for hyphal growth, surface hydrophobicity, conidium development as well as growth and infection mechanisms. A significant decrease by 10% and 8% were found in the rates of radial growth and submerged mycelial biomass of the transformants, respectively. No significant difference could be determined in the biomass, morphology and wettability of aerial hyphae produced. Foam produced by vigorous agitation of the cell free spent culture supernatants of the *AfpA* knock out mutants was significantly less stable then the foam of the wild type strain. Comparing the development of conidia, no significant difference in spore numbers was detectable but conidial length was significantly reduced in the disruption mutant. Furthermore, decreased germination rates of conidia were observed at a concentration of 6×10^5 spores/ml in the disruption mutants. Also a significantly reduced pathogenicity against wheat and barley seedlings was determined in *AfpA* knock out clones.

Furthermore, targeted gene disruption was compared with RNA silencing approaches to analyse the function of the alkaline foam protein A (AfpA) in *Fusarium graminearum*. Targeted gene disruption is the most established method to define gene function in filamentous fungi, but the characterisation of multicopy genes as well as essential genes makes the analysis difficult. For such genes RNA silencing can alternatively be applied. However, the varying degrees of silencing can make experimental results difficult to interpret. It could be shown that RNA silencing in *F. graminearum* was very effective. All significant differences, which occurred between knock out mutants and wild type strain, could be confirmed with the *AfpA* silencing transformants, whereas phenotypic differences between the silencing and the disruption of *AfpA* could not be found.

Furthermore, silencing of the *AfpA* gene was combined with silencing of the *polyketide synthase 12* gene (*PKS12*) as phenotypical marker in one co-silencing construct. The combination with PKS12 provided a simple method to detect transformants with an efficient gene silencing. This is the first time to demonstrate the usefulness of co-silencing of a pigment-coding gene for effective selection of silencing mutants in *Fusarium*.

6 Zusammenfassung

Oberflächenaktive Proteine haben ein breites Spektrum unterschiedlicher Funktionen für filamentöse Pilze und sind daher äußerst bedeutsam. Unter den oberflächenaktiven Proteinen sind die Hydrophobine die größte und auch wichtigste Gruppe. Sie spielen eine bedeutende Rolle bei der Ausbildung von Strukturen, die im direkten Kontakt mit der Luft stehen wie z.B. Luftmycel, Sporen und Fruchtkörper. Außerdem unterstützen Hydrophobine das Wachstum der Hyphen von der flüssigen Phase heraus in die Gasphase bzw. die Anheftung der Hyphen an hydrophobe Oberflächen, ein wichtiger Schritt während der Infektion. Aufgrund des amphiphilen Charakters sind Hydrophobine zudem in der Lage, Luftblasen in Flüssigkeiten zu stabilisieren sowie Eigenschaften von Oberflächen zu verändern. Aufgrund dessen werden Hydrophobine als mögliche Initiatoren von Gushing diskutiert, einem Phänomen, bei dem kohlensäurehaltige Getränke nach dem Öffnen spontan überschäumen. Da die Mechanismen dieses Phänomens noch nicht ausreichend verstanden sind, war das Ziel dieser Arbeit, das Hydrophobin 5 von *Fusarium culmorum* (FcHyd5p) in *Pichia pastoris* heterolog zu exprimieren, um für weitere Experimente größere Mengen diese Proteins zur Verfügung zu haben und somit die Bedeutung des Hydrophobins für das Gushing-Phänomen aufzuklären.

Einer weiteren Gruppe oberflächenaktiver Proteine aus Pilzen, den Fungispuminen, wurde ebenfalls ein Einfluss auf das Gushing-Phänomen nachgewiesen. Diese neu entdeckte Proteinklasse wurde aus Schaumfraktionen gewonnen, die aus zellfreien Kulturüberständen von *F. culmorum* stammen. Das “alkaline foam protein A” (AfpA) als Vertreter dieser Gruppe hat ähnliche Eigenschaften wie Hydrophobine, dazu gehören Oberflächenaktivität, Eingliederung in Gas-Flüssigkeit Interphasen, Hitzestabilität und die Fähigkeit, Aggregate auszubilden. Da jedoch bisher noch wenig über die Funktionen des AfpA für den Pilz bekannt ist, war es ein Ziel dieser Arbeit, mit Hilfe von AfpA knock out Mutanten mehr über die Rolle des AfpA zu erfahren.

Das Klasse II Hydrophobin FcHyd5p von *Fusarium culmorum* wurde in *Pichia pastoris* heterolog exprimiert und Transkription sowie Expression des rekombinanten Gens durch RT-PCR sowie SDS-PAGE und Western blot mit 6 x His-tag Antikörpern nachgewiesen. Die Aufreinigung des Hydrophobins FcHyd5p erfolgte unter Verwendung von Dialyse, isoelektrischer Fokussierung und Ultrafiltration. Es konnte gezeigt werden, dass FcHyd5p Eigenschaften von Oberflächen verändern kann. Hydrophobe

Oberflächen, welche mit dem Überstand einer FcHyd5p produzierenden *P. pastoris* Kultur benutzt wurden, waren anschließend hydrophil. Außerdem konnten aufgrund der Oberflächenaktivität des Hydrophobins Luftblasen in Flüssigkeiten stabilisiert werden. Der Kulturüberstand eines FcHyd5p produzierenden *P. pastoris* Klons, der mit Hilfe eines Homogenisators aufgeschäumt wurde, verblieb für 24 h trübe und der Schaum für mehr als 72 h stabil, wohingegen der Kulturüberstand des Wildtyps nach 10 min aufklarte und der Schaum nach 2 h zusammenfiel. Schließlich konnte gezeigt werden, dass FcHyd5p „Gushing", ein Phänomen, welches das spontane Überschäumen von karbonisierten Flüssigkeiten beschreibt, induzieren kann. Die Zugabe von 2 mg gefriergetrocknetem Kulturüberstand eines FcHyd5p produzierendem *P. pastoris* Klons führte zu einem Verlust von 252 ml ± 20 pro 500 ml Bier (50%) und 179 ml ± 7 pro 330 ml karbonisiertem Wasser (54%). Weder unbehandeltes Bier bzw. Wasser noch Bier bzw. Wasser, welches mit dem gefriergetrocknetem Kulturüberstand des *P. pastoris* Wildtyps behandelt wurde, zeigten Gushing. Weiterhin ergab die Zugabe von 215 µg gereinigtem FcHyd5p pro 500 ml Bier ein Volumenverlust von 77 ml ± 40 (15%).

Des Weiteren wurde das Gen von AfpA im Genom des Pilzes *Fusarium graminearum* zerstört, um mehr über die Funktion des Proteins für den Pilz zu erfahren. *F. graminearum* Wildtyp und *AfpA* knock out Mutante wurden miteinander in der Ausbildung von Hyphen, der Oberflächenaktivität, der Ausbildung von Konidien sowie im Wachstum und bei Infektionsmechanismen verglichen. Eine signifikante Abnahme von 10% wurde bei der Wachstumsrate und 8% bei der Masse des Substratmyzels festgestellt. Kein signifikanter Unterschied konnte bei der Biomasse, Morphologie und Benetzbarkeit des Luftmyzels festgestellt werden. Der Schaum des aufgeschäumten zellfreien Kulturüberstandes der *AfpA* knock out Mutanten war signifikant instabiler als der Schaum des Wildtyps. Bei der Zahl der Konidien zeigte sich kein signifikanter Unterschied. Die Länge der Konidien hingegen war bei *AfpA* knock out Mutanten signifikant geringer. Außerdem konnte eine geringere Keimrate der Konidien mit deletiertem AfpA bei einer Konzentration von 6×10^5 Sporen/ml festgestellt werden. Schließlich konnte gezeigt werden, dass *AfpA* knock out Mutanten eine geringere Pathogenität gegenüber Weizen und Gerste aufweisen.

Des Weiteren wurden *AfpA* knock out Mutanten mit Mutanten verglichen, deren *AfpA* Expression durch RNA Silencing inhibiert wurde. Der knock out von Genen ist die etablierteste Methode bei filamentösen Pilzen, um die Funktion von Genen zu analysieren, jedoch ist die Charakterisierung von mehrfach im Genom vorhandenen

Genen sowie essentiellen Genen mit dieser Methode schwierig. Für solche Gene eignet sich RNA Silencing. Allerdings kann die Effizienz des Silencing innerhalb der Klone variieren, was die Interpretation der Ergebnisse erschwert. Es konnte gezeigt werden, dass ein effektives RNA Silencing in *F. graminearum* möglich ist. Alle signifikanten Unterschiede, welche zwischen knock out Mutanten und Wildtyp festgestellt wurden, konnten durch RNA Silencing des *AfpA* Gens bestätigt werden, wohingegen keine signifikanten phänotypischen Unterschiede zwischen Silencing und knock out Mutanten gefunden werden konnten. Schließlich wurde die Polyketidsynthase 12 (*PKS12*) zusammen mit *AfpA* in einem Co-Silencing Konstrukt kombiniert. *PKS12* ist verantwortlich für die Synthese des Farbstoffes Aurofusarin in *F. graminearum* und wurde in diesem Konstrukt als phänotypischer Marker verwendet. Die Kombination von *PKS12* und *AfpA* erwies sich als besonders gut geeignete Methode, Transformanten mit einem effizienten Gen Silencing zu finden und konnte in dieser Arbeit zum ersten Mal für *Fusarium* etabliert werden.

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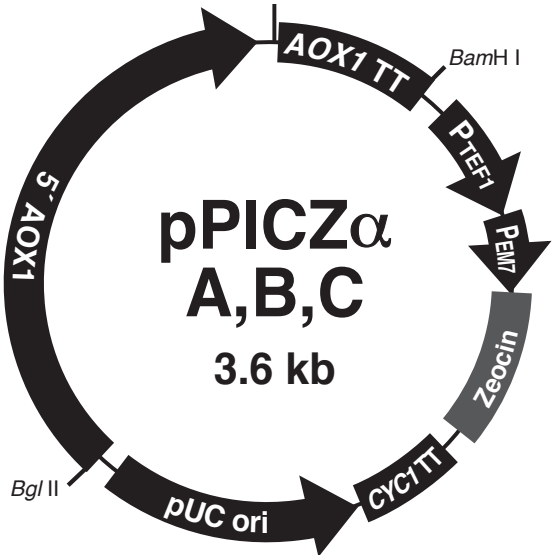
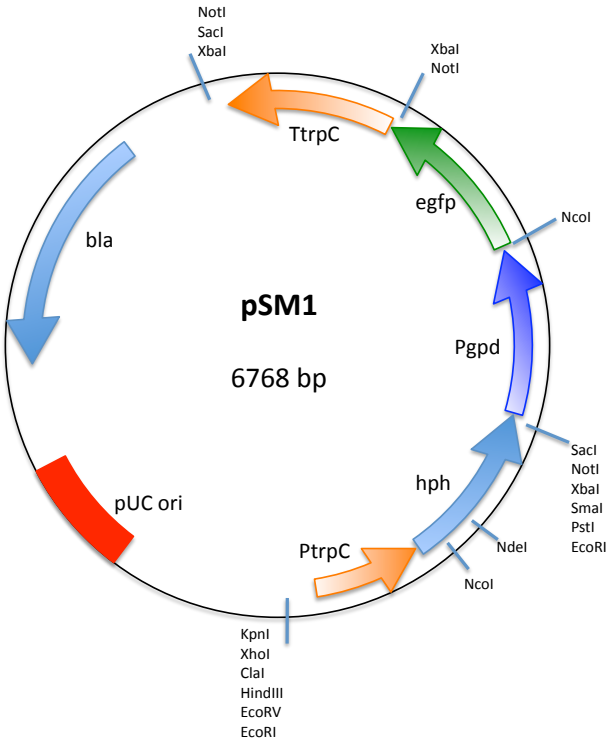
8 Appendix

8.1 Vector maps

8.1.1 pPICZαA



8.1.2 pSM1



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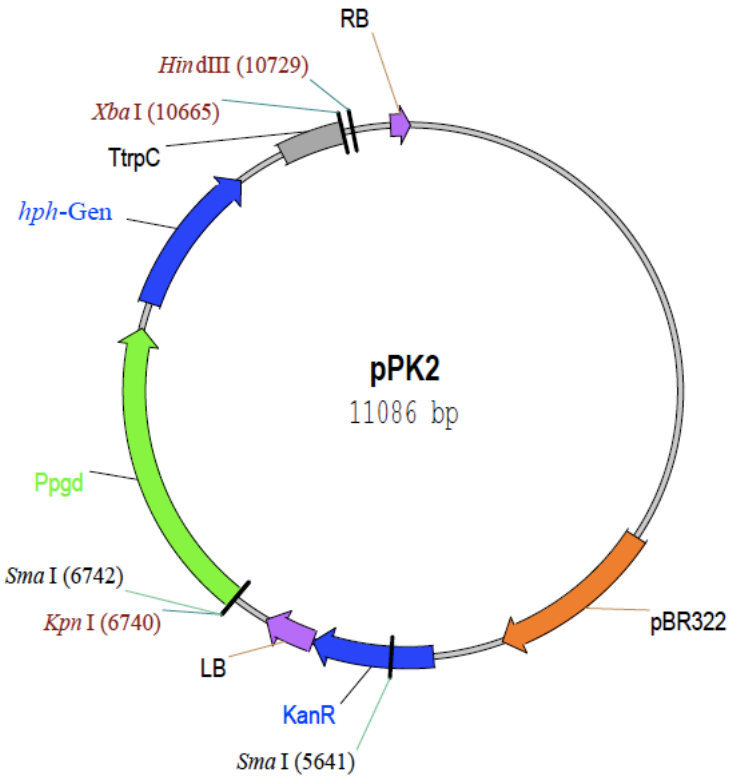
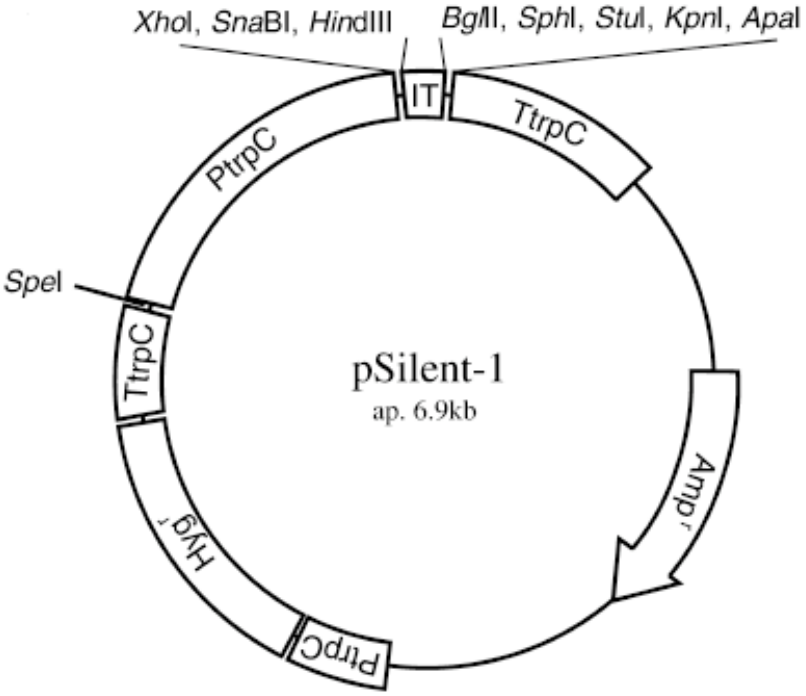


8.1.4 pSilent-1



8.2 Sequences

8.2.1 *FcHyd5* – genomic sequence (DQ449530)

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GCCTACATCCCTTGCAGTGGCCTCTACGGCACTTCCCAGTGCTGTGCTACTGATGTCTTGGGTGTTGCTGACCTC
GACTGTGGAAACCGTAAGTACTCGAGTCTGTCGATTGGAGATCAAGCTAACATGAACAGCCCCTTCGTCTCCCAC
CGACGCCGACAACTTCAGCGCTGTCTGCGCCGAGATCGGCCAGCGAGCTCGCTGCTGTGTTCTCCCCATCGTGAG
TTTATCTTTTGCATTGATGGTTAACATAACTAACATCTCTTCAGCTCGACCAAGGAATCCTCTGCAACACTCCTA
CTGGTGTCCAGGACTAA

8.2.2 *FcHyd5* – codon optimised for *Saccharomyces cerevisiae* (without intron):

ATGAAATTTTCTTTGGCTGCAGTTGCTTTGTTGGGTGCAGTAGTTTCTGCTTTGCCAGCTAACGAAAAAAGACAA
GCTTATATTCCATGTTCTGGATTATACGGTACTTCTCAATGTTGTGCTACAGATGTTTTGGGAGTTGCAGATTTG
GATTGTGGTAATCCACCATCTTCTCCAACAGATGCTGATAACTTTTCTGCAGTTTGTGCTGAAATTGGTCAAAGA
GCAAGATGTTGCGTTTTGCCAATTTTGGATCAAGGAATTTTGTGTAATACTCCAACAGGTGTTCAAGATTAA

8.2.3 *AfpA* from *Fusarium graminearum* (HM185071)

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8.2.4 *AfpA* from *Fusarium culmorum* (DQ336148)

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8.2.5 pPICZαA

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8.2.6 pSM1

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8.3 Publications

Stübner, M., Lutterschmid, G., Vogel, R. F., Niessen, L. (2010). Heterologous expression of the hydrophobin FcHyd5p from *Fusarium culmorum* in *Pichia pastoris* and evaluation of its surface activity and contribution to gushing of carbonated beverages. International Journal of Food Microbiology, 141, 110-115.

Lutterschmid, G., Stübner, M., Vogel R. F., Niessen, L. (2010). Induction of gushing with recombinant class II hydrophobin FcHyd5p from *Fusarium culmorum* and the impact of hop compounds on its gushing potential. Journal of the Institute of Brewing, 116 (4), 339–347.

Lutterschmid, G., Muranyi, M., Stübner, M., Vogel, R. F., Niessen, L. (2011). Heterologous expression of surface-active proteins from barley and filamentous fungi in *Pichia pastoris* and characterization of their contribution to beer gushing. International Journal of Food Microbiology, 147, 17-25.

Stübner, M., Lutterschmid, G., Vogel, R. F., Niessen, L. (2008). Heterologous expression of the hydrophobin FcHyd5p from *Fusarium culmorum* in *Pichia pastoris* and evaluation of its contribution to gushing. International Symposium for Young Scientists and Technologists in Malting, Brewing and Distilling. UCC. Cork. Ireland, poster presentation.

8.4 Lebenslauf

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