EXTRACELLULAR MATRIX PROTEINS IN GROWTH AND FRUITING BODY DEVELOPMENT OF STRAW AND WOOD DEGRADING BASIDIOMYCETES

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Summary

Fruiting body development in the homobasidiomycetous fungus *Coprinopsis cinerea* (*Coprinus cinereus*) is a complex process regulated by different environmental factors like temperature, light, humidity and nutrients. The combined action of various factors influences the mycelium growth and controls the formation of primordia and fruiting bodies. Within these multicellular structures, hyphae are closely attached to each other in a highly ordered manner. The extracellular matrix (ECM) surrounding the hyphae helps the fungus in multiple functions like surface attachment, cellular interaction and intercellular communication. The ECM proteins present in fungal cell walls and in the surrounding mucilage are thought to contribute to several of such functions during fungal development and differentiation. Hydrophobins and galectins are major groups of ECM proteins, which seem to an influential role in mushroom development. Previous studies revealed an interaction between galectins and hydrophobins of *C. cinerea* in *in-vitro* conditions, which raised many interesting question regarding their combined influence on growth and fruiting body development. Galectins are ß-galactoside carbohydrate binding proteins. Two fungal specific lectins i.e. galectins Cgl1 and Cgl2, have been described as molecular markers during the initial stages of fruiting body development. In this study, to understand the early stages of fruiting body development and to further analyze the localization of galectins, microscopy and reporter gene analysis were performed. Primary (small loosely attached hyphal aggregates) and secondary (compact globular 3-dimensional hyphal structures) hyphal knots were defined as the first stages of fruiting body development. Primary hyphal knots development was followed over the time and the influential role of light in the transition to secondary hyphal knots was determined. The *C. cinerea* laccase gene *lcc*1 under control of the promoters of galectin genes *cgl*1 and *cgl*2 was used to define places of primary and secondary hyphal knot formation in vegetative mycelium.

With the knowledge gained in understanding the early stages of fruiting body development in *C. cinerea*, a further focus was laid on the expression of hydrophobins at the stages where galectins were shown to be expressed. Hydrophobins are small fungal-specific proteins which are able to form amphiphatic films upon encountering different interfaces e.g. air-water interface. Hydrophobins are secreted at hyphal tips and the films formed by these proteins help fungi in reducing the water surface

tension thereby enabling the hyphae to emerge out from moist substrates. Furthermore, they cover outer fruiting surfaces and line air channels to protect the structures from water logging conditions and to enable gas exchange. Within the primordia developmental stage of *C. cinerea* where galectins are expressed, by protein analysis we identified four different hydrophobins (CoH14, CoH23, CoH24 and CoH25).

With the availability of genomic sequence of *C. cinerea*, a Blast search of the genome was performed with all known hydrophobins from other asco- and basidiomycetes, where 34 potential hydrophobin genes were found. This is the largest family of hydrophobins known so far in any fungus. Transcript analysis was performed for the deduced 34 hydrophobin genes using gene-specific primers. The expression patterns of multiple hydrophobin genes were analysed in different mono-, di- and homokaryotic mycelial samples and in various stages of fruiting body development by RT-PCR analysis. At advance fruiting stages, cap and stipe tissues were separately analyzed. In total, we found transcripts for 29 different hydrophobin genes expressed within the different strains and stages of *C. cinerea* development. Genes *coH*10 and *coH*32 were found to be mycelial specific whereas, transcripts for *coH*14, *coH*24 and *coH*25 were only seen in fruiting body specific tissues.

The surprising high number of hydrophobin genes in *C. cinerea* prompted the analysis of genomes of other basidiomycetes which are complete and publicly available. The same approach as used for screening *C. cinerea* genome was applied. In the well studied white-rot fungus *Phanerochaete chrysosporium,* 20 different hydrophobin genes were found, whereas in the ectomychorrhizal fungus *Laccaria bicolour* (genomic data is not published but restricted access has given to selected groups) 13 different genes, and in the maize pathogen *Ustilago maydis* two different genes were present. Interestingly, no hydrophobin gene was present in the genome of human pathogen *Cryptococcus neoformans*. In total, 68 potential hydrophobin genes were annotated from four different basidiomycete genomes.

In conclusion, this thesis gives a comprehensive analysis of different basidiomycete genomes and annotation of large number of hydrophobin genes with a main emphasis on *C. cinerea*. These studies pave a path for further studies on hydrophobins to understand physiological, ecological and developmental functions within fungi by applying modern molecular and proteomic techniques.

CHAPTER 1

Introduction

1.1 General introduction

Wood is a natural source used in constructions, paper industry, and in making products from wood such as furniture (Daniel 2003). As a biodegradable source, microorganisms may attack and degrade wood in order to utilize the organic materials to produce own biomass (Boddy and Watkinson 1994). Because of its ecological and economical importance, studies related to long term preservation of wood and protection against microbial attack is of much importance for the wood industry (Mai et al. 2004).

Wood is an organic material mainly composed of cellulose, hemicelluloses and lignin (Fengel and Wegener 1984). Microbial wood decay is defined as those significant changes in the physical and chemical properties of wood that are caused by the chemical (enzymatic) activities of the microorganisms (Kirk and Farrell 1987). Microorganisms break down the chemical complexes with the help of different enzymes (Leonowicz et al. 1999). The major groups of wood destroying organisms are fungi and certain bacteria, of which the wood-destroying basidiomycetous fungi are most intensively studied (Eriksson et al. 1990, Orth and Tien 1995). In spite of research over decades, the mechanism controlling the entry of wood-degrading fungi into living or dead wood has only poorly been elucidated (Gramss 1985). The major growth needs of wood inhabiting fungi are water, oxygen, a favorable temperature range, a digestible substrate, a favorable pH and chemical growth factors like nitrogen compounds, vitamins and essential elements (Zabell and Morrell 1992). Many fungi develop from minute airborne spores which can germinate on suitable substrate such as wet wood. The germinating spores produce hyphae, which collectively form a mycelium and start in this way colonization on wood. Subsequently, the hyphae penetrate into the wood, break down the wood cell walls and feed on cell wall components. Over the time, the invading fungi destroy the wood. Upon wood degradation under favorable environmental conditions, the mycelium may start forming fruiting bodies (Fig. 1) which on maturation release sexual spores into the environment, starting a new life cycle (van Wetter 2000).

Wood rotting fungi are mainly divided into two groups according to their effects on the wood. Brown rot fungi feed on cellulose as a wood cell wall component leaving a brown residue of lignin, the substance which holds the wood cells together. Infected wood will be greatly weakened, even before the fungi are recognized

Figure 1. Fruiting bodies of three white-rot species growing on straw block (left), tree branches and stems (middle and right) (modified from Hoegger and Kües 2006).

macroscopically. Advanced infestations of brown rot are evidenced by wood being more brown in color than normal, tending to crack across the grain. When dried, the infected wood will turn to powder, when crushed. In case white rots attack wood, they break down both lignin and cellulose causing the wood to lose its color and appear whiter than normal, therefore the name white rots. Wood affected by white rot normally shrink and collapse when severely degraded. Infested wood will gradually lose its strength and become spongy and defibred. White rot is a form of wood decay which results in bleaching of the wood. White rot basidiomycetes are especially important in the forest ecosystem since they are the only fungi capable of degrading all cell wall components (cellulose, lignin, and hemicellulose) of wood. Some fungi (white rot) may completely degrade the wood, producing weight loss up to 96-97%. Other fungi degrade only the carbohydrate portions of the wood cell walls and cause maximum weight losses of 60-70% (Eaton and Hale 1993).

Of the many white rot fungi, the best known and studied basidiomycetes are *Trametes versicolor*, *Pleurotus ostreatus,* and *Phanerochaete chrysosporium* (Fig. 1, Eaton and Hale 1993). In contrast, wood degradation by the white rot *Schizophyllum commune* is not at all understood (Peddireddi et al. 2005) albeit it is intensively used as a model species to study developmental processes in basidiomycetes. However, studies were now initiated with *S. commune* to follow hyphal growth on and in wood for examples with FTIR (Fourier transform infrared) spectroscopy (Naumann et al. 2005). One aspect of these studies addresses functions of hydrophobins, small fungal specific proteins (Wösten 2001, Wösten and Wessels 2006, see chapter I.2) in wood colonization and degradation (Peddireddi et al. 2005). Hydrophobins are present in the extracellular sheath of hyphae and are subject of study of this thesis (see below, for a

detailed review on hydrophobins please see chapter I.2 and for experimental work chapters 3 and 4).

Another model species in basidiomycetes is *Coprinopsis cinerea* (Fig. 2), which is a saprophytic fungus that grows naturally on horse dung but it also grows on straw and poorly on wood (Fig. 3, Rühl and Kües 2006, Navarro-González et al. 2003). Because of its short life cycle (two weeks under standard laboratory conditions), the possibility to perform Mendalian genetics and the ease of molecular genetic manipulations made the fungus a preferred model in basidiomycetes (Kües 2000). Accordingly, the genomic sequence of the fungus has been established by the Broad Institute (http://www.broad.mit.edu/ annotation/ fungi/coprinus_cinereus/) for use of the scientific community. Currently, there are three other genomes from basidiomycetes publicly available, i.e. of two heterobasidiomycetes, the maize pathogenic yeast *Ustilago maydis* (http://mips.gsf.de /genre/proj/ustilago/) and of the human pathogen *Cryptococcus neoformans* (http://www. broad.mit.edu/annotation/fungi/cryptococcus_neoformans/) and one homobasidiomycete, the white rot *P. chrysosporium* (http://genome.jgi-psf.org/ whiterot1/whiterot1.home. html; Martinez et al. 2004). However, this later species lacks laccase unlike most other white rot fungi (Gold and Alic 1993) and therefore can not serve as a standard model for white rot fungi. Furthermore, this species forms only a compact mycelium on the surface of wood as on organ for production of basidiospores (Breitenbach and Kränzlin 1986). For this reason, it is also not a model organism to study fruiting body development in basidiomycetes.

A more complex fruiting body consists of tightly aggregated hyphal cells arranged in distinct, differentiated twisted kinds of plectenchyma, i.e. interwoven hyphae in a tissue like appearance (Reijnders 1979). Fruiting bodies of various wood rotting fungi appear in bracket form with a more or less developed stalk on stems and branches of trees (Fig. 1). Other species including *C. cinerea* form fruiting bodies with the typical stipe and cap structures (Fig. 3). Fruiting body development in *C. cinerea* is most intensively studied for more than 100 years (Buller 1933, Kües 2000, Kües et al. 2004). Of all species, fruiting body development over the time is best described

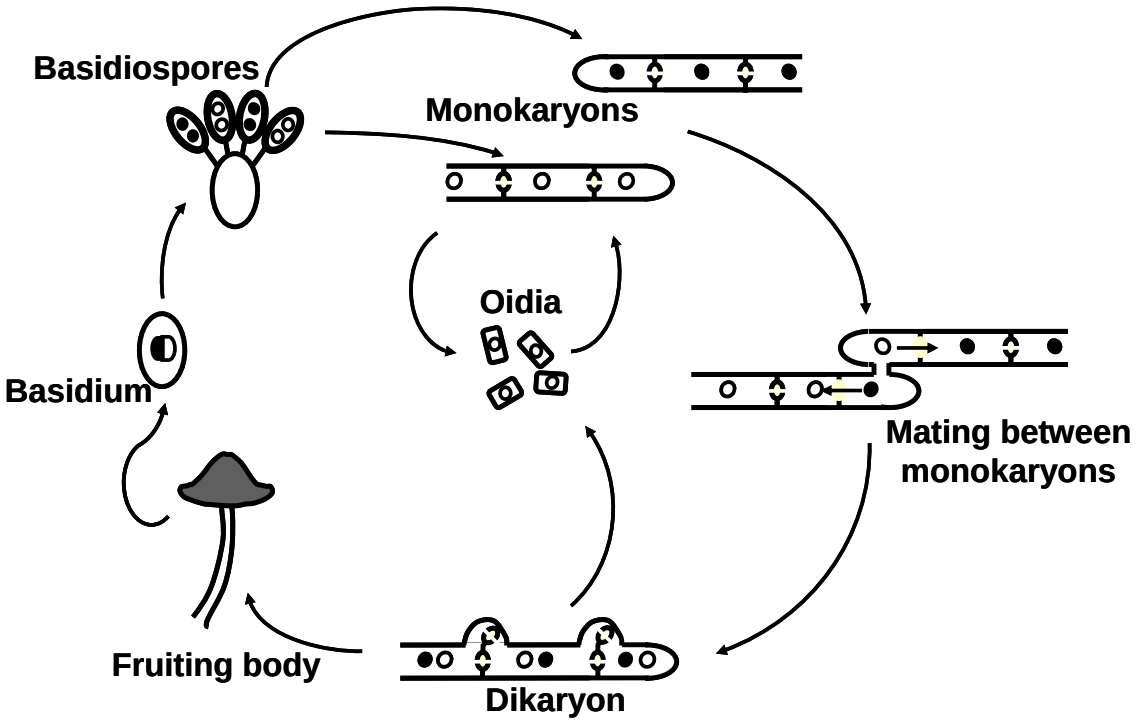

Figure 2. Life cycle of the model basidiomycete *Coprinopsis cinerea*. The life cycle starts with germination of the sexual haploid basidiospores into a mycelium with one hypha of haploid nuclei (monokaryon). If two monokaryons of different mating types (control mechanism of sexual compatibility between different strains) fuse, a dikaryon develops a mycelium with two haploid nuclei in the hyphal compartments, one from each monokaryotic parent. On the dikaryon, the fruiting bodies form. In the basidia, specific cells present on the surface of the gills of the fruiting body, karyogamy and subsequently meiosis occur to finally produce four basidiospores. On the monokaryon in a constitutive manner and on dikaryon in a light induced way, unicellular, uni-nuclear haploid spores (oidia) are produced (reproduced with kind permission from Kües 2000).

and thus, *C. cinerea* serves as the model species in basidiomycetes. Many environmental factors for the control of fruiting body development (light, temperature, humidity, nutrients and pH), and the major genetic control elements (mating type genes) are known (Kües 2000, Kües et al. 2002).

During fruiting body development, different extracellular proteins are expected to play a significant role in hyphal aggregation. Extracellular matrix (ECM) proteins secreted by fungal cells surround the hypha, may help in aggregating the hyphal structures, and also generally in attaching hyphae to surfaces (Walser et al. 2003). Within fruiting body tissues of *S. commune* a class of fungal specific extracellular small proteins called hydrophobins were first detected. Hydrophobins form amphiphatic films with hydrophobic and hydrophilic surfaces when exposed to different interfaces (eg. air-water). These hydrophobins are secreted by the hyphae

into the mucilage sheath that surrounds the hyphae, cover the emerging hyphae and help in aerial structure development in fungi. Particularly in fruiting bodies, they line air channels protecting them from water logging and therefore help in gas exchange within the fruiting body tissues (Wösten 2001, further details in chapter I.2).

Figure 3. Young fruiting bodies of *C. cinerea* grown on wheat straw as substrate (left) and on agar plate (right) (kindly provided by Wassana Chaisaena)

In *C. cinerea*, a mycelial specific hydrophobin CoH1 was isolated from vegetative mycelium. Unlike in the vegetative mycelium, expression of the gene (*coH*1) in the fruiting body was not detected (Ásgeirsdóttir et al. 1997). However, the specific function of hydrophobin CoH1 has still to be elucidated. Hydrophobins have been proven to be essential in hyphal colonization and penetration of plant material (e.g. leaves) and are postulated to have also a function in straw and wood colonization by the hydrophobic nature of lignocellulose cell walls and growth with vessels (Talbot et al. 1993, van Wetter et al. 2000, Peddireddi et al. 2005).

Apart from hydrophobins, galectins are also believed to be of importance in hyphal aggregations and inter-cellular communications. Galectins form a specific family of ß-galactoside binding lectins, which in animals are involved in cell growth, cell differentiation and regulation and in apoptosis (Cooper et al. 2002). Fungal galectins are well studied in *C. cinerea*, where two fruiting-specific galectins (CGL1 and CGL2) were isolated from fruiting bodies (Boulianne et al. 2000), their sugar binding capacities been elucidated and the crystal structural of CGL2 determined (Walser et al. 2004). The start of expression of galectins correlated with the early events in fruiting body development (primary and secondary hyphal knots, see further details in chapter 2). In the growing structure, the galectins are specifically found in the outer stipe and outer cap tissues that also contain galectin-specific ligands (Boulianne *et al*.

2000, Walser et al. 2005). In other species (*S. commune*, *Agaricus bisporus*, *Pleurotus ostreatus*), these tissues are shown to be specific for hydrophobin expression (Lugones et al. 1996, 1999, Penas et al. 1998). *In-vitro* analysis revealed an interaction between galectin CGL2 and hydrophobins isolated from fruiting bodies (Fig. 4), raising the possibility that such interaction also occurs *in-vivo* within the fruiting bodies (for further details see chapter I.2).

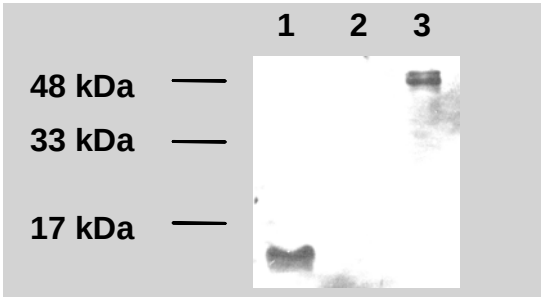

Figure 4. Western blot with isolated hydrophobins from *C. cinerea* fruiting bodies (1), horse radish peroxidase (2), and asilofetuin (3) treated first with galectins and secondarily with galectin antibodies (figure of unpublished results kindly supplied by Piers J. Walser). The band of approximate 14-15 kDa corresponds to typical sizes of hydrophobins indicating the interaction between hydrophobins and galectins.

1.1.1 Objectives of this thesis

Many basidiomycetes produce edible fruiting bodies and fruiting bodies with medical application. Commercial fruiting body production makes use of wood and straw as forest and agricultural wastes. Very few species are currently cultivated, mainly due to lack of understanding the processes involved in fruiting and how to manipulate these (Rühl and Kües 2006). Studies on *C. cinerea* are performed for easily gaining knowledge that subsequently might be possible to transfer to commercially interesting species that can be cultivated on lignocellulose waste.

The starting point of this thesis was the observation by Piers J. Walser that fruiting body specific galectins of *C. cinerea* interact with hydrophobins (Fig. 4). Little was known in *C. cinerea* on hydrophobins and the initial idea to this thesis was to identify and characterize hydrophobins from fruiting bodies, following the expression during development from early stages to fruiting body maturation and to study their interaction with the fruiting body specific galectins. However, due a surprisingly high number of hydrophobin genes in *C. cinerea* (a detailed description on the hydrophobin multi-gene family is given in chapter 3), the initial idea could not be followed up with the three year period of this PhD thesis.

The goals finally addressed in this thesis are listed in the following:

1. The early stages in fruiting body development are only poorly defined in literature (Liu 2001, Matthews and Niederpruem 1972, 1973 see chapter I.2 and chapter 2 for detailed descriptions on initial stages of fruiting body development). One objective was to define more exactly the onset of fruiting and to differentiate the morphology of the early stages by microscopy and reporter gene analysis making use of the promoters of the galectin genes *cgl1* and *cgl2*. This knowledge is needed for defining fruiting boy specific genes at the onset of hyphal aggregation for fruiting body development (see chapters 1.2, 1.3 and 2).
2. The second goal of the thesis was to isolate, purify and identify the hydrophobins from fruiting bodies by using suitable protein isolation techniques. Upon overcoming the unexpected difficulties in hydrophobin extraction by melanin contamination in later stages of fruiting bodies (see appendix Peddireddi et al. 2005) it became obvious that *C. cinerea* expresses more hydrophobins in the fruiting bodies as known from other basidiomycetes (Walser et al. 2003, see chapter-3). This was confirmed upon the release of the *C. cinerea* genome in June 2003 with the detection of the amazing number of 34 different hydrophobin genes (see chapter-3).
3. A third goal of this thesis concentrated therefore on expression analysis of all the 34 genes throughout vegetative and fruiting body development by RNA and protein analysis (details in chapter-4).
4. A fourth goal was to identify and annotate all hydrophobin genes in the available basidiomycetes genomes (including the genome of the ectomycorrhizal fungus *Laccaria bicolor* that was available since May 2005 to a few selected researchers) in order to study the evolution and development of hydrophobin multi-gene families in pathogenic, saprophytic, and white rot basidiomycetes fungi (chapter-5-general discussion).

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1.2 Extracellular matrix proteins in mushroom development

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The first two authors contributed equally to this review. Sections of the paper prepared by the author of this thesis: 1.2.1 Abstract, 1.2.2. Introduction, 1.2.5. Hydrophobins, 1.2.6. Other extracellular proteins from fruiting bodies, 1.2.7.Conclusion and future prospects, and respective parts of 1.2.9. Reference

1.2.1 Abstract

Mushrooms represent the most complex three dimensional structures that fungi produce and at the same time the most conspicuous manifestation of fungal differentiation. There are fundamental differences as to how fungi, on the one hand, and animals, on the other hand, go about constructing space-filling tissues. One of the most prominent features of fungi is the cell wall that gives hyphae their characteristic shape and appearance. The main difference between fungal hyphae and animal cells is that the former grows at the tip. This peculiar directionality leads to a characteristically fungal feature that requires individual hyphae to aggregate in order to form tissues that are then defined by a continuum of the cell wall, the interhyphal space or extracellular matrix (ECM). Thus, hyphal aggregation is regarded a prerequisite for tissue formation. The unity of cell wall and matrix ultimately joins one hypha to another. The gelatinous and hydrophilic matrix enables the propagation of molecules from one hyphe to its neighbour, therefore providing vital information on the hyphal environment, so very important since tissues emerge from pre-existing aggregates. Cell wall construction, maintenance and re-organisation are inseparably linked with differentiation of hyphae. Slowly, the players in the game are being identified and next to biosynthetic and lytic enzymes of the cell wall, two notable types of proteins are arising during differentiation: lectins, as potent elicitors and interpreters of cell-to-cell contacts exploiting the astronomical potential of glyco-coding, and hydrophobins, distinctly fungal proteins that upon aggregation form amphipathic films that confer the extraordinary hydrophobicity to certain fungal hyphae in emerging aerial mycelium and mushroom structures. The uncustomary nature of hydrophobins grants for many different facettes in function during cell wall assembly, protein trafficking through the cell wall matrix and rodlet formation on the surface. We here try to give an overview on the actions of these proteins with focus on mushroom development. However, much of our present knowledge is derived from studies with a few model fungal organisms that have and continue to lay the stepping stones into a vast field of research on the brink of dawn.

Keywords: ECMproteins-Hydrophobins-Galectins-Mushrooms-Fruiting body development

1.2.2 Introduction

The life cycle of higher basidiomycetes includes a homokaryotic and a dikaryotic mycelial growth phase. The homokaryotic or monokaryotic mycelium with one type of haploid nuclei arises from germination of meiotic haploid basidiospores, the dikaryotic mycelium with two different types of haploid nuclei by fusion of two homokaryons of different mating types (Casselton and Olesnicky 1998, Kües et al. 2002a). Mating types are determined by the *A* and *B* mating type genes. Homokaryotic mycelia are sexually compatible if they differ both in the *A* and *B* genes. The *A* genes encode two types of homeodomain transcription factors (HD1 and HD2), the *B* genes pheromones and pheromone receptors. For dikaryon development, HD1 and HD2 transcription factors of different *A* specificity have to interact and pheromones and pheromone receptors of different *B* specificity (Casselton and Olesnicky 1998, Hiscock and Kües 1999, Brown and Casselton 2001, Kothe 2001).

On the dikaryon, under defined environmental conditions, the mushroom or fruiting body develops. This represents the most complex aggregated structures known in the fungal kingdom. In the fruiting body, karyogamy and meiosis occur in basidia, specific cells found on the lower surface of the cap or, in case of puff balls, inside the mushroom. Usually four basidiospores bud from the basidia as the result of meiosis, closing the life cycle (Wessels 1993b, Kües 2000, Kües et al. 2004). Fruiting body development has been studied with the two model basidiomycetes *Coprinus cinereus* (Fig. 1) and *Schizophyllum commune*, and to some part with edible mushrooms such as *Agaricus bisporus*, *Lentinus edodes* and *Pleurotus ostreatus* (Wessels 1993b, Kües 2000, Kües et al. 2004, Umar and Griensven 1998, Kües and Liu 2000).

In *C. cinereus*, when nutrients are exhausted, development of a mushroom initiates at 25-28°C in the dark on one or a few neighboured hyphae with intense localised formation of short hyphal branches and higher order sidebranches, all characterized by short hyphal compartments and restricted tip growth. The resulting structure, a loose hyphal ball with yet little or no aggregation, is called the primary hyphal knot (Mattews and Niederpruem 1972, 1973, Boulianne et al. 2000, Liu 2001, Fig. 1a). When further kept in the dark, the primary hyphal knot matures into a sclerotium, a compact round to ovoid resting body with a dark rind of small melanized cells and an internal medulla of large, thick-walled, more or less spherical cells resembling chlamydospores (Kües et al.

2002b, Liu 2001, Waters et al. 1972, 1975). However, when a blue light signal is given, secondary hyphal knots arise within the cultures (Lu 1974a, Morimoto and Oda 1973, Tsusué 1969). Albeit cytologically not yet shown with a given structure, genetic evidence suggest primary hyphal knots to transform into the secondary hyphal knots which are compact aggregates of undifferentiated cells (Moore 1981, Kües et al. 1998, 2002b, Fig 1b). Further in development, cap and stipe tissues differentiate within the structure which now is called a primordium. On the outside, the primordium is covered by a veil of large, vacuolated cells. Primordium maturation takes about three days and needs further dark and light periods. During this time, in particular the gills differentiate with the basidia localized in the outermost gill layer called the hymenium (Kües 2000, Kües et al. 2004, Lu 1974b, Lu 1991, Moore et al. 1979, Moore 1998, Kamada 2002, Fig. 1 c-f). To induce karyogamy simultaneously within all the basidia and in parallel to initiate fruiting body maturation, another blue light signal is needed (Lu 1974a, Kamada et al. 1979, Lu 2000). Meiosis directly follows karyogamy (Lu 1974 (a, b), Lu et al. 2003). With proceeding meiosis, the stipe slowly begins to elongate, whilst the cap enlarges in size (Kües 2004, Liu 2001, Moore et al. 1979, Moore 1998, Fig. 1g, h). Subsequently, the veil ruptures. Within the still closed cap, basidiospores are produced and stain black by melanin incorporation at maturation. The stipe now rapidly elongates and the cap opens like an umbrella. The mature fruiting body is short-lived, existing merely for few hours. At the end of development, spores are quickly released by autolysis of the fruiting body (Kües 2004, Kües et al. 2004, Liu 2001, Moore et al. 1979, Kamada 2002, Iten and Matile 1970, Fig. 1i-p). As in formation of the dikaryon - under physiological conditions - mating type genes are crucial in fruiting body formation. *A* mating type genes of different specificity induce primary and secondary hyphal knot formation whilst heterologous *B* genes support the action of the *A* genes. Furthermore, *B* genes are needed at induction of fruiting body maturation. Development was found to arrest at the pre-karyogamy primordial stage when *B* genes of different specificity were lacking (Kües et al. 1998, 2002a, Tymon et al. 1992).

Fruiting body formation in *S. commune* also starts by intense local formation of short intertwined hyphal branches characterized by short cellular compartments (stage I). However, compared to primary hyphal knots of *C. cinereus*, these initial hyphal knots show little formation of higher order sidebranches. In *S. commune*, the short

hyphal branches tend to adhere to each other and grow in parallel upwards with their tips directed to the apical centre of the arising multicellular structure (stage II). Thus, the resulting structure resembles in shape a charcoal kiln or a corn shock and is called the stalk (Wessels 1993b, Leonard and Dick 1968, Schwalb 1978, van der Valk and Marchant1978, Raudaskoski and Viitanen 1982). Possibly, it is the analogue of the secondary hyphal knot of *C. cinereus*, albeit already formation of the stage I knot might positively be light-influenced (Raudaskoski and Viitanen 1982). Continuing development, hyphal cells at the periphery of the stalk partially swell, whilst the peripheral hyphae grow further inwards and attach more tightly together giving rise to a compact structure with a macroscopically visible dented centre. The resulting stage III structure is also referred to as apical pit. Gills are not visible albeit cellular hymenial differentiation commencing together with gill and basidia production. Only few such primordia will mature into the typical fan-shaped fruiting body by efferent opening which exposes the inner tissues and makes the gills macroscopically visible (stage IV). The young fruiting body continues to enlarge not by cellular inflation as in *C. cinereus* but by peripheral proliferation of hyphae (stage V). The fruiting body of *S. commune* is perennial. By cellular proliferation, over the time it may grow a few centimetres across, thereby continuously expanding the gilled surface. Unlike *Coprinus*, karyogamy, meiosis and spore production is not simultaneous. Onset of karyogamy, meisois and sporulation might be detected as early as stage III and can continue consecutively over years, but presence or absence of sporulation is not essential to any of the stages (Wessels 1993b, Leonard and Dick 1968, Schwalb 1978, van der Valk and Marchant 1978). Also in *S. commune*, the *A* and *B* mating type genes control onset of fruiting body development by regulating expression of fruiting specific genes (Wessels 1994b, section 4), but action of mating type products at later stages in development is not known.

In *A. bisporus*, fruiting body formation necessitates preliminary formation of thick mycelial cords. The mycelial cord is a first differentiated fungal tissue formed by an orderly, closely packed bundle of parallel growing hyphae. These adhere to each other by the virtue of a mucilaneous outer sheath that aids in creating a three-dimensional pseudoparenchymatous structure (Umar and Griensven 1998, section 2). Development of fruiting bodies initiates intimately linked to mycelial cords (Umar and Griensven

1997a), possibly by exploiting water and food resources present abundantly in form of glycogen in the cells of the mycelial cords and/or by communication of signals coming from differentiated tissues with a new gene expression profile compared to the vegetative individually growing, undifferentiated hyphae (Umar and Griensven 1998). Clémencon (1997) describes primary and secondary hyphal knot formation ("nodule" formation) as initial steps in fruiting that morphologically parallels the process in *C. cinereus*. On compost, within two days the nodules adopt a size of 2 mm (maximum of up to 6 mm), mainly by elongation. This developmental stage (called also "primordial undifferentiated stage") is characterized by somatic undifferentiated hyphal tissue enveloped by a universal veil. On day 3, first tissue differentiation is visible at the upper half of the young primordium as a result of cell death (variously called the "histo-organogenetic stage", "stage 1" or, following mushroom growers terms, "pin head"). Stipe and cap with primary lamellae are recognized on day 4 at a primordial size of 1 cm. As in *C. cinereus*, there is rapid stipe elongation by cellular expansion but also by some cell proliferation. Spores form on day 5 of development at a mushroom size of 5 cm, whilst the veil is still closed ("maturation stage", "stage 2" or "button stage" for mushroom growers). At the button stage, the cap to stipe diameter is about 2 to 3:1.At day 10 when the partial veil opens ("stage 3" or "cup stage"; "stage 4" when gills become visible from the outside) this relation increases to 3 to 4:1. A continuing process of spore formation and spore shedding takes place for at least the following 10 days ("spore-shedding stage"). Growth by cellular expansion continues till day 18 up to a cap size of 22 cm in diameter and a stipe to cap ratio of 4 to 6:1 ("open flats"). Around day 18, the "stage of senescence" starts, with introduction of small ruptures on the margins of the cap and central retractions, with tissue pigmentation. Due to tissue degeneration and break-down of mycelial cords at the point of fruiting body insertion, mushrooms dilapidate within further 18 days, marking the end of the fruiting body life-span (Umar and Griensven 1997 (a, b, c), Clémencon 1997, Flegg and Wood 1985, De Groot et al. 1996). Fruiting body development and associated tissue formations are so far best analysed in the three species described above. The processes are complex and by far not understood on the cellular level, let alone the molecular level. However, it is evident that cellular aggregation and interactions are central in all stages of fruiting body

development and first ideas on the molecular action of participating extracellular proteins are emerging.

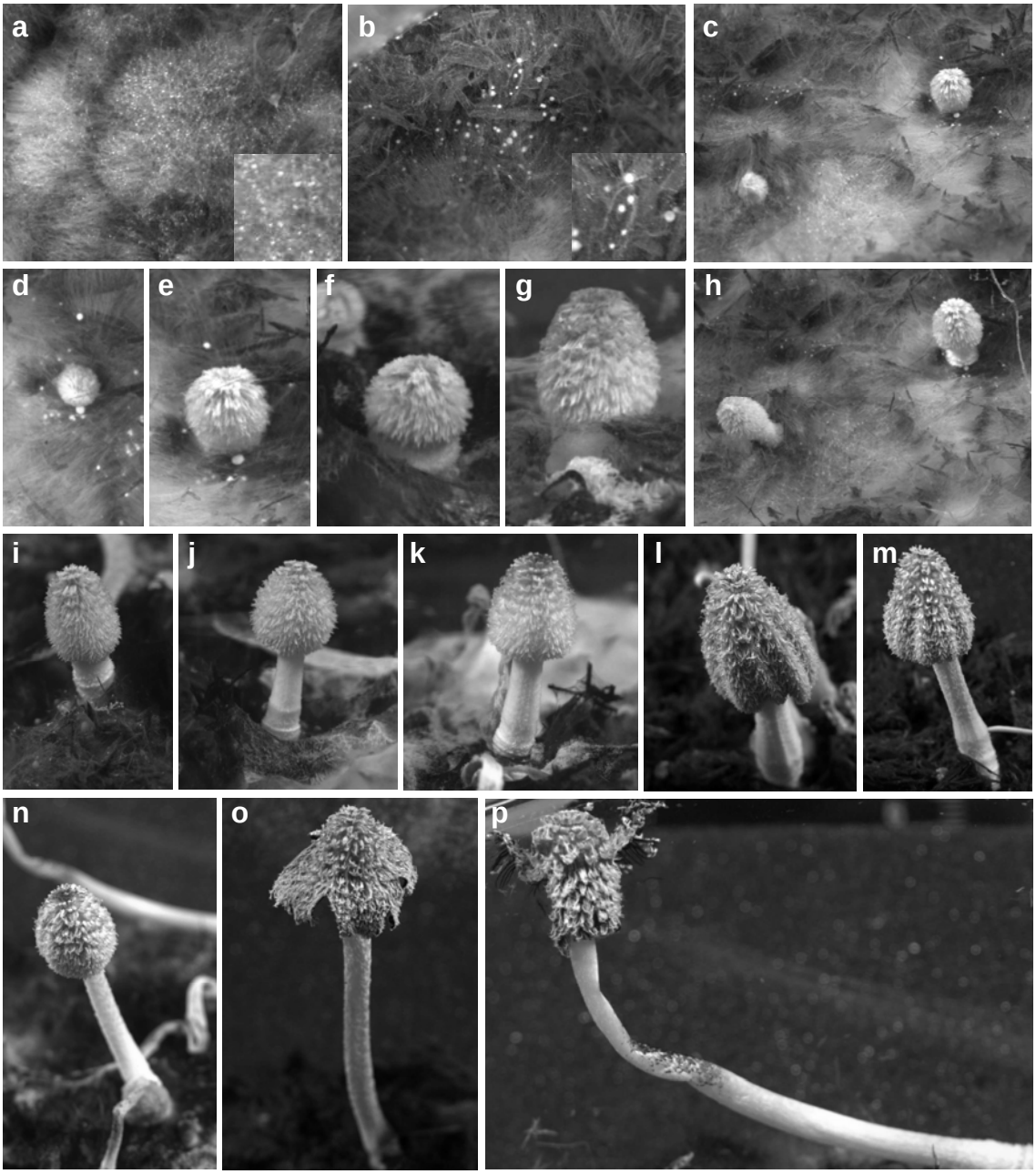

Figure 1. Fruiting body development in *Coprinus cinereus* strain AmutBmut on horse dung. Vegetative mycelium with primary hyphal knots (**B**) and with secondary hyphal knots (**A**), shown 2x enlarged in the insets. Primordia 1-day old (**D**), 2-days old (**C**,**E**). Fully developed primordium at karyogamy (**F**). Primordia at day 3 undergoing meiosis (**G**,**H**). (**C**) and (**H**) show the same primordia. Young slowly elongating fruiting bodies producing basidiospores (**I-M**). Rapid stipe elongation (**N**). Mature fruiting body with opened cap starting autolysis (**O**). Fruiting body collapsed due to autolysis (**P**).

1.2.3 The hyphal cell wall and aggregation

A crucial element in the development of fruiting bodies is hyphal aggregation, mediated by surface properties of the participating hyphal cells. Surface properties are determined by the characters of the cell wall and the surrounding extracellular sheaths. A prominent aspect of fungal cell walls is its architecture. By a combination of treatments with specific glycohydrolases, proteases and alkali, Hunsley and Burnett (1970) were able to demonstrate discrete layers in the ultrastructure of fungal cell walls from different fungal phyla using electron microscopy (Fig. 2). A given layer in the electron micrographs corresponded to mainly one type of polymer, with protein and β-glucan (α-1-3 linked glucose polymer) being dispersed between the layers. Within typical basidiomycetes, the skeletal structure of hyphal cell walls contains two types of structural polymers, chitin and β-glucan and interstitial components such as xylo-/galacto-/mannoproteins and α-1-3-glucan (Wessels and Sietsma 1981, Bartnicki-Garcia 1968, Fig. 2). Cell wall proteins are known to be covalently cross-linked to β-1-3-glucans (for reviews on this topic see (Sentandreu et al. 1994, Kapteyn et al. 1999, Smits et al. 1999, Stone and Clarke 1992), for an extensive review on fungal β-1-3-glucans see (Stone and Clarke 1992) and can therefore be considered integral structural elements of the wall matrix. Based on their solubility in alkali, glucans have been named S-glucan (soluble in alkali, equivalent to α-1-3-glucan in *S. commune*) and R-glucan (resistant to alkaline solubilisation, branched and cross-linked β-glucan in *S. commune*) (Wessels 1965). The S-glucan is mainly present beneath the water-soluble mucilage, but may also infiltrate other layers of the cell wall. R-glucan, similar to capsular polysaccharide in branching and linkages, but functionally very different, is restricted to the chitin-rich layer above the plasma membrane. Chitin is an unbranched polysaccharide of N-acetylglucosamine residues (GlcNAc) joined through β(1-4) linkages (Fig. 2). β-glucans, glucose polymers containing β1-3/1-6 linkages, are not present as individual polymers within the chitin-rich layer, but are covalently linked to each other and to chitin (Fig. 2), making the β-glucan insoluble in alkali and resistant to β-elimination (absence of a reducing end that would lead to degradation of the β-glucan in alkali) (Sietsma and Wessels 1979). It is thought, that precursors of the cell wall polymers are extruded at the hyphal apex where they elongate, become branched and cross-linked by transglycosylation, leading to the formation of chitin microfibril

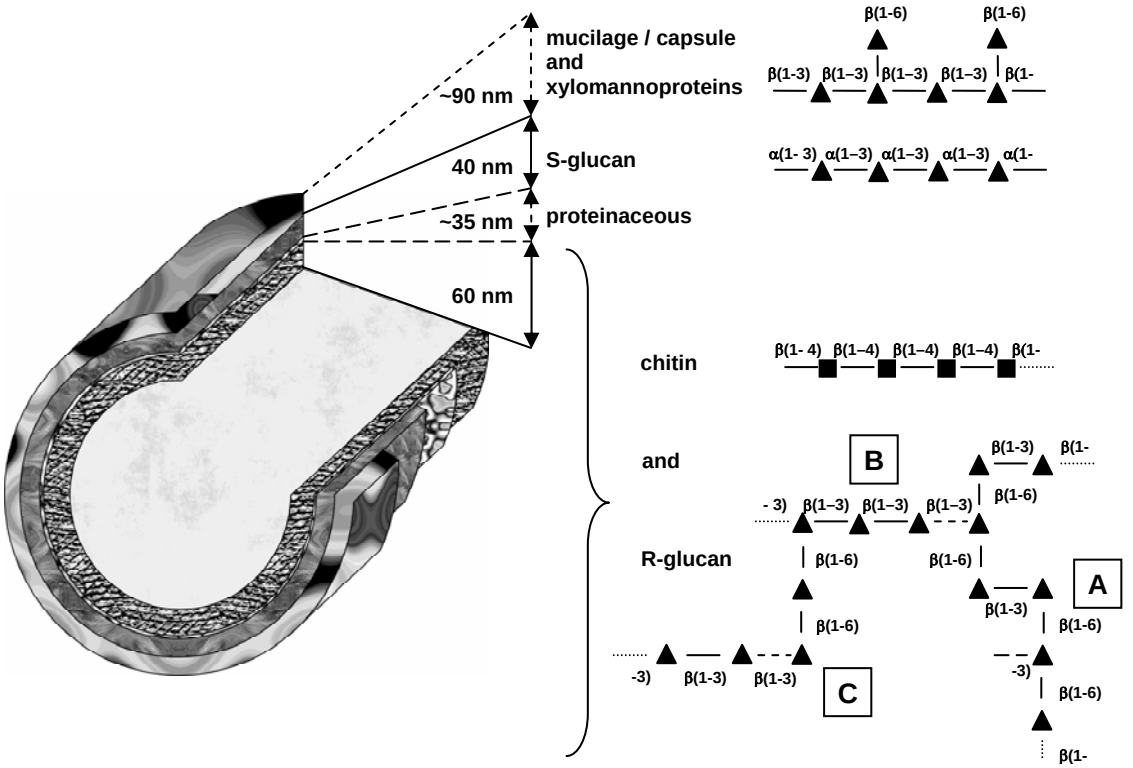

Figure 2. Layered structure of the basidiomycete cell wall [as proposed for *Schizophyllum commune* by Hunsley and Burnett [1970] and Wessels and Sietsma [1981]]. The innermost layer, adjacent to the plasma membrane, is composed of a rigid R-glucan-chitin complex, that also contains amino acids. This is followed by a layer of proteinaceous material, since protease treatment renders the innermost layer labile to attack by chitinase and subsequent extraction of the R-glucan. It is overlain and to some extent infiltrated by S-glucan that forms an outer, water-insoluble layer in the hyphal sheath, in parts microcrystalline to amorphous. To the exterior, the hyphae may present a gelatinous mucilage or capsule. This fraction is also known to contain xylose and mannose that may originate from interstitial glycoproteins. Approximate dimensions of these layers are given in nm, however, it must be noted, that this may alter significantly between species and also between cell types. Individual polymers may infiltrate different layers to varying extent, as indicated by dashed lines. Cell wall proteins are found in all layers. Illustrations of the glycans to the right are tentative approaches to the structure and linkages of the components. The length and branching pattern are expected to vary between specimens. Symbols: ▲, glucose; ■, N-acetylglucosamine. Dashed lines indicate possible cross-linking branches, dotted lines indicate continuation. (**A**) represents a mixed-linked β-glucan possibly attached to chitin. (**B**) shows a branched β1-3 glucan fragment with varying length of branches. (**B**) may be cross-linked to (**A**). (**C**) shows short β1-3-linked chains (varying in length) attached to β1-6 sidebranches of structure (**B**), but it may also be attached to (**A**).

attached to a glucan matrix (Wessels 1993a, Gooday 1995). This cross-linking process is thought to be mediated by activity of glucanosyltransferases that are located in/at the plasma membrane. As a consequence, rigidity of the cell wall increases with distance from the apex. Putative cross-linking proteins in the cell wall have been postulated for ascomycetes (for further reading see Chaffin et al. 1998, Popolo and Vai 1999).

A delicate balance between synthesis and breakage of cross-links and even whole polymers is hypothesised to enable the plasticity and morphological changes of the cell walls (Kamada et al. 1980). Relative percentages of cell wall polymers seem to differ significantly between monokaryotic and dikaryotic mycelia of *C. cinereus* (Marchant 1978) and during morphogenesis of fruiting structures (Kamada and Takemaru 1977a, 1983) or, not surprisingly, between species of fungi (Sietsma and Wessels 1981). Morphological differentiation of cell wall fibrils has been reported in relation to fruiting body development in *C. cinereus* (Kamada et al. 1991) and *A. bisporus* (Mol and Wessels 1990, Mol et al. 1990). The vegetative dikaryotic of *C. cinereus* hyphae show a random arrangement of chitin fibrils, whereas already in the secondary hyphal knot stage, chitin fibrils organise into either left- or right-handed helices. The amount of chitin present was shown to drop significantly in secondary hyphal knots and to increase again in later stages, parallel to an increase in transversal arrangement of chitin fibrils, and rearrangement to lesser degree of orientation (i.e. to random distribution of orientation relative to the hyphal axis) (Kamada and Tsuru 1993). Mol and co-workers (1990) presented a model of diffuse extension of the cell wall structure in *A. bisporus* fruiting body stipes based on electron-microscopic observations. Axial expansion (i.e. tip growth, polarised growth) was attributed to randomly organised chains of glucosaminoglycan (acid hydrolysis product of chitin) strongly interacting with an amorphous matrix in case of vegetative hyphae, versus transversal organisation in case of cell walls in stipe tissue. Exo-β1-3-glucanase treatment of (alkali extracted) stipe cell walls lead to complete axial contraction, indicating weak axial interactions of the chitin microfibrils, whereas vegetative walls retained randomly oriented microfibrils. Thus, isotropic growth of hyphae seems possible if the network of the cell wall polymers is loosened (or not rigidified in the first instance) and can be re-oriented. These data indicate that tissue re-modelling of fungi, as is the case during hyphal knot maturation, is accompanied by vigorous re-arrangement of cell wall polymers that give the fungal hyphae their characteristic shape.

Much of our current understanding of the fungal cell wall, particularly cell wall proteins, is based on experiments conducted with ascomycetes, usually *Saccharomyces cerevisiae* or *Candida albicans* (for reviews on this topic see (Kapteyn et al. 1999, Chaffin et al. 1998), for a comprehensive review on cell wall organisation of pathogenic

fungi including the importance in interaction with the host see Munro and Gow 2001. However, there seem to be themes and basic concepts shared between fungi (e.g. cross-linking), indicating that some model cell walls may well be appointed when trying to define variations on motives common to all fungal cell walls. Fungal cell wall proteins have been grouped based on their solubility (see Table 1). It has become evident that there is a large degree of cross-linking between cell wall proteins themselves and the matrix (Lipke and Ovalle 1998). Cysteine-rich proteins seem to create a disulphide-bonded layer within the cell wall that forms an efficient diffusion barrier to molecules of Stokes radii (i.e. the effective radius a hydrated molecule has in solution due to its rotation) equivalent to 400 kDa globular proteins or poly-cations with large hydrodynamic radii (such as DEAE-dextran). Treatment with EDTA had a similar effect as reducing agents on permeability of the cell wall of *S. cerevisiae*. It is hypothesised that the scavenging of divalent cations from salt-bridges between polyphosphates on mannans of yeast mannoproteins or anionic stretches on proteins were responsible for the observed increase in porosity (De Nobel et al. 1989, 1990). A functional role of disulphide layers implies the action of disulfide isomerases in cell wall construction since arrays of intermolecular disulfides are expected to form extracellularly. In analogy to this diffusion barrier, chitinase or zymolyase (glucanase) was observed to only be effective if the proteinaceous shield surrounding the fibrillar layer above the plasma membrane has been attacked by proteases (Hunsely and Burnett 1970, Zlotnik et al. 1984). In accordance with this, cell wall proteins seem to be heavily glycosylated which in turn partly protects them from proteolytic attack. Of the various types of protein present in fungal cell walls, functions in cellular aggregation are only slowly emerging.

Formation of a mycelial cord has been postulated the first step in tissue formation in fruiting body development of *A. bisporus* (Umar and Griensven 1997b, c, Clémencon 1997). During the initial steps of differentiation, excretion of vast amounts of mucilage is observed. This primes the contact between individual hyphae by creating a continuum hitherto referred to as the extra-cellular matrix (ECM). It is clear that the place of contact between two hyphal cells is this semi-liquid glucan layer surrounding the S-glucan (Fig. 2). Many mushroom hyphae have a characteristically hydrophobic surface, especially those exposed to the outside or air-filled cavities (see section 4). Hyphae in the interior of tissues, however, are hydrophilic with respect to their surface properties.

It has been postulated that a hydrophilic interhyphal environment (or interhyphal space, IHS), such as observed in fruiting bodies of *A. bisporus*, would be a prerequisite for tissue formation, since this should permit the propagation of (water-soluble) signals from cell-to-cell-neighbours, as against diffusion through the hyphal coenocytium or syncytium due to frequent anastomoses, which is also possible, but hardly vectorial (Umar and Griensven 1999). Hormone-type substances have been extracted from fruiting bodies of various species (Kamada et al. 1980, Novak Frazer 1994). The nature of such "morphogens" is however mostly unkown, but of great interest from a mechanistic point of view of tissue construction (Umar and Griensven 1999). Association of Schizophyllan (the high molecular weight gelatinous β1-3 glucan from *S. commune* with single β1-6 branches) with the *Schizophyllum* hydrophobin SC3 (small protein that forms amphipathic films on air exposed hyphal cells rendering them hydrophobic; section 4) was demonstrated by Martin and co-workers (Martin GG et al. 1999, 2000). Attachment to the polymer lead to stabilisation of small SC3 oligomers, whereas enzymatic or chromatographic removal of the polysaccharide resulted in immediate phase separation by aggregation of large protein multimers. It was therefore concluded that the β-glucan was necessary to catalyse amphipathic film formation in an aqueous environment, since the protein aggregates in a concentration dependent matter and film formation is in consequence drastically reduced (Martin et al. 1999, 2000). Mutants unable to produce hydrophobin SC3 on the other hand, show distorted cell wall formation with an increased mucilage production (van Wetter et al. 2000) indicating an influence of hydrophobins on cell wall and ECM structure. The exact role of cell wall polymers and hydrophobin assembly is however not understood to date. It is clear that the monomeric hydrophobin (or small multimers) must transverse the cell wall in order to reach the surface (Wösten et al. 1994a). Different roles of constantly changing cell wall polymers (continuous assembly and cross-linking) and, in addition, distinct oligomeric states of aggregating proteins make this process in cell wall assembly highly interesting but at the same time experimentally very challenging to handle (section 4) Fimbriae, fine surface hairs, in the heterobasidiomycete *Microbotryum violoaceum* are made up of collagen-like glycoprotein subunits of 74 kDa and surprisingly of 30 bp

Table 1. Characterisation of fungal cell wall proteins (CWP) into groups based on the mode of extraction from the cell walls

Protein type	Extractable	Examples & Reference
Soluble	Aqueous solutions	Lectins, see section 3
Detergent extractable	Mainly boiling in 2 % SDS (will also be extracted by chaotropic agents)	many yeast mannoproteins (Valentin et al. 1984)
Disulphide-linked to cell wall components	Reducing agents (β-ME, DTT)[c]	Yeast a-agglutinin Aga2p (Cappellaro et al. 1994)
GPI-remnant linked to cell wall components (GPI-CWP)[a]	After treatment with β-glucanases, phosphodiesterases or HF[d]	Yeast α-agglutinin (Lu et al. 1994, Kapteyn et al. 1996)
Pir-Proteins[b]	Mild alkaline treatment (30 mM NaOH)	Yeast Ccw5p to Ccw8p[e] (Mrša and Tanner 1999)
Hydrophobins	TFA, formic acid	Class I hydrophobins, see section 4
Transglutaminated	Only in combination with one of the above	Exogenous and endogenous substrates (Iranzo et al 2002)

[a] GPI: glycophosphatidylinositol. [b] Pir: proteins with internal repeats. [c] β-ME: β-mercaptoethanol; DTT: dithiotreitol. [d] HF: hydrofluoric acid. [e] Ccwp : covalently linked to cell wall.

single-stranded RNA molecules of yet unknown function (Celerin et al. 1994, 1996). Collagen is the principal component of the animal extracellular matrices in connective tissues of animals (for a review on collagens see Van der Rest and Garrone 1991). Digestion of *M. violaceum* fimbrial protein with Peptide-N-glycosidase F yields a 47 kDa aglycone (Celerin et al. 1995). Mannose is the predominant sugar since α-mannosidase treatment produces almost completely deglycosylated protein (Castle et al. 1996). Antibodies raised against the denatured fimbrial subunits but not antibodies raised against the native fimbriae reacted with fimbrial preparations of other fungi including *C. cinereus* and *S. commune* (Celerin et al. 1995, Castle and Boulianne 1991). This indicates a conserved protein structure in fungal fimbriae on the one hand, and species specific modifications such as unique glycosylation pattern on the other, e.g. which would function in distinct cell-cell recognition or interaction. Fimbrial-dependent cell-cell interaction in mating of *M. violaceum* could be demonstrated by dose dependent inhibition through α-fimbrial antibodies. Furthermore, the mannose-specific lectin Concanavalin A inhibited mating, whereas wheat germ agglutinin (recognising N-acetylglucosamine (GlcNAc) and β-linked polymers of such, e.g. chitin) did not have adverse effects on mating. Simple monosaccharides were also effective in inhibition, confirming that a lectin is involved in cell-to-cell attachment or recognition in the mating process (Castle et al. 1996).

1.2.4 Mushroom lectins

Lectins or agglutinins are a widespread type of carbohydrate-binding protein, comprising several protein classes. Historically the term was coined for proteins of legume origin as agglutinins of erythrocytes, but in the meantime, proteins with lectin properties have been isolated from most kingdoms and the definition of "a lectin" has been adapted (Goldstein et al. 1980). The study of lectins in fungi started in 1907/10 with toxicological investigations into to presence and activity of hemolytic agglutinins encountered in mushroom fruiting bodies that were frequently found amidst edible species (Ford 1907, 1910). To date, a vast number of lectins has been purified and partially characterised from vegetative fungal mycelia and from fruiting structures in particular. The prevalence of lectins from fruiting structures might reflect, however, the ease of accessing mushroom tissues and the difficulties in obtaining pure vegetative mycelium from natural sources. Guillot and Konska (1997) and Wang et al. (1998a) made exhaustive attempts to summarise the data on mushroom lectins. Here, we will focus on lectin physiology with particular interest in mushroom development.

By nature of their function, lectins are expected to be extracellular, with the exception of lectins involved in fundamental intracellular aspects of eukaryotic cells such as glycoprotein processing (Schrag et al. 2003), regulation of cell cycle and apoptosis, or even nuclear functions in pre-mRNA splicing (Liu et al. 2002). Many extracellular functions, e.g. binding to ECM, cell-to-cell adhesion have been proposed for animal lectins (for a recent overview of animal lectin function see Kilpatrick 2002). Some possible roles of lectins have also been ascribed to interactions of the fungal organism with its surrounding, e.g. symbiotic or parasitic relationships or predation (Table 2).

There are fundamental differences as to how mushrooms build tissues in contrast to animals or plants. As described in section 1, the mushroom fruiting body is constructed within a globular structure, the hyphal knot, built from a seemingly disordered mycelial mass that gives rise to the primordium. The main difference to animals is that virtually all cells of the primordium are totipotent (Money 2002). Thus, tissues do not originate from meristematic stem cells (e.g. epidermal stem cells), but rather differentiate from existing or growing hyphae, intimately linked to the unique nature of apical cell growth of fungi (Reijinders 1993). This feature requires exactly concerted mapping of where

Table 2. Lectins implicated in interactions of fungi with other organisms

Relationship	Interaction	Evidence	Reference(s)
Symbiotic	Fungal-plant (mycorrhizae)	Cell wall localisation by indirect immunofluorescence; in situ binding of lectin to plant root hairs	(1)
	Fungal-algae (lichen)	In situ binding of lectin to phytobiont	(2)
Predatory	Fungal-nematode	Hapten (GalNAc) inhibition of nematode capture; inhibition of capture with lectin pre-incubation	(3-5)
Defensive	Fungal-virus	Heterologous, antiviral activity against tobacco mosaic virus	(6)
Parasitic	Fungal-fungal	Growth phenotype of mycoparasite on lectin-coated nylon fibres	(7)

(1) Giollant et al. 1993, (2)Petit et al. 1982 , (3) Nordbring and Mattiasson 1979, (4) Jasson and Nordbring 1984, (5) Balgh et al. 2003, (6) Sun et al. 2003, (7) Inbar and Chet 1992

and when which type of cell should be formed. Therefore, all cells differentiating must gain vital information of their surrounding. Due to the enormous combinatorial potential of glyco-codes, lectins appear exquisitely suitable to mediate or interpret inside-out signalling of cells or appropriate cell-cell contacts (Gabius 2002). The research into lectins of mushroom origin has so far been mostly restricted to purification, usually by means of affinity chromatography with the carbohydrate of interest, and characterisation by the oligosaccharide specificity of the obtained lectins. Next to traditional purification and hemagglutination assays, a screening method for novel lectin specificities (from mushroom extracts, but equally applicable to any lectin source) has recently been presented, applying hybrid glycoproteins and neoglycoproteins (Matsumoto et al. 2001). The main focus has historically been set on lectins that would be suitable as probes for histochemical detection or clinical investigations such as blood group typing Veau et al. 1999, for review in Blood Group reactivity see Wang et al. 1998a), recognition of transplantation antigens (Winter et al. 2002) and cancer (Irazoqui et al. 1999, Wang et al. 2000). Compared to the impressive number of mushroom lectins isolated and (partially) characterised, the physiological data concerning function of these proteins are at best meagre and restricted to a handful of studies. Lectins have been implicated in growth and morphogenesis of mushrooms. Main evidence for this arises from studies with *C. cinereus* galectins, *L. edodes* lectin and *Pleurotus cornucopiae* lectins. A lectin was found to appear in *Lentinus* mycelia mainly during formation of "brown films" after the originally white mycelium gets pigmented and before encrustation of the

"brown skin", typical of Shiitake before primordia appear (Tsivileva et al. 2001). Lectin activity decreased significantly after this developmental stage during primordial maturation. Kaneko and co-workers (1993) found a lectin that appeared concomitantly with the primordial stage of *P. cornucopiae* and continued to increase in expression (as judged by hemagglutination) during primordium maturation. This lectin activity was later referred to as PCL-F and shown to exist functionally in three forms: 16 and 15 kDa isolectins, subsequently called PCL-F1 and PCL-F2, which form homodimers of around 32 and 30 kDa, respectively, and a heterodimer (Yashida et al. 1994, Iijima et al. 2002). Prior to fruiting, PCL-M appears in cultures grown on solid medium and then decreases in expression during the stages of primordia formation and maturation (Oguri et al. 1996). Mycelia grown in submerged cultures did not show lectin activity nor binding of erythrocytes to hyphae, in contrast to mycelia from solid media. This binding was stated to be inhibited by asialofetuin, EDTA or α-PCL-M antibodies. Dikaryotic, but not monokaryotic mycelia produced the lectin. Western blot analysis revealed stage specific expression of both, PCL-M and PCL-F lectins. Interestingly, antibodies did not cross-react, indicating that the two lectins are immunochemically distinct. It was therefore suggested, that PCL-activity is involved in stage specific steps of fruiting, PCL-M at the onset of fruiting and PCL-F during later stages. Furthermore, the two lectins seem to differ in their carbohydrate recognition. A PCL-F deficient strain (KC-2) of *P. cornucopiae* has been generated and it shows no phenotype in fruiting. The function of these developmentally regulated lectins thus still remains elusive. PCL-F1 shows extensive sequence homologies (52%) with a lectin isolated from *A. bisporus* fruiting bodies (ABL) (Presant and Kornfeld 1972, Crenshaw et al. 1995, Fig. 3).

ABL from *A. bisporus* is one of the most extensively characterised mushroom lectins with respect to carbohydrate binding and therapeutic properties (Irazoqui et al. 1999, Sueyoshi et al. 1985, for review see Wang et al. 1998a). ABL (tetramer with 16 kDa subunits) binds β-galactosides of the Thomsen-Friedenreich-type (Gal β1-3GalNAc) and cannot be inhibited by galactose and only very poorly by β-galactosides with glucose or N-acetylglucosamine at the reducing end (lactose-type). Both lectins show very little homology (ABL 29%, PCL-F 21%) to another β-galactoside binding lectin, CGL2, isolated from *C. cinereus* (Cooper et al. 1997). Merely the tryptophane residue - essential for galactose recognition (Weis and Drickamer 1996, Elgavish and

Shaanan 1997) - in the carbohydrate recognition domain (of CGL2, putatively of ABL and PCL-F) is conserved between these three lectins (Fig. 3). Table 3 shows a summary on β-galactoside binding lectins from mushrooms.

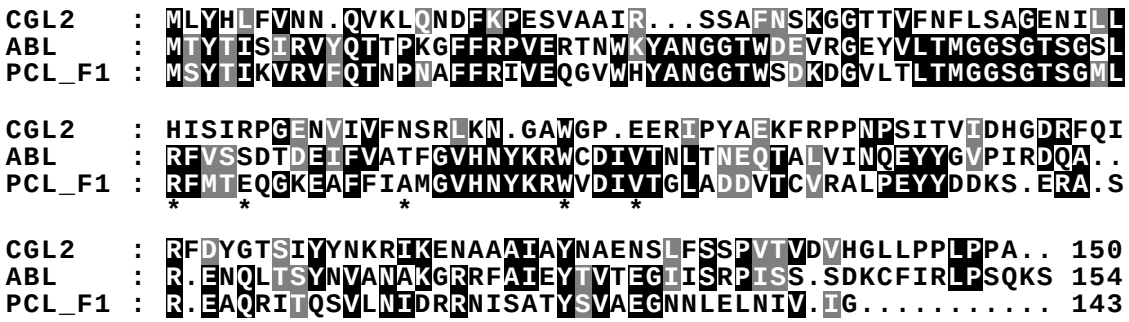

Figure 3. ClustalW alignment of β-galactoside binding lectins from *Coprinus cinereus* (CGL2), *Agaricus bisporus* (ABL) and *Pleurotus cornucopiae* (PCL F1). ABL and PCL F1 share extensive sequence homology, whereas CGL seems to be distinct. Sequences with dark shadings represent identical residues shared by at least 50% of the sequences. Light shaded residues represent conservative substitutions. Asterisks mark residues known to interact with the □-galactoside in galectins (carbohydrate recognition domain). ClustalW: http://www.ebi.ac.uk/clustalw.

Galectins are a large family of β-galactoside binding lectins, originally isolated from the animal kingdom (Barondes et al. 1994). Similar to their distribution, very widespread functions have been ascribed to galectins (Perillo et al. 1998). They are characterised by conserved amino acids in the sugar binding site and usually form canonical dimers, either covalent with a linker peptide or non-covalent by protein dimerisation. The *Coprinus* galectins have been shown to exist as functional dimer (Cooper et al. 1997) or higher order multimer (Walser et al., in preparation). Boulianne and co-workers (2000) demonstrated developmental expression of two isolectins, CGL1 and CGL2. The latter was expressed within aerial mycelium in a "fruiting-competent" outer zone (where one finds primordia in later stages) of the mycelial culture. No or very little galectin was detected (by Western blot analysis and RT-PCR of transcripts) in younger cultures. With the onset of fruiting development, CGL2 was detected in fruiting as well as in non-fruiting zones. Conditions that inhibit mushroom development such as constant light, non-functional *A*-mating type proteins or carbon and/or nitrogen repression also abrogated galectin expression. Furthermore, formation of hyphal knots (inhibited by constant light) also correlated with galectin transcription and protein expression. CGL1 transcripts and protein appeared during later stages of development

Table 3. β-galactoside binding lectins isolated from mushrooms: physico-chemical properties

Mushroom	Lectin name[a]	M_r (kDa), oligomeric state[b]	Type of oligomer[c]	Glyco-sylated	pI	Reference (s)
Agaricus bisporus	ABL	16 tetramer	A_4	Yes	5.5–6.7	(1)
Agrocybe aegerita (cylindracea)	ACG - AAL	15 dimer 16 & 15 15.8 dimer	A_2 A_1B_1 A_2	No - No	5.0[e] - 3.8	(2) (3) (4)
Amanita pantherina	APL	22 dimer	A_2	Yes	-	(5)
Auricularia polytricha		23 monomer	-	Yes	10.7	(6)
Coprinus cinereus	CGL1 CGL2	16.5 dimer	A_2 (A_1B_1?)	No	9.3[e]	(7)
Flammulina velutipes	FVA	11 dimer	A_2	No	5.4	(8)
Ischnoderma resinosus	IRA	16 dimer	A_2	Yes	~ 5	(9)
Lactarius deliciosus	LDL	18 and 19 dimer	A_1B_1	-	6.7	(10)
Lactarius deterrimus	lDetL	18 dimer	A_2	No	6.5	(11)
Lactarius lignyotus		22 tetramer	A_4[d]	Yes	-	(12)
Laetiporus sulfureus	PSL	36 & 60 tetramer	A_2B_2	No	5.2	(13)
Mycoleptodo-noides aitchisonii		16 tetramer	A_4	Yes	4.5	(14)
Pholiota aurivella	PAA	18 polymeric	$A_{>5}$	Yes	5.2	(15)
Pleurotus cornucopiae	PCL-F	15 & 16 dimer	A_2 & B_2 A_1B_1	Yes	6.1[e]	(16)
Tricholoma mongolicum	TML-1 TML-2 TML-F	17.5 dimeric - -	A_2 A_2 -	No No Yes	- - -	(17) (18)
Xerocomus chrysenteron		17-22 monomer	A_1	Yes	-	(12)

[a] For simplicity, abbreviations have frequently been given to lectin by the authors. [b] Monomeric lectins are very rare, but have been reported. [c] The type of oligomer refers to hetero- (i.e. A_nB_n) or homo-oligomerisation (i.e. A_n). [d] Intermolecular disulphide bond reported. [e] Predicted pI.

(1) Presant and Kornfeld 1972, (2) Yagi et al. 1997, (3) Wang et al. 2002, (4) Sun et al. 2003, (5) Zhuang et al. 1996, (6) Yagi and Tadera 1988, (7) Cooper et al. 1997, (8) Yatohgo et al. 1998, (9) Kaawagishi and Mizuno 1988, (10) Guillot et al. 1991, (11) Giollant et al. 1993, (12) Sychrova et al. 1985, (13) Konska et al. 1994, (14) Kawagishi et al. 2001, (15) Kawagishi et al. 1991, (16) Yoshida et al. 1994, (17) Wang et al. 1995, (18) Wang et al. 1998a, b

and could not be detected in non-fruiting zones. Expression continues throughout primordial development with highest activity prior to meiosis in order to decline at the developmental stage of early meiosis (Boulianne et al. 2000, Charlton et al. 1992). Thus, this galectin was proposed to be specific to the primordium. Cellular localisation applying indirect immunofluorescence of the galectins showed a marked accumulation in veil (outer stipe tissue) and libsanoblema (outer stipe tissue) of the primordium (Fig. 4). These two tissue types are expected to be subject to the most tension during rapid stipe elongation of fruiting body maturation (Kamada and Takemaru 1977b, Reijnders 1979).

Immuno-electron microscopy revealed a subcellular localisation of the galectins, with the lectin accumulating within the extracellular matrix of stipe cells as well as along the cell surface. Most staining was achieved in veil cells and the "connective" tissue of the libsanoblema, consistent with immunofluorescence findings. In addition to extracellular localisation, strong labelling was also seen in membrane associated vesicles of stipe cells, possibly linked to the secretion of galectins. Galectins have no typical secretion signal (Fig. 5). Heterologously expressed in yeast, galectin secretion occurs not through the classical secretory pathway at the hyphal apex but by a novel, yet to be defined pathway that may not be exclusively restricted to the hyphal tip. The distinct expression of the *Coprinus* galectins within specialised tissues of the fruiting structure combined with the demonstration of extracellular localisation and multimeric organisation may indicate that these lectins function in cell-to-cell contact (Boulianne et al. 2000, Fig. 4). So far, no galectin deficient strains of *C. cinereus* have been generated and the precise function of these lectins is still unknown.

Yagi and co-workers (1997) purified a lectin from *Agrocybe cylindracea* (synonym *aegerita*) and could recently show that this lectin (ACG) too falls into the family of galectins (Yagi et al. 2001). Based on physico-chemical properties, amino acid composition and partial overlap in the N-terminus, ACG is possibly the same lectin as AAL isolated from *A. aegerita* fruiting bodies by Sun et al. 2003 (Zhao et al. 2003). The

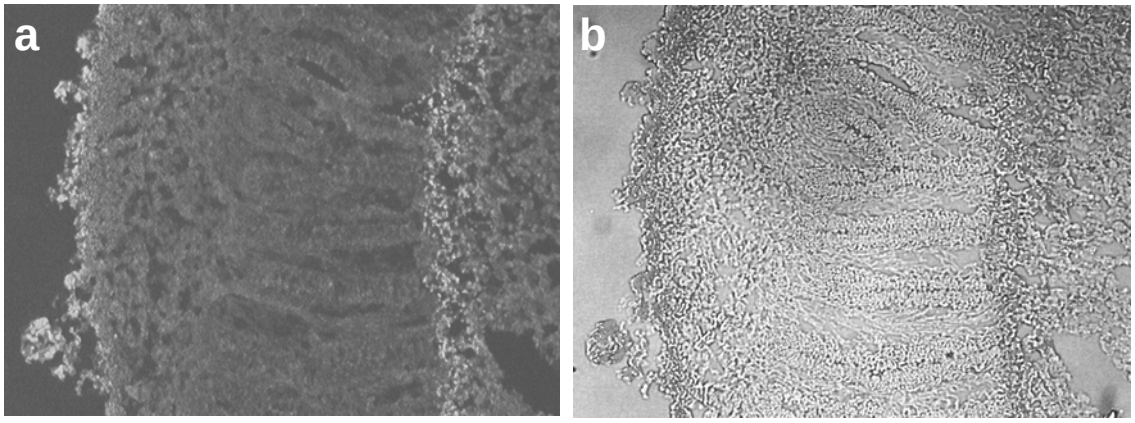

Figure 4. Differential localisation of galectins in *Coprinus* primordia (strain AmutBmut). A. radial section of a fruiting body of strain AmutBmut incubated with rabbit αCGL antibodies and detected with goat-α-rabbit rhodamine conjugate. B. Superposition of fluorescent micrograph with bright-field picture. Marginal staining is observable in the gill tissues and subcutis (tissue layer underneath the veil) relative to expression levels in veil and stipe cortex.

```
CGL2     :  ....MLYHLFVNNQ..VKLQNDFKPESVAAIRSSAFNSKG......GTTVFNFLSAG
ACG      :  STTSAVNIYNISAGASVDLAAPVTTGDIVTFFSSALNLSAGAGS.PNNTALNLLSEN
F8-5B11  :  ....MFYLLPVGHEHCVTLKDELKKDSLIVFRSDKYRFKKDCHPDTDHSCVRLYDCK

CGL2     :  ENILLHISIRPGENVIVFNSRLKNGAWGPEERIP.YAEKFRPP..NPSITVIDHGDR
ACG      :  GAYLLHIAFRLQENVIVFNSRQPNAPWLVEQRVSNVANQFIGSGGKAMVTVFDHGDK
F8-5B11  :  ENIVLHIGFRRGQNKIVFNSKTAKGAWGAEESCA.LDGAFKGE..DVTITVYDHGDH
                 *    *         *        *   *

CGL2     :  FQIRFDYGTSIYYNKRIKENAAAIAYNAEN..SLFSSPVT.VDVHGLLPPLPPA 150
ACG      :  YQVVINEKTVIQYTKQISGTTSSLSYNATEGTSIFSIVVEAVTYTGLA...... 161
F8-5B11  :  FQILCDYRTVHYYKKQCNENIKVISSTLTRQ..................... 138
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Figure 5. Sequence alignment of three basidiomycete galectins (ClustalW). CGL2 *Coprinus* galectin 2, ACG *Agrocybe cylindracea* galectin, F8-5B11 translation [nucleotides 38-451] of Genbank BM346915/BM34916 cDNA from *Heterobasidion annosum*. CGL2 and ACG share 41 % homology, CGL2 and F8-5B11 44%, ACG and F8-5B11 35%. Sequences with dark shadings represent identical residues shared by at least 50% of the sequences. Light shaded residues represent conservative substitutions. Asterisks mark residues known to interact with the β-galactoside in galectins.

latter is not only fascinating due to its anti-viral and anti-tumor properties, but its application on vegetative mycelium of *A. aegerita* and also on vegetative mycelium of the heterologous species *Auricularia polytricha* induced production of fruiting structures (Sun et al. 2003). Karlsson and co-workers (2003) submitted expressed sequence tags (ESTs) from the pine root rot fungus *Heterobasidion annosum* challenged by *Pinus sylvestris* seedling roots, that show significant homology (all residues involved in galactoside binding conserved among galectins) to the other basidiomycete galectins, thus confirming that galectins are more widely distributed in mushrooms and reports of additional galectin proteins from fungi are to be expected (Fig. 5). *Agrocybe* galectin

ACG (as well as lectin AAL) has been shown to exist in a homodimeric form (Barondes et al. 1994, Sun et al. 2003). Oligosaccharide specificity of ACG was assessed by frontal affinity chromatography and revealed strong interaction (K_d's < 3 μM) with acidic oligosaccharides that have α2-3NeuAc attached to the β-galactoside at the non-reducing end (e.g. as found on gangliosides types GD1a/GM1b or sialylated mammalian protein linked glycans) and Galili pentasaccharide (Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc(NAc)) or Blood Group A (GalNAcα1-3[Fucα1-2]Galβ1-4Glc(NAc), K_d ~10 μM)oligosaccharides (Hirabayashi et al. 2002).

Many fungal lectins have been purified with specificities to glycans previously not known to exist in these organisms. Recent studies have revealed that α and also β-galactosides as well as α-fucosides, as found on mammalian glycans and towards which many of the isolated mushroom lectins show activity, are abundant in glycoinositol-phosphosphingolipids that exhibit remarkable heterogeneity within and among basidiomycetes (Jennemann et al. 2001). Kawai and co-workers purified glycosphingolipids of the cerebroside type from *S. commune* (Kawai and Ikeda 1983) and later from *L. edodes* (Kawai 1989) that stimulated fruiting of these mushrooms in vitro. Induction of fruiting was also reproducible by application of glycosphingolipids of plant origin (Kawai et al. 1986). Length of the fatty acid moiety proved to be pivotal in these experiments, the glycoside seemed not to be essential. However, all active compounds purified from mushroom sources were glycosphingolipids (Kawai 1989).

1.2.5 Hydrophobins

Hydrophobins are small secreted, highly stable fungal proteins typically of about 100 amino acids (mature proteins) that self-assemble at hydrophilic-hydrophobic interfaces such as a water/air interface into highly insoluble amphipathic membranes with a hydrophobic and a hydrophilic side (Wessels 1999, Wösten and Willey 2000a, Talbot 2001, Wösten 2001, Whiteford and Spanu 2002, Fig. 6). The amphipathic films attach with their hydrophilic side onto hydrophilic cellular surfaces giving them a hydrophobic character when fungi try to escape their aqueous environment. Amphipathic hydrophobin layers on water/air interfaces lower the water tension from 72 to maximal 24 mJ m^{-2}, allowing hyphal tips to break through the water surface into the air (Wösten et al. 1999). Hydrophobins are also able to attach as films on

hydrophobic surfaces such as Teflon. Compared to binding to hydrophilic surfaces, the two sides of the hydrophobin films are interchanged rendering the surface hydrophilic and thus wettable (Wösten and de Vocht 2000b).

Two types of hydrophobins are distinguished mainly based on solubility characteristics and also on hydropathy patterns, reflecting divergence in sequences. Aggregates of type I hydrophobins are insoluble in aqueous solvents like hot-SDS solutions. They can only be dissolved by harsh treatments using organic solvents such as 99% TFA (tri-fluoro acetic acid) or FA (formic acid). In contrast, type II hydrophobins are readily dissolved in solvents such as 60% ethanol or pure H_2O (Wessels 1994, 1997). So far, type I hydrophobins have been described in ascomycetes and basidiomycetes, whereas type II hydrophobins are only known from ascomycetes (Wösten 2001, Whiteford and Spanu 2002, Wessels 2000).

On the cell wall of aerial fungal structures, self-assembly of class I hydrophobins gives the characteristic picture of a layer of rodlets, arranged as a mosaic of parallel protein bundles (Wösten and Willey 2000a, Wösten et al. 1993, 1994, Lugones et al. 1996, 1998, van Wetter et al. 2000b, Trembley et al. 2002a). Rodlets are sized 5-12 nm in diameter and some 50 to 150 nm in length (Wösten et al. 1993, 1994a, Lugones et al. 1996, 1998, Trembley et al. 2002a, Gunning et al. 1998). Hydrophobins from different fungi and different hydrophobins from the same species are on the whole not well conserved in sequence. However, all have eight characteristic cysteine residues at conserved positions. The consensus sequence for matured hydrophobins (class I and class II) reads X_n—C^1—X_{5-10}—C^2—C^3—X_{11-44}—C^4—X_{8-23}—C^5—X_{5-9}—C^6—C^7—X_{6-18}—C^8—X_m. In class I hydrophobins, the cysteine doublets are followed by hydrophilic amino acids (Wösten 2001, Whiteford and Spanu 2002, Wessels 1997, Kershow and Talbot 1998, Fig. 6). In class I hydrophobin SC3 of *S. commune*, no free SH groups were present in either the monomeric or the assembled form, indicating that all cysteines are involved in intramolecular disulphide bridges (de Vries et al. 1993, de Vocht et al. 2000). Following secondary structure analysis in the class II hydrophobin CU from *Ophiostoma ulmi*, four disulfide bridges are predicted for class I hydrophobins: C^1 is bonded to C^2 or C^3, C^2 or C^3 is bonded to C^4, C^5 is bonded to C^6 or C^7 and C^6 or C^7 is bonded to C^8 (Yaguchi et al. 1993). Such interaction results in two domains with each two loops (Wösten and de Vocht 2000b, de Vocht et al. 2000).

Hydropathy patterns support the presence of these two domains (Wessels 1996). The mature proteins are moderately hydrophobic with calculated Kyte and Doolittle hydrophobicity indices in the range of 0.01 to higher than 0.8 (Wessels 1997, Penas et al. 2002). In detail, the mature proteins are characterized by a typical hydropathy pattern: a neutral to sometimes slightly hydrophilic N-terminus is followed by a succession of a hydrophobic core, a short hydrophilic domain and a hydrophobic C-terminus. Within this general structural profile, the conserved cysteine residues are not placed to particularly fixed positions (de Groot et al. 1996, Wessels 1994a, Lugones et al. 1996, Trembley et al. 2002a, Schuren and Wessels 1990, Tagu et al. 1996, Ásgeirsdóttir et al. 1997, Ng et al. 2000, Santos and Labarère 1999, Ando et al. 2001).
In accordance with classical secretion at the hyphal tips (Wessels 1993a, 1997, Wösten et al. 1994a), unprocessed proteins have an N-terminal secretion signal, 16-26 aa in length (Fig. 6, Table 4). The theoretical isoelectric points (IPs) of the secreted proteins vary considerably, for example CoH2 from *C. cinereus* vegetative mycelium (Ásgeirsdóttir *et al.* 1997) has a calculated IP of 3.27 and POH2 from *P. ostreatus* vegetative mycelium (Ásgeirsdóttir et al. 1998) of 7.47. Since these proteins are very small, a single amino acid exchange from a positive to a negative charged residue has an enormous influence: the allelic proteins Vhm3-1 and Vmh3-2 from *P. ostreatus* var. *florida* differ by one arginine to glutamic acid substitution (Peñas et al. 2002) and the calculated IPs are 7.42 and 5.04, respectively. Despite a general structural similarity, hydrophobins are only partially interchangeable in function (van Wetter et al. 2000a, Kershaw et al. 1998). Unlike SC1 and SC4 of *S. commune*, SC3 was in no way able to complement a defect in conidiation and appressoria formation in the rice-pathogen *Magnaporthe grisea* (Gunning et al. 1998). Likewise, SC4 from fruiting bodies of *S. commune* cannot fully substitute SC3 in its function in vegetative mycelium. SC4 membranes show lower affinity for cell walls of emergent hyphae than films of SC3 (van Wetter et al. 2000a). Whether their quite distinct IPs (SC1 and SC4: 6.87, SC3: 4.23; Table 4) have part in this is not known. Different lectin specificity of the hydrophobins (Wösten 2001, Wösten et al. 2000b, van Wetter et al. 2000b) and different glycosylation (Kershaw et al. 1998) have been proposed before as possible reasons.

Glycolytic digestion showed SC3 to be O-glycosylated with 16-22 mannoses, probably at threonine residues at the N-terminal part of the protein. Glycosylation appears to enhance attachment to hydrophobic surfaces, possibly by influencing protein conformation during the process of selfassembly (see below, de Vocht et al. 1998). Potential glycosylation sites are present to varying degree in the known hydrophobins of basidiomycetes (as determined by the CBS prediction servive; http://www.cbs.dtu.dk; Penas *et al.* 2002). Experimental evidence proved *Pleurotus* POH2 (Ásgeirsdóttir et al. 1998) and Vmh3 (Peñas et al. 2002) and indicated at least hydrophobin DGH1 from the lichen *Dictyonema glabratum* (Trembley *et al.* 2002a) to be glycosylated. In contrast, SC4 from *S. commune* (van Wetter et al. 2000a) and ABH1 and ABH3 from *A. bisporus* (Lugones et al. 1996, 1998) are not. In conclusion, glycosylation appears not to be crucial to the principle hydrophobin function. However, hydrophobin proteins migrate differentially in SDS gels of different polyacryl amide concentrations (presumably due to oligomerisation, Table 4), making molecular weights also of the non-glycosylated proteins difficult to determine. This variable behaviour in migration likely reflects features in protein structure.

SC3 of *S. commune* is structurally best analyzed. The hydrophobin is secreted into the growth medium in a water-soluble, monomeric form (Wösten et al. 1993, 1994a, b, 1999). In the process of self-assembly, the protein undergoes three different conformational changes. The water soluble monomeric state ("soluble-state hydrophobin") is characterized by ~40% β-sheet structure (de Vocht et al. 1998). Disulfide bridges appear to keep hydrophobin monomers soluble whilst not directly involved in self-assembly (de Vocht et al. 2000). Moreover, Schizophyllan may stabilize hydrophobins in solution (Martin GG et al. 1999, 2000). A critical hydrophobin concentration is needed for self-assembly (Martin GG et al. 2000, Gunning et al. 1998). Upon interaction with a hydrophobic solid support, the protein undergoes a structural intermediate ("α-helical-state") characterized by an increase of α-helix which is proposed to act as an anchor in attaching to the hydrophobic surface. The region of α-helix formation is likely between the third and fourth of the conserved cysteine residues (de Vocht et al. 1998, 2002). The same α-helix formation is also postulated to occur during the self-assembly process at water/air interfaces (Wösten and de Vocht 2000b). The α-helical-state during self-assembly at water/air interfaces is only intermediate

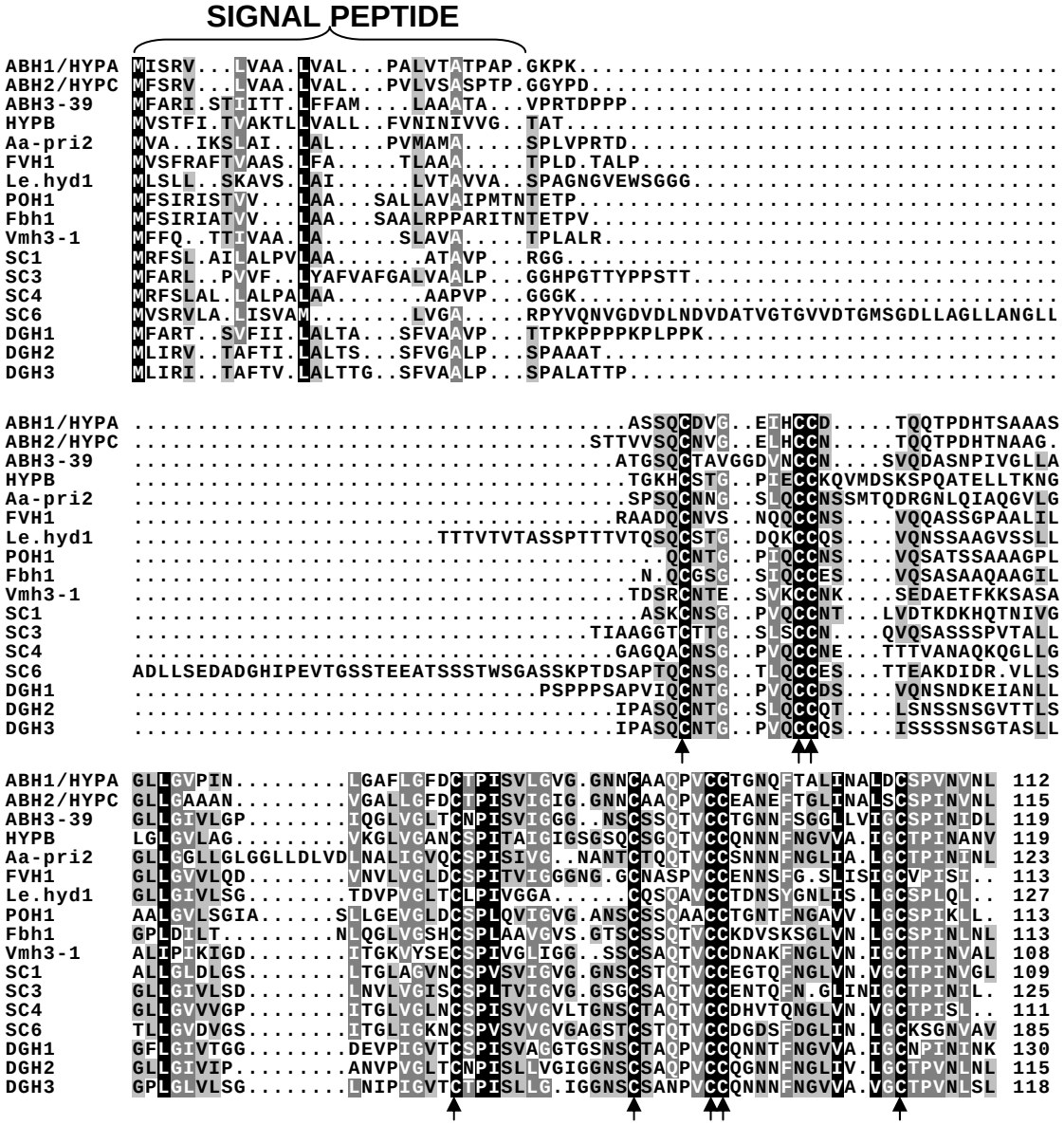

Figure 6. ClustalW alignment of hydrophobins found in aggregated fungal structures of basidiomycete. Sequence alignments were edited in the GeneDoc program (psc.edu/biomed/genedoc). Arrows indicate the positions of the eight conserved cysteine residues. Signal peptides, respectively the amino termini of the mature proteins have been verified in case of ABH1/HypA (Lugones et al. 1996), POH1 (Ásgeirsdóttir et al. 1998), Fbh1 (Peñas et al. 1998), SC3, SC4 (Wessels et al. 1991), DGH1 (Trembley et al. 2002a). GenBank accession numbers are *A. bisporus* ABH1/HYPA: CAA03494, ABH2/HYPC: P49073, HYPB: CAA03496; ABH3-39: CAA74939.1; *A. aegerita* Aa-pri2: AAD41222; *P. ostreatus* POH1: CAA12391; *P. ostreatus* var. *florida* Fbh1: CAC95144, Vmh3-1: CAD12831; *F. velutipes* FVH1: BAB17622; *L. edodes* Le.hyd1: AAG00900; *S. commune* SC1: P04158, SC3: P16933, SC4: P16934, SC6: O74300; *D. glabratum* DGH1: CAC86002, DGH2: CAC86005, DGH3: CAC86006.

whilst in case of self-assembly on a hydrophobic solid support, the α-helical state arrests until a combined treatment of heat and detergent. Upon self-assembly at the water/air interface, the α-helical state rapidly converts to the "β-sheet-state" characterized by an increase in the β-sheet structure up to 65%. β-sheet stacking helps to reduce the surface

tension and to organize into the characteristic insoluble amyloid-like hydrophobin fibrils. Initially upon transition from α-helical to β-sheet state, aggregated hydrophobins form an amorphous stable film (β-sheet state 1). Within a few hours it converts into the typical rodlet pattern (β-sheet state 2) that is only dissolved by harsh treatments such as TFA or FA (de Vocht et al. 2000, 2002, Butko et al. 2001). At water/organic solvent (hexan) interfaces, in a dynamic folding-refolding process, monomeric SC3 undergoes folding to an elongated planar structure with extensive β-sheet secondary elements within ~100 ns. Particularly at the initial stage of the process the dynamic appears to be driven by the two-dimensional interface. This is thought to juxtapose the perpetually alternating hydrophilic and hydrophobic residues of the protein´s primary sequence in a manner appropriate for giving a final amphipathic structure. Further to these initial structural characterisations, tertiary structures of hydrophobins are not yet available by difficulties in stabilizing the soluble forms due to aggregation of the proteins (Zangi et al. 2002).

Numerous class I hydrophobins (respectively their genes) have been isolated from basidiomycetes, representing proteins expressed in vegetative mycelium of monokaryons and/or dikaryons, in mycelial cords, in various stages of fruiting body development and in mantle tissue and Hartig´ net of ectomycorrhiza (see Fig. 7, Table 4). Phylogenetic sequence analysis suggests hydrophobins of basidiomycetes and those of ascomycetes to have diverged from each other after separation of the two clades (Whiteford and Spanu 2002, Wessels 1997, 2000). With the first hydrophobin sequences of basidiomycetes established, cluster analysis suggested proteins from vegetative mycelium and proteins present in fruiting bodies to represent two clearly separated groups (Wessels 2000, Trembley et al. 2002a,b, Ásgeirsdóttir et al. 1998). With increasing numbers of proteins, a cladistic division of hydrophobins according to place of expression is less clear. As shown in Fig. 7, Le.hyd1 of *L. edodes*, mainly expressed in fruiting stages as well as the fruiting-linked FVH1 of *F. velutipes* cluster together with various hydrophobins from vegetative mycelia and with three hydrophobins from fruiting bodies of the lichen *D. glabratum*. Vmh3 hydrophobins from *P. ostreatus* var. *florida*, highly expressed in vegetative mono- and dikaryotic mycelium and weakly in mushrooms, group together with fruiting body specific *S. commune* hydrophobins. In addition, hydrophobins from ectomychorriza (a cellular

interaction between fungal cells and root cells of the plant host (Duplessis et al. 2001) whose formation appears to have at least some functional requirements in common with fruiting body development (Fischer and Kües 2003, Nehls et al. 1999) are found amongst hydrophobins from fruiting bodies of *A. bisporus* and *A. aegerita*. Bootstrap values in most cases are low (Fig. 7), reflecting little general sequence conservation and, in consequence, uncertainty in the proposed protein relations. Prediction by cluster analysis of functional similarity of hydrophobins, respectively of place of actions for example in aggregated structures has therefore its limitations. This is further supported in analysis, when leaving out the only known hydrophobin of the heterobasidiomycete *Ustilago maydis* (Hum2, Bohlmann 1996). In the dendrogram without Hum2, the allelic proteins Vmh1-1 and Vmh1-2 from vegetative mycelium of *P. ostreatus* var. *florida* shift into the vicinity of the fruiting body specific protein Aa-pri2 from *A. aegerita* (not shown). Our current knowledge on hydrophobins linked to fruiting body development is given in Table 4. In a number of studies, total primordia tissues or total fruiting bodies or vegetative mycelium together with developing structures were used to isolate mRNA for Northern blot analysis, not allowing statements on expression in specific tissues (de Groot et al. 1996, Ng et al. 2000, Santos and Labarere 1999, Ando et al. 2001, Ásgeirsdóttir *et al.* 1998, Penas et al. 1998).

Often, the specific fruiting stage analyzed is not well documented in the literature (Ng et al. 2000, Santos and Labarere 1999, Ando et al. 2001, Mankel et al. 2002), probably due to unsatisfying descriptions of the individual courses of fruiting body development. Confusion in nomenclature of developmental stages can explain discrepancies in experimental results (Ng et al. 2000, Nishizawa et al. 2002). Furthermore, mature fruiting bodies might not be the best to test for mRNA production when wanting to know whether specific hydrophobins relate to fruiting. At that point of completed development, structural genes such as those for hydrophobins might not anymore be expressed as documented for instance for *hypB* in *A. bisporus* (de Groot et al. 1999) and *Aa-pri2* of *A. aegerita* (Santos and Labarere 1999). mRNA-based reports on lack of expression in fruiting bodies (Penas et al. 2002, Ásgeirsdóttir et al. 1997, Ásgeirsdóttir et al. 1998, Mankel et al. 2002) therefore might require a more particular analysis in the future. There are several stages in fruiting body development (see section

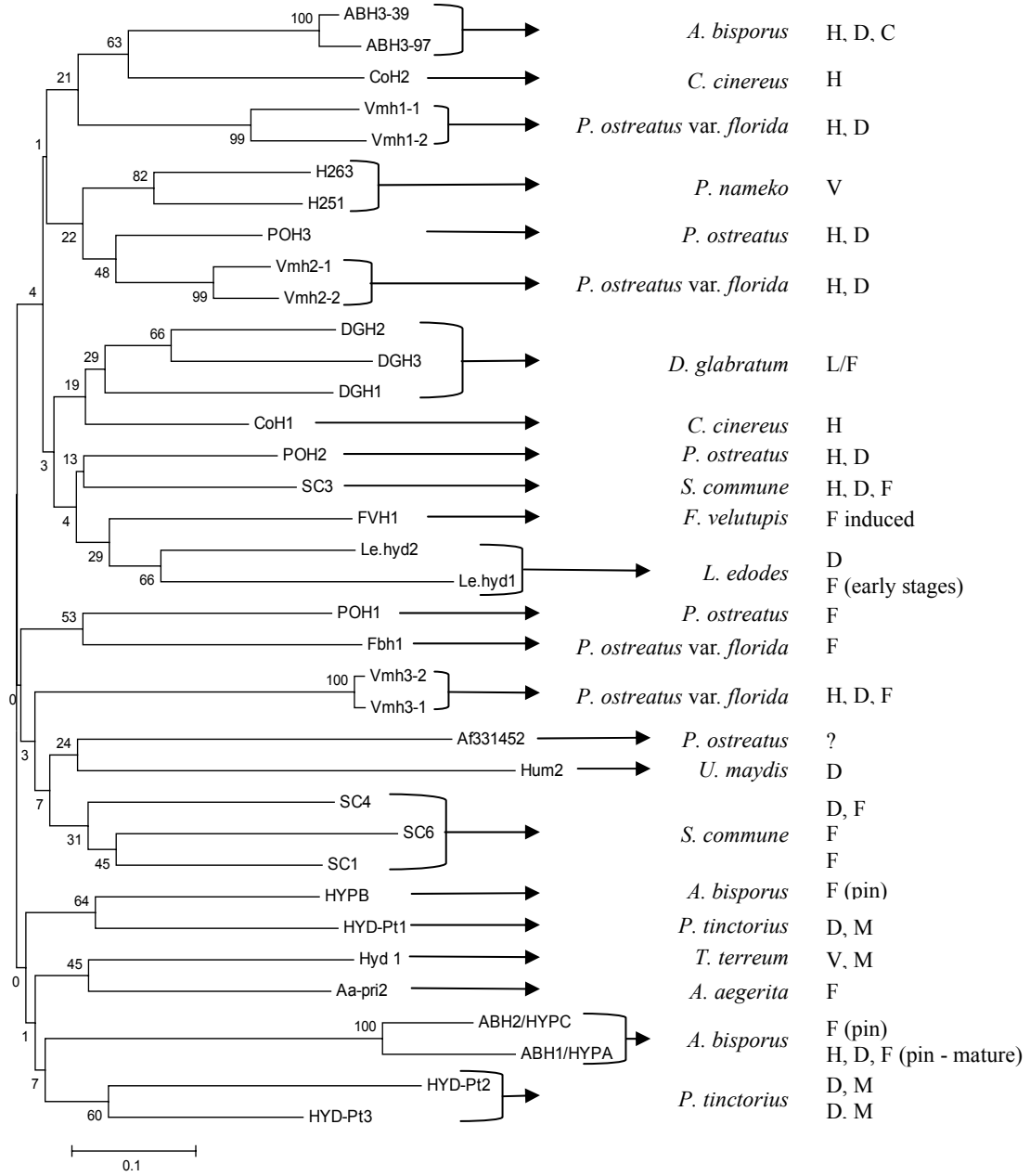

Figure 7. Phylogenetic tree of class I hydrophobins from basidiomycetes. Using MEGA Version 2.1 (http://megasoftware.net), the tree was constructed from ClustalW alignments of the core hydrophobin domains (sequences between the first and the last conserved cysteine residue Wösten 2001). The scale bar indicates the distance equivalent to 0.1 amino acid substitution per site, numbers are bootstrap values that were calculated using 500 replications. GenBank accession numbers are: *A. bisporus* ABH3-97: CAA74940.1; *C. cinereus* CoH1: CAA71652, CoH2: CAA71653; *L. edodes* Le.hyd2: AAG00901; *Pholiota nameko* H251: BAB84545, H263: BAB84546; *Pisolithus tinctorius* HYD-Pt1: P52748, HYD-Pt2: P52749, HYD-Pt3: AAC95356; *P. ostreatus* POH2: CAA74987, POH3: CAA76494, Af3311452: AAL57034; *P. ostreatus* var. *florida* VMH1-1: CAD12829, VMH1-2: CAD12830, VMH2-1: CAD12833, VMH2-2: CAD12834, VMH3-2: CAD12832; *Tricholoma terreum* Hyd1: AAL05426.1; for *U. maydis* Hum2 see (Bohlmann 1996); for others see Fig. 6. For gene expression/protein localisation, see (De Groot et al. 1996,Lugones et al. 1996, 1998, 1999, van Wetter et al. 2000a, Trembley et al. 2002a, Peñas et al 2002, Schuren and Wessels 1990, Tagu et al. 1996, Ásgeirsdóttir et al. 1997, Ng et al. 2000, Santos and Labarère 1999, Ásgeirsdóttir et al. 1998, Peñas et al 1998, Wessels et al. 1991, Butko et al. 2001, Bohlmann 1996, Mankel et al. 2002, Nishizawa et al. 2002, de Groot et al. 1999, Wessels et al. 1995, de Groot et al. 1997, Ruiters and Wessels 1989, Tasaki et al. 2002, Trembley et al. 2002a, b,

Wessels et al. 1991, van Wetter et al. 1996b, Tagu et al. 1998, 2001, Duplessis et al. 2002, Mamkel et al. 2000). C = mycelial cords, D = dikaryotic mycelium, H = homo- or monokaryotic mycelium, F = fruiting body, L = lichen fruiting body, M = ectomycorhizza (hyphal mantle, Hartig´ net), V = vegetative mycelium.

1) and, as the examples of *A. bisporus* and *S. commune* show, different hydrophobins might be expressed at different times according to differentiation of specific tissues (de Groot et al. 1996, 1999, Wessels et al. 1995). In developing, respectively mature fruiting bodies of *S. commune* and *A. bisporus*, antibodies and/or mRNA analysis (in situ mRNA hydridisation, Northern analysis of mRNA from selected tissues) localise SC3 (Wessels et al. 1995), SC4 (Lugones et al. 1999, van Wetter et al. 2000a), ABH1/HYPA and HYPB (de Groot et al. 1996, Lugones et al. 1996, 1999, de Groot et al. 1997, 1999) to discrete tissues. Next to be abundantly expressed in aerial vegetative mycelium of homo- and dikaryon, SC3 is found on individual aerial hyphae covering the *S. commune* fruiting bodies (stage IV) and it is present in cells of the hymenium (Wessels et al. 1995). The fruiting body specific SC4 and *A. bisporus* ABH1/HYPA localize in the inner plectenchymatic tissues at boundaries of the ECM and air channels that traverse the plectenchyma (Lugones et al. 1996, 1999). Thus, by lining the air channels, these hydrophobins give them a hydrophobic surface and prevent them from collapsing and filling with water under wet conditions. At least SC4 is not needed for air channel construction since air channels are found in SC4 knock-out mutants, albeit easily water-fillable (van Wetter et al. 2000a). In addition to inner plectenchymatic tissues (for example in the stipe at the botton and the cap stage), ABH1/HYPA is strongly expressed in the outer regions of pileus (peel tissue) and stipe and in the partial veil but not in gills, in particular also at the time of enlargement of the mushroom cap (de Groot et al. 1996, Lugones et al. 1996, Wessels et al. 1995). A function in expansion and/or maintenance of the fruiting body is postulated (de Groot et al. 1996), for example in prevention of inflow of water from the outside and protection of the fruiting body against bacterial and fungal parasites (Lugones et al. 1996). Compared to *ABH1*/*hypA*, expressed at constant levels throughout mushroom development, the expression pattern of *hypB* is totally different. *hypB* is most highly expressed during the initial stages of fruiting body development up to stage 1 and subsequently decreases in expression. mRNA expression is found in the inner tissues of the developing structure (pin stage), at a high level in pileus tissue and the transitional zone between stipe and cap and at a

lower level within the inner stipe but not in gills. Functions in lining air channels and in protection against bacterial infections have also been postulated for HYPB (Penas et al. 2002). In *L. edodes*, Le.hyd1 acts probably also by lining up air channels. *Le.hyd1* is expressed throughout primordial development in all tissues but the top parts of the cap and the prehymenophore (Nishizawa et al. 2002). Likewise, Fbh1 from *P. ostreatus* var. *florida* is expressed in all tissues of developing fruiting bodies except for gills (Peñas et al. 1998). In young fruiting body tissues of the lichen *D. glabratum*, the three hydrophobins *DGH1*, *DGH2* and *DGH3* are comparably expressed and may be functionally redundant in lining gas-filled spaces. However, in older stratified parts, *DGH1* and *DGH2* are expressed in mycobiont hyphae that surround the photobiont whereas transcripts of *DGH3* occur in hyphae attached to the hymenium (Trembley et al. 2002b).

The examples may indicate that the various hydrophobins do indeed have specific functions, in particular when different hydrophobins occur in distinct tissues (Wösten 2001). In all so far described cases, hydrophobins were found in fruiting structures on air-exposed tissues or air-exposed cells within tissues, thus excerting the function of creating a hydrophobic, air-interacting surface. The fact, that lack of SC3 production causes increased water-soluble β–1-3 glucan mucilage production in vegetative mycelium of *S. commune* and a decrease in alkali-resistant, chitin-linked glucan (van Wetter et al. 2000b) gives another interesting thought to tissue formation. Tissues of fruiting bodies are rich in extracellular mucilage that may function in cellular attachment (section 2 and 3) and the lack of hydrophobin expression within cells of the inner tissues might positively influence its production. In biological systems, hydrophobins may also function on hydrophobic surfaces, for example when spores of pathogenic fungi attach to the hydrophobic surfaces of plant leaves (Wösten 2001, Talbot *et al.* 1996). There is no evidence in fruiting body development for hydrophobins acting on hydrophobic surfaces, for example in attachment of hydrophobin coated hyphae (Wessels et a. 1991, Wessels 1997).

Incomplete EST sequences (GenBank BM346867, BM346868) from *H. annosum* challenged by *Pinus sylvestris* seedling roots (Karlsson et al. 2003) suggest that next to galectins, hydrophobins have their part in the process of root attachment, attack and/or

Table 4: Hydrophobins associated to fruiting body development of basidiomycetes and of basidiomycetous lichens

Unprocessed protein	Mature protein	Expression/protein localisation	Reference (s)
Agaricus bisporus			
ABH1/HYPA: length 112 aa, signal peptide 23 aa, cleavage site PAP-GK[1]	8.9 kDa[2], 8-9 kDa on 15%, 16 kDa on 12.5% SDS-PAGE, IP: 4:32[2], not glycosylated[1]	Fruiting body specific; comparable transcription from stage 1 to 4: highly expressed in veil/peel and pileus, poorly in stipe, not in gills; protein lining air channels in the outer tissues of pileus and stipe (annulus/partial veil) and in the core of stipe, not found in gills	(1-6)
HYPB: length 119 aa, signal peptide 26 aa, cleavage site VVG-TA[2]	9.2 kDa[2], IP: 7.79[2],	Fruiting body specific; primarily expressed in pins, less as maturation goes on; in stage 1 and 2 highly in the inner regions of the cap, diffuse in the stipe, not in the gills, in stage 4 poorly in inner cap tissues, high at the transitional zone between stipe and cap	(5)
ABH2/HYPC: length 115 aa, signal peptide 23 aa, cleavage site PTP-GG[2]	9.1 kDa[2], IP: 3.87[2],	Fruiting body specific; poorly expressed in pin stage; no data available at later fruiting stages	(1-2)
ABH3: length 119 aa, signal peptide 23 aa, cleavage site ATA-VP[2]	9.2 kDa[2], IP:2.68[2], not glycosylated[1]	Forms outer coating of mycelial cords, not expressed during pin stage; no data available at later fruiting stages	(3)
Agrocybe aegerita Aa-pri2: length 123 aa, signal peptide 18 aa, cleavage site AMA-SP[2]	10.7 kDa[2], IP: 4.02[2]	Fruiting body specific: expressed in primordia, not in earlier aggregates, not in mature fruiting bodies	(7)
Coprinus cinereus	11 kDa on 15% SDS-PAGE	Proteins in primordia undergoing meisois and in young elongating fruiting bodies	Fig. 8

[1] confirmed, [2] predicted

Table 4 (continued)

Unprocessed protein	Mature protein	Expression/protein localisation	Reference (s)
Dictyonema glabratum DGH1: length 130 aa, signal peptide 22 aa, cleavage site AVP-TT[1]	11 kDa[2], 14 kDa on 15% SDS-PAGE, IP: 6.08[2], possibly glycosylated	Expressed in mycobiont hyphae surrounding the photobiont in younger and older regions of the fruiting bodies; proteins on fungal cell walls lining gas-filled spaces in the photobiont layer and lower tissues	(8, 9)
DGH2: length 115 aa, signal peptide 22 aa, cleavage site ALP-SP[2]	9.1 kDa[2], IP: 5.46[2]	Expressed in mycobiont hyphae surrounding the photobiont in younger and older regions of the fruiting bodies	(8, 9)
DGH3: length 118 aa, 23 aa signal peptide, cleavage site ALP-SP[2]	9.0 kDa[2], IP: 5.46[2]	Less abundantly expressed in mycobiont hyphae surrounding the photobiont in younger regions of the fruiting bodies, in older regions expression in hyphae attached to the hymenium	(8, 9)
Flammulina velutipes FVH1: length 113 aa, signal peptide 20 aa, cleavage site AAA-TP[2]	9.2 kDa[2], IP: 3.59[2]	Transcription under fruiting conditions at the mycelial “brown film” and “brown skin” stages prior to appearance of visible fruiting structures; decrease in transcription with primordial development, little respectively no transcripts in enlarging and mature fruiting bodies	(10)
Lentinula edodes Le.hyd1: length 127 aa, signal peptide20 aa , cleavage site VVA-SP[2]	12.4 kDa[2], IP: 3.72[2]	Poorly expressed in mono- and dikaryon, highly during primordia development (from first tissue differentiation till completion; stage I, II, III) except for the top parts of the cap and the prehymenophore, poorly in mature fruiting bodies	(11-13)

[1] confirmed, [2] predicted

Table 4 (continued)

Unprocessed protein	Mature protein	Expression/protein localisation	Reference (s)
Pleurotus ostreatus			
POH1: length 113 aa, signal peptide26 aa, cleavage site TNT-ET[1]	8.4 kDa[2], 9 kDa on 15%, 15 kDa on 12.5% SDS-PAGE, IP: 4.00[2]	Fruiting body specific (mature fruiting bodies?)	(14)
P. ostreatus *var.* ***florida***			
Fbh1: length 113 aa, signal peptide 26 aa , cleavage site TNT-ET[1]	8.6 kDa, 12 kDa on 15% SDS-PAGE, IP: 4.61[2]	Fruiting body specific; transcription in all tissues of mature fruiting bodies except gills, highly in middle outer regions of the stipe, irregular in inner tissues of pileus	(15)
Vmh3: lenght 108 aa, signal peptide 23 aa, cleavage site ALR-TD[2]	11.2 kDa[2], IP: 7.48[2], glycosylated	Transcripts in mono- and dikaryons, and in fruiting bodies	(16)
Schizophyllum commune			
SC1: length 109 aa, signal peptide 18 aa, cleavage site ATA-VP[2]	10.8 kDa[2], IP: 6.87[2]	Expressed at early fruiting stages with a peak at stage IV, poorly expressed at stage V	(17-19)
SC3: length 125 aa, signal peptide 24 aa, cleavage site ALP-GG[1]	9.8 kDa[2], 28 kDa on 12.5% SDS-PAGE, IP: 4.23[2], glycosylated	Up to 1% of total mRNA in mono- and dikaryon; low expression in early fruiting stages till stage IV; protein present in hyphae covering fruiting bodies and in cells of the hymenium, not present in inner plectenchymatic tissue	(18-23)
SC4: length 111 aa, signal peptide 16 aa, cleavage site PVP-GG[1]	8,7 kDa[2], 19 kDa on 12.5% SDS-PAGE, IP:6.87[2], not glycosylated	Low expression in dikaryon; well expressed at early fruiting stages with a peak at stage IV, still high at fruiting body enlargement; proteins lines up air cavities in the inner plectenchymatic tissues of fruiting tissues	(3, 18, 19, 21-23)
SC6: length 185 aa , signal peptide 17 aa, cleavage site VGA-RP[2]	16.7 kDa[2], IP:3.70[2]	Negligible expressed before, moderately at stage IV, less during fruiting body expansion	(18, 19, 22)

[1] confirmed, [2] predicted
(1) de Groot et al. 1996, (2) Lugones et al. 1996, (3) Lugones et al. 1998, (4) Lugones et al. 1999, (5) de Groot et al. 1999, (6) de Groot et al. 1997, (7) Santos and Labarere 1999 (8) Trembley et al. 2002(a), (9) Trembley et al. 2002(b), (10) Ando et al. 2001, (11) Ng et al. 2000, (12) Nishizawa et al. 2002, (13) Kaneko and Shishido 2001, (14) Ásgeirsdóttir et al. 1998, (15) Penas et al. 1998, (16) Peñas et al. 2002, (17) Wessels et al. 1995, (18) Ruiters and Wessels 1989, (19) Mulder and Wessels 1986, (20) van Wetter et al. 2000a, (21) Wessels et al. 1991(a, b), (22) Wessels et al. 1995, (23) Schuurs et al. 1998

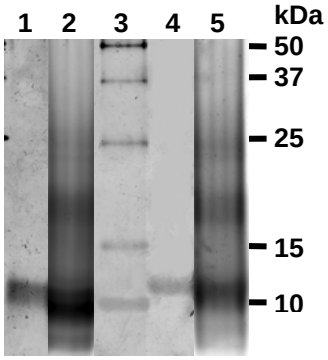

Figure 8. Hydrophobins from *C. cinereus* strain AmutBmut isolated from primordia undergoing meiosis (lane 1 and 4) and from young elongating fruiting bodies producing basidiospores (lane 2 and 5) and separated by 15% SDS-PAGE.
Note that isolation of hydrophobins from later stages of fruiting body development is hampered, presumably by presence of melanins in the sample. Proteins have been isolated from 200 mg tissue per sample following the method of Lugones et al. [161]. Isolated proteins have been dissolved in 100 □l of sample buffer and aliquots of 10 □l were loaded into the slots of the gel. Protein samples in lane 1 and 4 have been reduced by addition of 10 □l of 20% 2□-mercapto-ethanol and boiled for 4 min directly before loading. Protein samples in lane 2 and 5 were diluted with 10 □l sample buffer, boiled for 4 min and loaded. Lane 3: precision protein standards 161-0362 (Biorad, München).

wood degradation. Up to 8 different hydrophobins are known to be expressed in mantle tissue of ectomycorrhizae and the inner Hartig´ net of fungus-marbled root tissue but whether they act in binding the hyphae to the root surface, in aggregation of hyphae or in root-tissue penetration by fungal hyphae remains to be established (Mankel et al. 2002, Tagu et al. 1998, 2001, Duplessis et al. 2001, Mankel et al. 2001, Voiblet et al. 2001, Tagu et al. 2002). It is not clear whether hydrophobins from aggregated structures (fruiting bodies, mycelial cords and ectomycorrhizal mantle tissue/Hartig´ net) have specific minute sequence requirements different from those of non-aggregating vegetative mycelium. However, hydrophobins can differ in lectin specificity and this may determine particular functions in aggregated structures (van Wetter 2000b, Wösten 2001, Wösten and de Vocht 2000b). Similar cytological processes, operations and, respectively, places of action may ask for analogous regulation of genes of interlinked functions (Nehls et al. 1999). Fruiting body specific hydrophobins follow a different regulatory pattern than those present in the vegetative mycelium. In *S. commune*, *SC1* and *SC4* are regulated by mating type pathway in a very different way as to *SC3*. The *A* and the *B* mating type proteins together have positive effects on *SC1* and *SC4*, while the products of the *B* mating type genes act negatively on SC3 production (Wessels et al. 1991,1995, Schuurs et al. 1998, Tagu et al. 2003). Not surprisingly, mutations in genes known to interfere with fruiting (*FBF*, *THN*, *FRT1*) also affect expression of

hydrophobin genes (Wessels et al. 1995, Wessels 1992, Horton et al. 1999). In *C. cinereus*, compatible *A*, respectively compatible *B* mating type proteins appear both to down-regulate monokaryon-specific CoH1 and CoH2 (Ásgeirsdóttir et al. 1997) *C. cinereus* also expresses hydrophobins in the fruiting bodies (Fig. 8) but the corresponding genes have not yet been identified and their regulation not determined. The fruiting body-specific hydrophobins react in vitro with the *A* mating type-regulated, fruiting body specific galectins of *C. cinereus* (P.J. Walser, unpublished) which brings forth an additional interesting reason to study them in terms of expression profile and tissue localization, in particular to elucidate whether the hydrophobins pinpoint to the same fruiting body tissues as the galectins do (Boulianne et al. 2000, Fig. 4).

1.2.6 Other extracellular proteins from fruiting bodies

Dons *et al.* (1984) isolated various cDNAs of differentially expressed transcripts genes from fruiting bodies of *S. commune*. Amongst these were cDNAs from the hydrophobin genes *SC1*, *SC3*, *SC4* and *SC6* (section 4). In addition, the two closely linked genes *SC7* and *SC14* were found specifically expessed in *S. commune* during fruiting, in particular at stage V when the fruiting body expands (Mulder and Wessels 1986, Ruiters and Wessels 1989, Schuren et al. 1993). Their predicted products are 204, respectively 214 amino acids in size and share 87% similarity in sequence. The hydrophilic proteins form a family together with secreted pathogenesis-related proteins (PR1) from plants whose biological role in defense towards pathogens is still unknown. This family of proteins is rich in aromatic amino acids and has four conserved cysteines. Both, SC7 and SC14 have N-terminal secretion signals. Antibodies proved SC7 to be secreted. The protein is loosely associated with hyphal walls and present in spaces between hyphae in plectenchymatic tissues of the fruiting body. A role in hyphal-hyphal interactions for tissue formation is postulated (Wessels et al. 1995, Schuren et al. 1993).

Other ECM proteins known from mushrooms, respectively aggregated fungal structures (ectomycorrhiza) are also cysteine-rich but space their cysteine residues differentially to hydrophobins. Aa-pri3 from *Agrocybe aegerita* is a 95 amino acids long protein (10 kDa) with a 20 amino acids long N-terminal secretion signal. The mature protein contains 8 cysteines as in hydrophobins but differentially spaced and in addition 8 glycines. The *Aa-pri3* gene is expressed in *Agrocybe* primordia, not in mycelium, hyphal aggregates and mature fruiting bodies. A proposed function for the protein is

interaction with other primordial secreted proteins or with cell wall components like chitin (Sirand-Pugnet and Labarere 2002). The 26 kDa PriA from *L. edodes* has cysteine-rich motifs (in total 19 cysteine residues) resembling zinc fingers typified by transcription factor TFIIIA, zinc clusters observed in metallothionines (Kajiwara *et al.* 1992, Ishizaki *et al.* 1999) and epidermal growth factor (EGF)-like domains found in extracellular matrix proteins and integral membrane glycoproteins (Kües and Liu 2000). The serine, threonine and proline-rich PriA protein has a length of 285 amino aacids with a hydrophobic N-terminal signal peptide of 27 amino acids and a C-terminal CAAX-motif (C = cysteine; A = aliphatic residue; X = any residue) known to promote membrane interactions of proteins (Kajiwara et al. 1992). Thus, an extracellular function is most likely. When heterologously expressed in *Escherichia coli*, PriA increases heavy metal sensitivity (Ishizaki et al. 1999). When overexpressed in *L. edodes* monokaryons, accumulation of zinc ions decreases (Ishizaki et al. 2000). Under normal conditions, *priA* is well expressed in primordia and subsequent enlargement of the immature fruiting structure whereas preprimordial mycelia produces few *priA* transcripts and mature fruiting bodies neglectable amounts (Kajiwara et al. 1992, Ishizaki and Shishido 2000). Leung et al. (2000) identified a related *LePriA* sequence of *L. edodes* transcribed in primordia but also expressed in young and mature fruiting bodies. Moreover, *priA*-like genes have been detected in the EST-libraries from shaken culture of *P. ostreatus* (GenBank AT004194; Lee et al. 2002) and from *Pinus* challenged *H. annosum* (GenBank BM346898; Karlsson et al. 2003).

Aa-Pri1 of *A. aegerita*, 145 amino acids in length and 16 kDa in molecular weight, originally has been proposed to allow hyphae to aggregate, following protein binding to specific membrane receptors. The acidic, hydrophilic protein has 39% identity and 47% similarity with the Asp-hemolysin of *Aspergillus fumigatus*. The protein has a typical N-terminal secretion signal of 20 amino acids with the cleavage site at LHN-VG (Fernandez Espinar and Labarere 1997). As *Aa-Pri3*, *Aa-Pri1* is specifically expressed in primordia, not in mycelium, hyphal aggregates and mature fruiting bodies (Fernandez Espinar and Labarere 1997, Berne et al. 2002). A related gene is known to be expressed during fruiting in *P. ostreatus* (Lee et al. 2002). Aegerolysin and ostreolysin, 16 kDa sized proteins corresponding to these genes have been isolated from fruiting bodies of *A. aegerita* and *P. ostreatus*. Both proteins have hemolytic activities on mammalian

erythrocytes by a colloid-osmotic mechanism, compatible with the formation of pores of 4 nm by protein aggregates. Protein action (membrane-binding and pore-formation) is modulated by presence of lysophospholids (1-α-lysophosphatidylinositol, 1-α-lysophosphatidic acid/oleoyl). Moreover, ostreolysin was not active on vesicles produced from *Pleurotus* mushrooms. It has been proposed that in vivo in the fungus, these types of proteins contribute to cell signaling since lysophospholids are well known signaling molecules in other biological systems (Berne et al. 2002, Sepcic et al. 2003). Whether there is also lytic activity in mushroom development, for example in the now variously described events of programmed cell death (Umar and Griensven 1997c, 1998, Lu 1974b, Lu et al. 2003), remains to be shown (Kües and Liu 2000).

The developmentally regulated gene *SC25* was isolated from *Amanita muscaria*. The protein product resembles weakly in sequence extensions (Nehls et al. 1999), a major group of gylocoproteins from plant cell walls (Jose-Estanyol and Puigdomenech 2000). Consistent with an extracellular function, the 184 amino acids long SC25 protein has a 17 amino acids N-terminal signal-peptide. Compared to vegetative mycelium, SC25 expression is both 30-folds increased in ectomyccorhizal fungal tissues as in the fruiting bodies (Nehls et al. 1999). Martin and coworkers (Voiblet et al. 2001, Martin F et al. 1999, Laurent et al. 1999) describe another interesting class of ECM proteins that they detected in *P. tinctorius* ectomycorrhoriza formation. SRAPs (symbiosis-regulated acidic polypetides), a family of at least six different members sized about 30 kDa produced during ectomycorrhiza formation, contain an RGD cell-adhesion motif. The proteins were observed on the hyphal surface and in the *Eucalyptus globulus* root cell walls and suggested to form part of a cell-cell adhesion system needed for aggregation of hyphae in ectomycorrhiza. As the *C. cinereus* galectins (Boulianne et al. 2000), they do not have typical secretion signals and are possibly secreted by a non-classical secretion pathway (Martin F et al. 1999, Laurent et al. 1999). Similar ECM proteins have not yet been described from basidiomycete fruiting bodies.

SC15, a highly abundant, secreted protein of 15 kDa (191 amino acids including a 22 amino acids long N-terminal signal peptide) of *S. commune* of unknown function, coprecipitates from medium together with hydrophobin SC3, when sparging the medium with air (Wösten et al. 1993). The protein is devoid of S-containing amino acids and rich in leucine, alanine, glycine and valine residues. The N-terminal half of

SC15 is rather hydrophilic, whereas the C-terminal half is highly hydrophobic. SC15 shows no homology to any other known protein. Its predicted structure contains large segments of α-helical structure that matches no other structure in the protein structure data bank. SC15 is localised in the ECM and in the cell walls of aerial hyphae of mono- and dikaryons that also express SC3 and appears to be regulated in expression as *SC3*, for example co-ordinately by the *B* mating type genes and the *THN* gene (Lugones 1998). Little SC15 protein is found in fruiting dikaryons (Schuren et al. 1993). It is not present in the ECM or cell walls of fruiting body plectenchymatic tissues that also lack SC3. Information on occurrence of SC15 in aerial hyphae covering fruiting bodies and in cells of the hymenium has to be provided together with information whether SC3 and SC15 interrelate in function (Lugones 1998).

MFBA is a 2157 amino acid sized protein detected by cDNA cloning from mature fruiting bodies of *L. edodes*. It contains a cell-surface attachment-promoting RGD motif at position 812-814 (Kondoh et al. 1995). A short MFBA peptide containing this sequence has been shown to mediate cell-adhesion of mammalian cells, aggregation of mycelial fragments of *S. commune* and floc formation of *Saccharomyces cerevisiae* cells (Kondoh et al. 1995, Yasuda and Sshishido 1997, Yasuda et al. 1997). *mfbA* is expressed in late-fruiting body development, mostly within the gill-less regions of the cap at a stage when most tissues of the mushroom have already been established (Yasuda et al. 1997), arguing against a function as general aggregation factor. Furthermore, not only the enormous length of the complete protein, but in particular the presence of a PHD finger and a SET domain, large domains typical for transcription factors regulating chromatin structure in a developmental manner, sheds doubt on an extracellular function of MFBA in mediating aggregation (Kües and Liu 2000, Shishido 2002). Incidental RGD motifs occur frequently in proteins with functions unrelated to cellular aggregation (Ohno 1995).

1.2.7 Conclusion and future prospects

The ECM as the outer surrounding of hyphae present in aggregated structures is a site of multiple functions including surface attachment, cellular interaction and intercellular communication. Few proteins present in ECM and contributing to ECM functions are so far recognized. Of these, lectins and hydrophobins are partially

understood. In future, the already known and also new proteins have to be further elucidated and their specific functions within the ECM to be confirmed or newly established. To this end, their exact places of production and action have to be known. A first hindrance to be overcome is the ambiguity of how developmental stages are referred to by terms such as “primordium” and “fruiting body”. Up to now, the same developmental stage might be differently called or same term might be used for different stages (Liu 2001, Nishizawa et al. 2002). Mushrooms as well as other aggregated structures contain several different types of tissues and within the tissues even different types of differentiated cells. Morphological-cytological descriptions are currently still far too imprecise to fully understand and compare the different developmental stages within a given species and between species.

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1.2.9 References

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1.3 Spatial and temporal expression of laccase in *Coprinopsis cinerea* using galectin promoters

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The contents and text of this conference paper was compiled by the author of this thesis from literature and from computer analysis of publicly available sequence data. S. Kilaru, K. Kaur and J.K. Pemmasani performed the experiments described in section “2. Reporter constructs” and supplied Fig. 7.

1.3.1 Abstract

Galectins are β-galactoside binding lectins defined by a conserved sequence. In mammals, there is a family of galectins that interact with glycoproteins in both extracellular and intracellular milieu and regulate various biological phenomena including cell growth, cell differentiation, cell adhesion, and apoptosis. Outside of the animal kingdom, genes for galectins are known in *Arabidopsis* and a few basidiomycetes. In fungi, galectins have been studied in detail in *Coprinopsis cinerea*. The *C. cinerea* galectins Cgl1 and Cgl2 are specific to the fruiting body. When nutrients are exhausted, Cgl2 is expressed under dark conditions in the mycelium at places of fruiting body initiation and primary hyphal knot formation. Cgl2 expression continues within the initial stages of fruiting body development till primordia formation is completed. In contrast, expression of Cgl1 starts later with the light-induced formation of secondary hyphal knots. Cgl1 expression also continues during primordia development to end at the stage of meiosis. In the genome, there is a gene for a third galectin (*cgl3*) but nothing is yet known about its expression. In this study, we establish the *C. cinerea* laccase gene *lcc1* as a reporter gene to study spatial and temporal regulation of galectin gene promoters during fruiting body development.

Keywords: Fruiting bodies-Lectins-Hyphal aggregation-Tissue-specific expression-Laccase

1.3.2 Introduction

1.3.2.1 *Mushroom lectins*

Lectins have originally been detected and defined from plant origin as agglutinins of erythrocytes. Subsequently, lectins have been isolated from most kingdoms and the lectin definition has become more generalized as a protein other than enzymes and antibodies that binds tightly but reversibly to a specific sugar or sugars. Lectins comprise several different protein classes defined by sugar-binding specificities and sequence similarities (Goldstein et al. 1980, Ambrosi et al. 2005). Lectins are most often found extra cellular with suggested functions in binding to the ECM (extra cellular matrix), cell-to-cell adhesion and defense of pathogens (Kilpatrick 2002). Other lectins have fundamental intracellular roles such as in glycoprotein processing (Schrag et al. 2003), in regulation of cell cycle and apoptosis, in nuclear pre-mRNA splicing (Liu et al. 2002, Wang et al. 2004) and possibly in nitrogen storage (Law 2000).

The study of lectins in fungi started in 1907 with toxicological investigations on hemolytic agglutinins from edible fruiting bodies and from the fly agaric (Ford 1907, 1910). Since then, a large number of lectins have been purified and characterized from vegetative mycelia and, mostly, from fruiting bodies of basidiomycetes (Guillot and Konska 1997, Wang et al. 1998). Lectins have been implicated in growth and morphogenesis of mushrooms (Richard et al. 1994, Walser et al. 2003, Swamy et al. 2004, Wösten and Wessels 2005), may function in storage (Kellens and Peumans 1990) and in symbiotic and parasitic relations including mycorrhiza (Guillot et al. 1994) and lichen associations (Elifio et al. 2000, Lehr et al. 2000), plant-pathogenic interaction (Rudiger 1998), insect defense (Birck *et al*. 2004), virus defense (Sun et al. 2003) and mycoparasitism (Inbar and Chet 1992, 1994). One aspect in research concentrates on the medicinal and pharmacological potential of lectins from mushrooms. Lectins from basidiomycetes have been demonstrated to have anticancer activities, mitogenic activities and immunomodulatoric activities (for examples see Wang *et al*. 2000, 2003, Lee et al. 2003, Ngai et al. 2003, Ho et al. 2004, Kawamura et al. 2004, Sze et al. 2004).

Currently, the best understood fungal lectins are the two fruiting body-specific galectins Cgl1 and Cgl2 of *Coprinopsis cinerea* (Cooper et al. 1997, Boulianne et al. 2000, Walser et al. 2004, 2005).

1.3.2.2 *Galectins*

Galectins are a large family of β-galactoside binding lectins that are characterized by conserved amino acids in the carbohydrate recognition domain (CRD; Fig. 1). Originally, galectins were isolated from various phyla of the animal kingdom (mammals, birds, amphibians, fish, nematodes and sponges) but galectins or at least genes for galectins are now also known from plants (*Arabidopsis*) and basidiomycetes (Fig. 2). In humans, several different galectins are known with widespread functions. Each galectin exhibits a specific pattern of expression in various cells and tissues, and expression is often closely regulated during development. In the extracellular compartment, galectins are thought to act by cross-linking β-galactoside containing glycoconjugates, resulting in modulation of cell adhesion and cell signaling. Within cells, galectins have been shown to regulate cell cycle, cell growth and apoptosis and to act in pre-mRNA splicing (Leffler 1997, Cooper 2002, Wang et al. 2004**).** Understanding the roles of galectins in basic biological processes is vital for possible applications of galectins in diagnosis and therapy of cancer, autoimmunity and transplant-related disease (Hughes 2001).

Whilst highly similar to each other (21 to 86 % identity, 38 to 92 % similarity), fungal galectins have only 7 to 19 % amino acid identity and 15 to 21 % similarity to human galectins (Table 1). In similarity cluster analysis, they form a separate branch from the animal galectins (Fig. 3). Nevertheless, fungal galectins might be very useful for medical purposes but still have to be tested for any medical application. In support of this idea, the β-galactoside binding lectins ABL from fruiting bodies of *Agaricus bisporus* and XCL from fruiting bodies of *Xerocomus chrysenteron* have been shown to

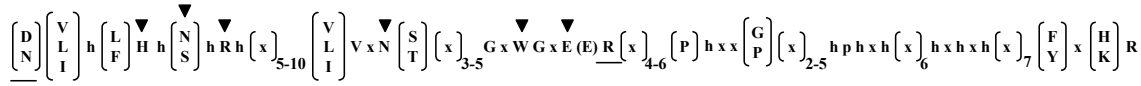

Figure 1. Conserved sequence elements within the carbohydrate recognition domain of the galectin family. Arrows mark residues with invariant bonding to carbohydrate ligands. Underlined positions indicate residues that coordinate ligands in extended binding site (e.g. acetamido group of N-acetyllactosamine or substituted ß-galactosides). Furthermore, several positions of hydrophobic amino acids (h) are conserved that possibly help in stabilizing the galectins fold. x: any amino acid. After Barondes et al. (1994a, b).

Figure 2. Sequence comparison of human and fungal galectins. Sequences of human and of fungal proteins were aligned separately from each other in order to better maintain the impression of sequence similarities between the fungal proteins. Arrows mark residues with invariant bonding to carbohydrate ligands (see Fig. 1). Protein and gene accession numbers in the NCBI GenBank: Cgl1 of *Coprinopsis cinerea* (Q06100), Cgl2 of *C. cinerea* (Q9P4R8), AAL of *Agrocybe aegerita* (Q6WY08), F8-5B11 of *Heterobasidion annosum* (BM346916), human Gal-1 (NM_002305.2), human Gal-2 (NM_006498.2), human Gal-3 (NM_002306.1). Cgl3 was deduced from contig 1.28 (position 715943 to 715452) from the *C. cinerea* genome sequence (http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/). Note that the sequence of AAL has been deduced from a partial cDNA and that Gal-3 is N-terminal truncated by 96 amino acids.

have antiproliferative effects on human epithelial cancer cells, without having any apparent cytotoxicity (Marty-Detravas et al. 2004, Carrizo et al. 2005). These lectins and the related β-galactoside binding lectin PCL F1 from *Pleurotus cornucopiae* fruiting bodies and a lectin expressed during mycorrhiza in *Paxillus involutus* however distinguish from the galectins by their specific carbohydrate binding sites (Fig. 4). The proteins define a new class of lectins, the actinoporin-related family of fungal lectins (Birck et al. 2004). Moreover, a third class of fungal β-galactoside binding lectins have recently been detected in fruiting bodies of the mushroom *Laetiporus sulphureus* (Tateno and Goldstein 2003) that is neither related to galectins nor to the family of actinoporin-related lectins (not shown).Lectins of the actinoporin-related family and the *L. sulphureus* lectins (LSLa, LSLb and LSLc) are pore-forming proteins and seem to act as toxins (Tateno and Goldstein 2003, Trigueros et al. 2003, Birck et al. 2004).

Table 1. The amino acid identity and similarity between fungal and human galectins

Galectin	Cgl1	Cgl2	Cgl3	AAL	F8-5B11	Gal-1	Gal-2	Gal-3
Cgl1	100 % 100 %	86 % 92 %	32 % 50 %	27 % 44 %	34 % 46 %	15 % 31 %	17 % 30 %	8 % 15 %
Cgl2		100 % 100 %	32 % 47 %	27 % 43 %	32 % 44 %	16 % 32 %	19 % 32 %	8 % 16 %
Cgl3			100 % 100 %	29 % 44 %	31 % 48 %	13 % 24 %	14 % 28 %	7 % 16 %
AAL				100 % 100 %	21 % 38 %	14 % 27 %	16 % 29 %	8 % 17 %
F8-5B11					100 % 100 %	15 % 25 %	15 % 25 %	7 % 16 %
Gal-1						100 % 100 %	41 % 59 %	11 % 23 %
Gal-2							100 % 100 %	13 % 21 %
Gal-3								100 % 100 %

In basidiomycetes, galectins are known in *C. cinerea* and in *Agrocybe aegerita* (Boulianne et al. 2000, Yagi et al. 2001; Fig. 2). Furthermore, EST sequences suggest galectins to also occur in *Heterobasidion annosum* (Walser et al. 2003; Fig. 2, 3).

Expression of the galectin genes *cgl1* and *cgl2* in *C. cinerea* correlates with fruiting body development (Charlton et al. 1992, Boulianne et al. 2000). Within Petri-dishes on complete medium, the genes are expressed in the outer zone of the culture in the youngest aerial mycelium, once the fungus covers the whole plate. Western blot and RT-PCR analyses showed very little or no expression of galectins in younger, actively growing cultures. With the onset of fruiting body development, Cgl2 was detected in fruiting zones and, in low amounts, also in non-fruiting zones. Formation of primary hyphal knots (lose compact structures occurring at the early stages of fruiting body

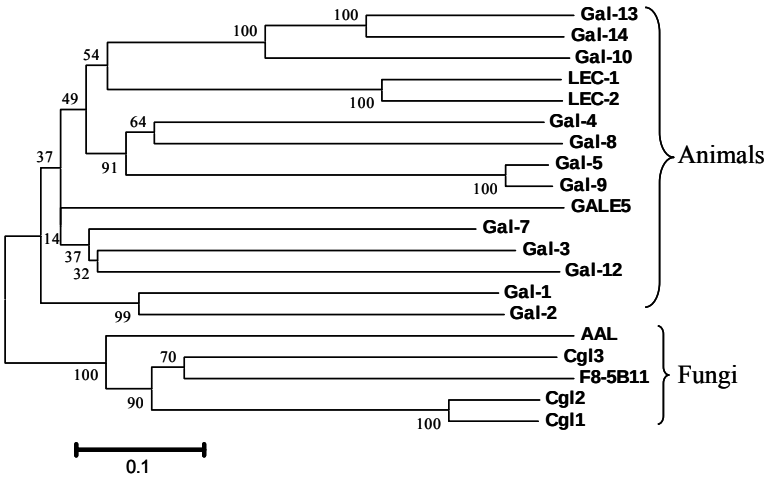

Figure 3. Phylogenetic tree of galectins from humans (GenBank accession numbers: Gal-1: NM_002305.2, Gal-2: NM_006498.2, Gal-3: NM_002306.1, Gal-4: NM_006149.2, Gal-5: AAH73889.1, Gal-7: NM_002307.1, Gal-8: NM_201545, Gal-9: NM_002308.2, Gal-10: Q05315, Gal-12: AAG40864.1, Gal-13: NM_013268.2, Gal-14: Q8TCE9), the roundworm *Caenorhabditis elegans* (LEC-1: NP_496801.2, LEC-2: NP_496165.2), the mosquito *Anopheles gambiae* (GALE 5: XP_309359.2) and fungi. For origin of fungal proteins and accession numbers see legend of Fig. 2.

development; Walser et al. 2003, Kües et al. 2004) was correlated with galectin gene transcription. *cgl1* transcripts and Cgl1 protein appeared during later stages of development, starting with the stage of light-induced secondary hyphal knots (in which cap and stipe tissues differentiate; Walser et al. 2003, Kües et al. 2004). Expression continues throughout primordia development and declines at early meiosis at the stage of prophase I (Charlton et al. 1992, Boulianne et al. 2000).

Galectins in C. cinerea are secreted and found in cell walls and the extracellular matrix of mushroom tissues. Cellular localization of galectins showed a marked accumulation of the proteins in the veil, the outer cap and stipe tissues of the primordium. These tissues are subjected to strong tensions during rapid stipe elongation and cap opening in the process of fruiting body maturation. Therefore, a function in hyphal-hyphal aggregation and tissue formation has been proposed for the galectins (Boulianne et al. 2002, Walser et al. 2005). In support of this argument, the outer cap and stipe tissues also contain galectin ligands. Possible ligands are also detected in the hymenium, the outer spore-bearing cell layer of the gills not expressing the known galectins. Currently, it is not clear whether β-galactoside binding lectins other than Cgl1 and Cgl2 are expressed in these tissues. At least one candidate exist in *C. cinerea* (see

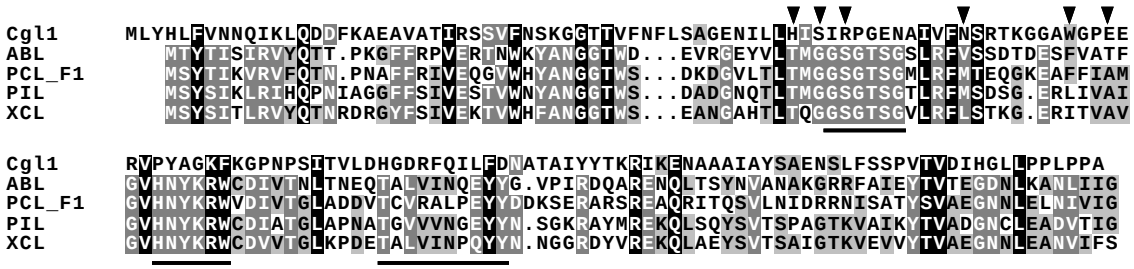

Figure 4. Comparison of fungal β-galactoside binding lectins from various basidiomycetes. Cgl1 is a galectin from *Coprinopsis cinerea*. All other lectins belong to a recently defined family of fungal lectins that have structural similarities to actinoporins (Birck et al. 2004). Arrows above the sequences mark residues in galectin Cgl1 with invariant bonding to carbohydrate ligands (see Fig. 1). Regions with possible residues for carbohydrate binding in the actinoporin-related family of fungal lectins are underlined (Birck et al. 2004). Protein and gene accession numbers in the NCBI GenBank: Cgl1 of *C. cinerea* (Q06100), ABL of *Agaricus bisporus* (Q00022), PCL F1 of *Pleurotus cornucopiae* (BAB63922.1), PIL of *Paxillus involutus* (AAT91249.1), XCL of *Xerocomus chrysenteron* (AAL73236.1).

results). Remarkedly, the galectin ligands are of glycolipid nature (Walser et al. 2004, 2005).

The β-galactoside binding lectin SRL from sclerotial bodies (mycelial aggegrates serving in dormany) of *Sclerotium rolfsii* has also been described to bind glycosphingolipids (Swamy et al. 2004).

1.3.2.3 *Laccase as reporter for tissue-specific expression of galectins genes*

One way to further understand the role of β-galactoside binding lectins and their ligands in mushroom development is to look more deeply into temporal and spatial expression of their genes. To this end, for *C. cinerea* we are developing a reporter system based on enzymatic laccase activities.

Laccases are phenoloxidases that belong to the multi-copper oxidase (Mco) family. These enzymes are versatile redox-enzymes that oxidize various phenolic compounds and aromatic amines (Messerschmidt 1998). The colorless compound ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) is an artificial substrate for laccases. Upon oxidation by a laccase, ABTS gives rise to a colored product that can be used in photometric tests as well as in plate tests to assess enzyme activities. Using the *C. cinerea lcc1* gene as reporter, we previously have established a laccase reporter assay to test activity of various homologous and heterologous constitutive promoters in *C. cinerea* (Kilaru et al. 2005 and submitted). It is now our goal to test whether gene

lcc1 can also serve in studying developmental regulated promoters. As first promoters,the regulatory sequences of the fruiting body specific galectin genes *cgl1* and *cgl2* will be analyzed.

1.3.3 Material and methods

pYSK2 (Kilaru et al. 2005) is a yeast shuttle-vector that contains the yeast 2µm *ori* and *URA3* selection marker, the ColE1 *ori* (*ori Ec*) and amp^R from *E. coli*, the phage f1(+) *ori*, the *C. cinerea* genes *pab1* (for *para*-aminobenzoic acid synthesis; James et al. 2002) and *lcc4* (for laccase Lcc4; Hoegger et al. 2004). This plasmid was used in *in vivo*-recombination (Raymond et al. 1999) in *Saccharomyces cerevisiae* strain RH 1385 (Mösch et al. 1990) to replace *lcc4* sequences with gene *lcc1* under control of galectin gene promoters. Promoter sequences were amplified with chimeric primers from genomic DNA of *C. cinerea* homokaryon AmutBmut (Granado et al. 1997). Gene *lcc1* was obtained from plasmid pESK1 (Kilaru et al. submitted). The constructs were isolated from yeast, transformed for plasmid amplification into *Escherichia coli* strain XL1-Blue (Stratagene) and isolated from the bacterium by standard protocols (Sambrook et al. 2001). Constructs were used in transformation of *C. cinerea* homokaryon strain AmutBmut (*A43mut*, *B43mut*, *pab1-1*). Plasmid pPAB1-2 with the *pab1* wildtype gene (James et al. 2002) was used as a control in transformation. 0.5 mM ABTS was added to regeneration agar and YMG/T complete medium to detect laccase activity. Media receipies, growth and fruiting conditions are given by Granado et al. (1997). tblastn searches were performed with the *C. cinerea* genomic sequence (http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/).

1.3.4 Results and discussion

1.3.4.1 *Promoter comparison*

Genes *cgl1* and *cgl2* are highly similar (87 % sequence identity in the coding regions, 65 % sequence identity in the promoter regions and 63 % sequence identity in the terminator regions) and are found in tandem arrangement in a distance of 1366 bp (Boulianne et al. 2000; Fig. 5). Deduced from alignments with the *cgl1* promoter region and with the *cgl2* terminator region, 495 bp of the *cgl1-cgl2* interim region present the *cgl1* terminator sequence and 871 bp the *cgl2* promoter region (Fig. 6). The

corresponding promoter (*cgl1*) and terminator (*cgl2*) sequences are 841 bp and 522 bp long, respectively.

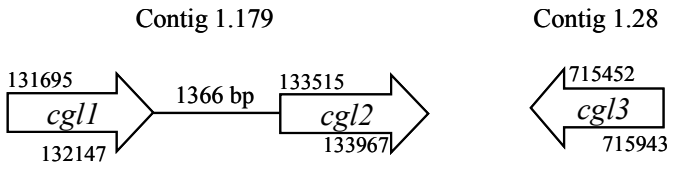

Figure 5. Galectin genes: localization in the genome of *Coprinopsis cinerea*. Sequences were identified by tBlast searches of the *C. cinerea* Okayama 7 genome (http://www.broad.mit. edu/ annotation/fungi/coprinus_cinereus/) with Cgl1 from strain AmutBmut. Identical results hits were obtained in tblastn searches with Cgl2 and Cgl3.

Bertossa et al. (2004) found minimal *cgl2* promoter activity to reside within 627 bp. The authors defined a number of potential promoter elements for *cgl2*, of which at least direct repeats of the sequence TGGAAG, (a) CRE-like binding sequence(s) and a Sp1-like motif seem to participate in promoter regulation. A sequence resembling mating-type protein binding sites of other fungi (*hsg*-like motif) was found to be not essential. When comparing the *cgl1* and *cgl2* promoter sequences (Fig. 6), large regions of high similarity are obvious. Interestingly in these regions, few potential regulatory elements were defined. In contrast, regions with identified or postulated elements are more dissimilar and, often, elements are unique to the *cgl2* promoter. Most of the non-conserved elements locate in a 120 bp sequence that mediates induction of *cgl2* expression in the dark (Bertossa et al. 2004; Fig. 6). *cgl1* and *cgl2* are differentially regulated by light and dark signals. *cgl2* is dark induced, whilst *cgl1* is light induced (Boulianne et al. 2000) and may need other, yet to be defined regulatory elements.

1.3.4.2 *Reporter constructs*

In order to study the temporal and spatial expression of galectins within mycelial cultures and different primordia tissues and for future definition of individual promoter elements, we developed a reporter system using the enzymatic activities of *C. cinerea* laccase Lcc1 as a marker.

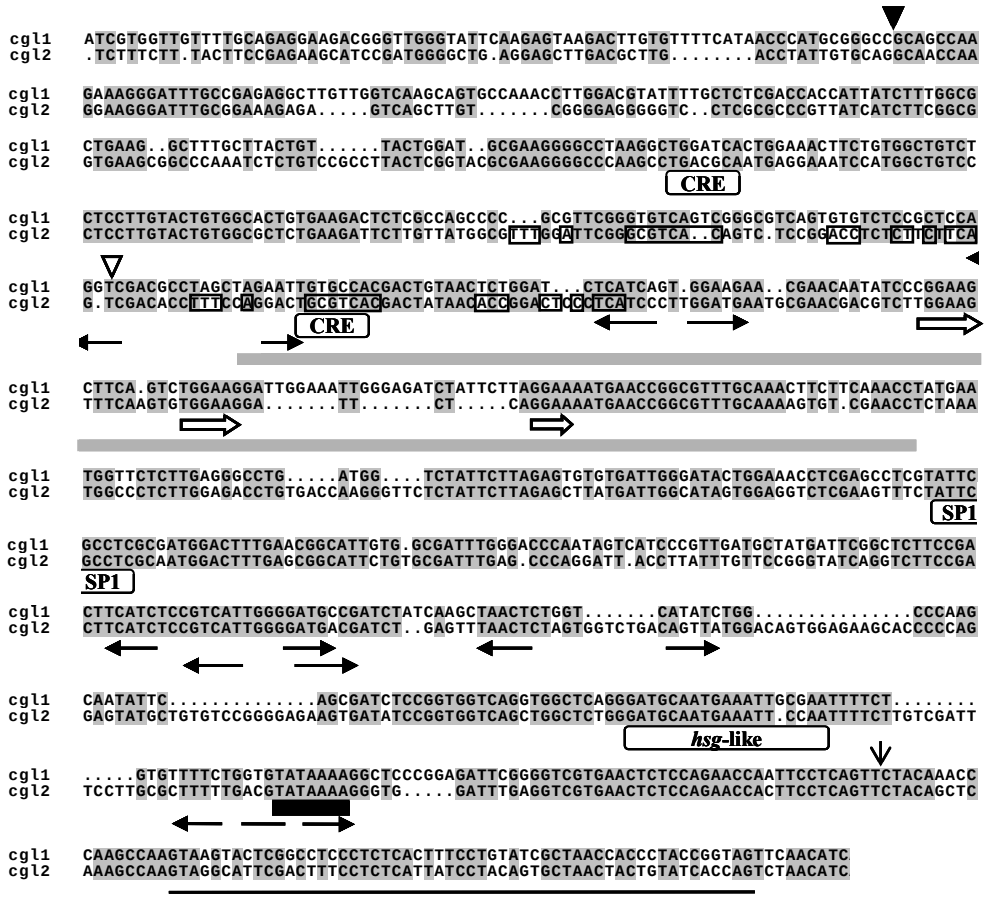

Figure 6. Comparison of the *cgl1* and *cgl2* promoter sequences. A black triangle indicates the maximal extension of the *cgl2* promoter – upstream sequences belong to the *cgl1* terminator region. The minimal *cgl2* promoter sufficient to confer regulated expression is marked by an open triangle. The 120 bp sequence needed for dark induction of the *cgl2* promoter is indicated by a grey bar. Within the *cgl2* promoter sequence, two identical, equal-spaced sequence stretches are outlined by open boxes. Boxes underneath the *cgl2* promoter sequence indicate motifs resembling known binding sites of eukaryotic transcription factors (CRE, SP1, *hsg*-like). Direct repeats are marked by open arrows, indirect repeats by inverse oriented black arrows. A (non-essential) TATA box is marked by a strong black bar [further details on promoter elements are given in Bertossa et al. (2004)]. A potential transcription start site (Charlton et al. 1992) is marked by a vertical arrow. A black line indicates an intron in the 5′-untranslated region of *cgl1* and *cgl2* transcripts (Boulianne et al. 2000).

By *in vivo*-recombination in yeast, we subcloned the promoters of the galectin genes c*gl1* and *cgl2* in front of laccase gene *lcc1* (see Fig. 7). The resulting *cgl1* and *cgl2* promoter constructs were called pYNS2 and pYSK36, respectively. These constructs and as a control pPAB1-2 were transformed into *C. cinerea* homokaryon AmutBmut. 77 different transformants were obtained for construct pYNS2, 23 different transformants for pYSK36 and 176 different transformants for pPAB1-2. On regeneration agar containing uncolored ABTS, none of the transformants caused green staining of the agar

which is indicative for oxidation of ABTS by laccase activity. Some clones were cultured on YMG/T plates with ABTS. It appears that positive *lcc1* transformants with the *cgl2* promoter produce laccase at the edges of the plates when cultures are kept in dark. In contrast, transformants of the *cgl1* promoter seem to produce laccase at the outer edge of the cultures in a day/night rhythm under fruiting conditions. Later during incubation, laccase activity is seen within all cultures including those of pPAB1-2 transformants. Each time, laccase activity starts from the inoculum in the middle of the plates. However, there appear to be differences in quality (intensity of staining) and timings, raising possibility that both promoters are active at a senescent mycelial stage (not shown). Former work revealed that Cgl1 and Cgl2 are expressed specifically at outer colony edges at places of fruiting body initiation and within fruiting structures (Boulianne et al. 2000, Bertossa et al. 2004). Our preliminary analysis of transformants with *lcc1* reporter constructs seems to confirm the results of *cgl1* and *cgl2* expression at the initiation of fruiting body development. Ongoing studies target at tissue specific localization of laccase activity during different stages of fruiting body development.

1.3.4.3 *Genes for β-galactoside binding lectins in sequenced genomes of basidiomycetes*

In addition to *cgl1* and *cgl2*, within the genome of *C. cinerea* there is a gene for a third galectin (Fig. 5), Cgl3 (Fig. 2, 3) found by tblastn searches with Cgl1 and Cgl2. Gene *cgl3* is less similar to the other *C. cinerea* galectin genes (55/55 % sequence identity in the coding region, 46/47 % sequence identity in the promoter region and 48/48 % sequence identity in the terminator region compared to *cgl1*/*cgl2* sequences) and it is found at another chromosomal location (Fig. 5). Temporal and spatial regulation of *cgl3* expression might be tested in future by the *lcc1* reporter gene system. We also searched the *C. cinerea* genome with lectins ABL from *A. bisporus*, PCL F1 from *P. cornucopiae*, PIL from *P. inovolutus*, XCL from *X. chrysenteron* and LSLa, LSLb and LSLc from *L. sulphureus* but without hitting a gene. Apparently, *C. cinerea* does not produce lectins belonging to these two other families of β-galactoside binding lectins. tblastn searches of the established genomes of *Phanerochaete chrysosporium* (Martínez et al. 2004), *Cryptococcus neoformans* (http://www.broad.mit.edu/annotation/fungi/cryptococcus_neoformans/) and *Ustilago maydis* (http://www.broad.mit.edu/

annotation/fungi/ustilago_maydis/) with the *C. cinerea* galectins suggests that these species have no galectins. Searches with the other fungal β-galactoside binding lectins also gave no positive result. This does not exclude that there are other types of β-galactoside binding lectins, both in the analyzed fungi and/or in other basidiomycetes.

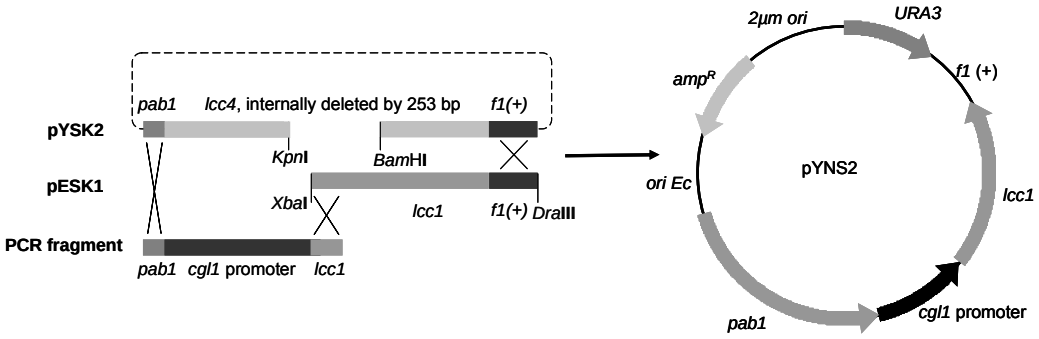

Figure 7. For *in vivo*-recombination in *Saccharomyces cerevisiae*, the yeast-*Escherichia coli* shuttle vector pYSK2 (Kilaru et al. 2005; only simplified map) was digested with *Bam*HI and *Kpn*I that cut within the *C. cinerea* gene *lcc4*. The linearized and purified vector was mixed with i. a 3.0 kb *Xba*I-*Dra*III fragment from plasmid pESK1 (Kilaru et al. submitted) containing the *lcc1* gene of *C. cinerea* monokaryon AT8 and ii. a 3.0 kb DNA fragment obtained by PCR from homokaryon AmutBmut genomic DNA with the chimeric *pab1-cgl1* and *cgl1-lcc1* primers. Upon yeast transformation, positive clones were identified by colony-PCR using the chimeric primers for DNA amplification. Following plasmid amplification in *E. coli*, the identity of the construct was conformed by restriction enzyme analysis (for explanation of other elements on the construct see Materials and Methods). To obtain pYSK36 with *lcc1* under control of the *cgl2* promoter, an analogous strategy with chimeric *pab1-cgl2* and *cgl2-lcc1* primers was followed.

1.3.5 Conclusions

Various types of β-galactoside binding lectins are by now described within the basidiomycetes. In most instances, they are implicated with developmental processes. However, species differ in the scenario of lectins they are equipped with. In *C. cinerea*, there are genes for three different galectins. Using laccase Lcc1 activity as reporter, we now can follow the temporal and spatial regulation of expression of all galectin genes under *in vivo*-conditions. The laccase reporter system provides both qualitative and quantitave information.

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CHAPTER 2

Microscopic observations and reporter gene analysis during early stages of fruiting body development in *Coprinopsis cinerea*

2.1 Abstract

Initial cytological events during fruiting body development in the basidiomycete *Coprinopsis cinerea* are little understood. *C. cinerea* undergoes different morphological changes, starting with a single hypha or a few hyphae forming a network with the surrounding hyphae making hyphal knots and thereby initiating the fruiting body development. Hyphal knots are hyphal-hyphal aggregates in which the hyphae are very loosely attached with each other in the early stages (primary hyphal knots) of fruiting body development to subsequently transform into compact globular three-dimensional structures (secondary hyphal knots). Formation of these hyphal knots are mainly regulated by light, that also decides upon the fate of further development in *C. cinerea* fruiting. Genes for fruiting-body specific galectins (*cgl*1 and *cgl*2) were found in earlier work to be differentially expressed in secondary and primary hyphal knots, respectively. In this study at a cytological level, we documented the events undergoing at the early stages of fruiting body development and at molecular level, using *C. cinerea* laccase (*lcc*1) as reporter gene under the influence of the fruiting-specific galectin gene promoters, the spatial and temporal regulation of expression of galectins was analyzed during fruiting body development.

Keywords: Hyphal knots-Galectins-Reporter gene-Laccase-Fruiting body development

2.2 Introduction

Fruiting body development in *Coprinopsis cinerea* is a very complex cellular process that is influenced by several environmental and genetic factors (Kües 2000). Under the influence of external environmental factors such as light, temperature humidity and nutrients, on the vegetative mycelium, fruiting body formation starts on a single hypha or on a few neighbored hyphae, with intense localized formation of short hyphal branches with restricted tip growth (Kües 2000, Liu 2001). Hyphal-hyphal interaction between the branches leads to the formation of hyphal aggregates (hyphal knots) which develop further by tissue differentiation into compact aerial structures called primordia. Once the tissues containing these primordia are fully established, within these primordia, karyogamy and meiosis occur in specialized cells (basidia) and this initiates their maturation into fully developed fruiting bodies (Kües 2000). In *C. cinerea*, hyphal knots, as the early stages of fruiting body development, are divided into dark-dependent primary hyphal knots and light-induced secondary hyphal knots (Kües 2000, Liu 2001). Within the small globular structures of the primary hyphal knots, hyphae are loosely attached with each other. Secondary hyphal knots are very compact 3-dimensional structures with tightly attached hyphae forming a pseudoparenchyma. Tissue differentiation of cap and stipe starts within these structures (Kües 1998, Boulianne et al. 2000). In the initial stage of fruiting body development, the switch between primary hyphal knots to secondary hyphal knots is considered as a critical light-controlled step in *C. cinerea* (Kües et al. 1998, 2002). If no light source is given for a long time after primary hyphal knot formation, these structures will mature into long-lasting resting bodies called sclerotia (Moore 1981, Clemencon 1997, Kües et al. 1998). In contrast, if too much light is given, no further development of primary hyphal knots occurs (Liu 2001).

In literature, the developmental processes of primary and secondary hyphal knot formation are poorly described and little documented by appropriate pictures. Matthews and Niederpruem (1972, 1973) microscoped the early stages and presented pictures from a hyphal lattice on which primary hyphal knots arised and the authors documented in light-microscopic photos primary hyphal knots (termed early primordia by these authors) and secondary hyphal knots (termed stage II primordia by these authors). However, these pictures are momentary documentations of different structures. Transition of a specific primary hyphal knot into a secondary hyphal knot was not shown, and possibly up to date, nobody might have ever observed the whole

process of development of a given single structure. Liu (2001) and Göbel (2003) tried to follow under the microscope the developmental processes from vegetative mycelium over primary hyphal knot to secondary hyphal knot formation over the time on a given place within the mycelium. Thereby, these authors encountered several technical difficulties. Observation of mycelium under the microscope easily caused desiccation of hyphae, leading to a stop in development. Therefore, cultures need to be kept as much as possible in between observation periods in a humid chamber. Since growth of the fungus occurs best at 37°C and fruiting body formation at 28°C, this can cause water condensation upon transfer of cultures from growth conditions to lower room temperature for microscopy. Water condensation hinders the clear visualization of hyphae by the microscope. In consequence, strong changes between temperatures during the observation periods have to be avoided. Most interestingly, since light influences the fate of development, the white light stream of microscope causes a block in maturation of primary hyphal knots and induces asexual spore production (oidia formation) (Polak et al. 1997, Liu 2001, Göbel 2003). A yellow filter within the light path of the microscope prevents this (Liu 2001), since it is blue light that is active in *C. cinerea* in controlling developmental processes (Kertesz-Chaloupková et al. 1998, Kües et al. 1998). To further avoid unwanted light on the fungal cultures, microscopy should be performed in a dark room (Liu 2001).

In this study, the microscopic techniques to observe given structures over the time under the microscope as first established by Liu (2001) and Göbel (2003) were further developed. Also in this work, the transition of primary hyphal knots to secondary hyphal knots could not be documented. However, for the first time secondary hyphal knots could reliably be produced in cultures for microscopy. In addition, further details on development of primary hyphal knots were documented and learned from this study.

In *C. cinerea*, within primary and secondary hyphal knots, two fruiting-specific galectin genes were shown to be expressed, *cgl*2 and *cgl*1, respectively (Boulianne et al. 2000). Galectins are ß-galactoside-binding lectins that in animals regulate various biological phenomena including cell growth, cell differentiation, cell adhesion, and apoptosis. Other than in animals, galectins are only found in some mushroom fungi (Walser et al. 2003). In *C. cinerea*, differential expression of galectins during early stages of fruiting body development was shown (Charlton et al. 1992, Boulianne et al. 2000). A function in hyphal-hyphal aggregation and in tissue formation has been

proposed for the secreted galectins, as they are expressed in the hyphal knots and in the extracellular matrix of the outer cap and stipe tissues of primordia (Boulianne et al. 2002, Walser et al. 2005). Within the same type of primordium tissues of mushrooms of other species, expression of hydrophobins (small fungal specific extracellular proteins) was reported (Walser et al. 2003). These secreted proteins are supposed to line air channels for gas exchange in fruiting body tissues, by protecting these tissues from water logging and they possibly function in cementing hyphal cells in the mushrooms tightly together (Walser et al. 2003). P. J. Walser observed that the *C. cinerea* galectins interact with hydrophobins from *C. cinerea* in *in-vitro* conditions (see chapter 1). Localization of galectins and hydrophobins in the same tissues makes possible that the two types of proteins interact with each other in order to perform a common physiological or mechanical functions.

For better understanding of hydrophobin and galectin expression patterns in the early stages of fruiting and their influence on fruiting body development, and of possible interaction between these two proteins *in-vivo*, a focus was therefore laid on studying the early stages of fruiting body development in *C. cinerea*. Spatio-temporal expression of galectins genes during the fruiting body development was analyzed using suitable reporter gene construct as presented in chapter 1.3.

The reporter constructs contain the *C. cinerea* laccase gene *lcc*1 (Hoegger et al. 2004) under the control of promoters of *C. cinerea cgl*1 and *cgl*2 genes, respectively (Chapter 1.3). Gene *cgl*2 is first expressed in established, fruiting-competent vegetative mycelium kept in the dark. *cgl*2 expression starts with the development of primary hyphal knots and continues during secondary hyphal knot and primordia formation till tissue development is completed. *cgl*1 expression starts somewhat later in light together with secondary hyphal knot formation and also continuous further during primordia development (Charlton et al. 1992, Boulianne et al. 2000). Expression of the *cgl*1 and *cgl*2 genes in the past were followed by transcripts in PCR and Northern blot approaches and by proteins in Western blot analysis (Boulianne et al. 2000). For this type of analysis, RNA and galectins were isolated from mycelium containing primary and secondary hyphal knots.

To better localize expression of galectin genes to the minute structures of hyphal knots within mycelium, here use of a color reaction by an enzyme action is made. Laccases are phenoloxidases oxidizing the colorless compound ABTS (2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) as an artificial substrate giving rise to a

colored product (Matsumura et al. 1982) that can be measured photometrically as well as seen in plate tests to assess enzyme activities (Kilaru et al. 2005).

2.3 Materials and Methods

2.3.1 Strains and culture conditions.

Coprinopsis cinerea strain AmutBmut (*A43mut*, *B43mut*, *pab-1*) is a self-compatible homokaryon which produces fruiting bodies without mating with another compatible strain (Swamy et al. 1984). The fungal cultures were grown on solid YMG/T agar plates in Petri dishes of 5 cm Ø for 3 days at 37°C in the dark in light proofed ventilated black boxes (Granado et al. 1997, Kertesz-Chaloupková et al. 1998). For microscopic studies, observation windows were made within the fungal cultures by taking out a piece of agar with the dimensions of approx. 1 x 1 cm (Fig. 1). Cultures were further incubated at 28°C in the dark for about 24 h to allow the hyphae to grow within the observation window onto plastic of the petri dish. The hyphal development within the window was monitored carefully, using an inverse Axiovert (Zeiss) microscope. Observations were done at regular intervals of 3 to 4 hours.

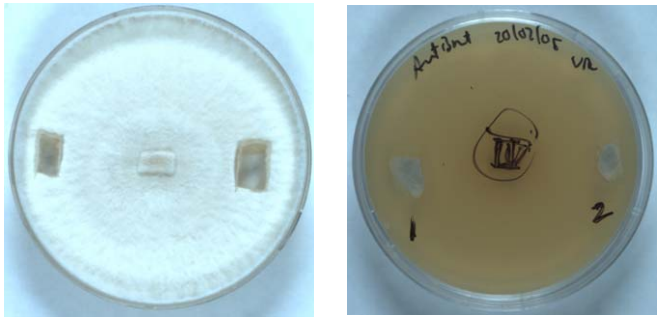

Figure 1. YMG/T culture plates with observation windows for the microscopic study of the hyphal growth under controlled conditions at 28°C. At the left, a mycelial culture is shown from the top after 3 days of growth at 37°C in the dark, cutting the window and further incubation for 2 days at 28°C in the dark. At the right, the same plate is shown from below to demonstrate that fungal mycelium grew from the agar onto the plastic surface of the Petri dish.

For details on the gene reporter construct and DNA transformations see the introductory chapters in this thesis (see chapter 1.3 and Granado et al. 1997). Transformants of homokaryon AmutBmut with constructs *cgl1* and *cgl2*, respectively, were grown on YMG/T agar with ABTS in Petri dishes of 9 cm in Ø for 5 days in the dark at 37°C in light proofed ventilated black boxes and subsequently transferred into a climate chamber for fruiting body development in a 12 h dark/12 h light regime at 28°C (Granado et al. 1997). For laccase testing within tissues, primordia were harvested at the developmental stage where primordia undergo karyogamy. Primordia

were longitudinally cut into two halfs and these halfs were incubated at room temperature for up to 3 days in 120 mM sodium acetate, pH 5.6 with 50mM ABTS.

2.4 Results

C. cinerea strain AmutBmut is a self-compatible homokaryon that due to mutations in both mating type loci (*A* and *B*) is capable of forming fruiting bodies without prior mating to another strain (Swamy et al. 1984). The strain was used in this study for analysis of early steps in the fruiting body development. Microscopic observations were made by using an inverted microscope (Axiovert 25 from Zeiss) fixed with a camera for better analysis of the minute structures of primary and secondary hyphal knots.

2.4.1 Hyphal knot formation: initial experiences in observations

In first series of experiments, the focus was laid on getting familiar with handling the cultures. First, one had to know how the fungus generally grows, how branching occurs, how hyphae interact and how to recognize the first signs at places, where a hyphal knot will later be formed. For hyphal knot formation, the inoculated agar culture plates were incubated at 37^{o}C continuous dark with humidity in well ventilated black boxes. Soon after the cultures covered the entire agar plate, the observation windows were made and parallel sets of plates were incubated at 28^{o}C and 37^{o}C under dark conditions, respectively. We observe a marked influence of temperature on the growth rates of hyphae between the two temperatures. Hyphae grow very fast and dense into the observation window at continuous 37^{o}C incubation, whereas the cultures at 28^{o}C grow slower with few hyphae entering the free space in the window. The dense growth at 37^{o}C made documentation of single hyphal growth more difficult although the whole developmental process from growth of mycelium to primary hyphal knot formation happened within 35-40 h. In 28^{o}C cultures, the process needed about 60 h during which the mycelium was never so densely grown so that single hyphae could not at all be followed. Therefore, 28^{o}C was chosen as the ideal growth temperature for further observations of mycelium in windows. Thereby, the hyphal growth was monitored initially after every 3-4 hour intervals, and when they started forming primary hyphal knots, observations were made at every 2 hours time intervals.

Hyphal knots will never appear on fast growing leader hyphae, i.e. those hyphae that grow out straightforward from a culture to occupy a new surface area (Glass and Fliessner 2006). Within the observation windows of mycelial cultures, the hyphal growth was monitored carefully from the initial stage, where a single hyphae starts to develop and makes sub-apical branches of 1st order and then 2nd order (Fig. 2a) 24h after making the windows. 6 hours later, side branches from hyphae grew towards each other (Fig. 2b), some of which subsequently fused in anastomosis (Fig. 2c, 5 hours later than Fig. 2b). After 10 hours within the same culture, at localized places, hyphae start to interact and get closer to each other (Fig. 2d). Still at this stage, places of later hyphal knot formation are not recognized. A young forming primary hyphal knot is seen however 24 h later (Fig. 2e).

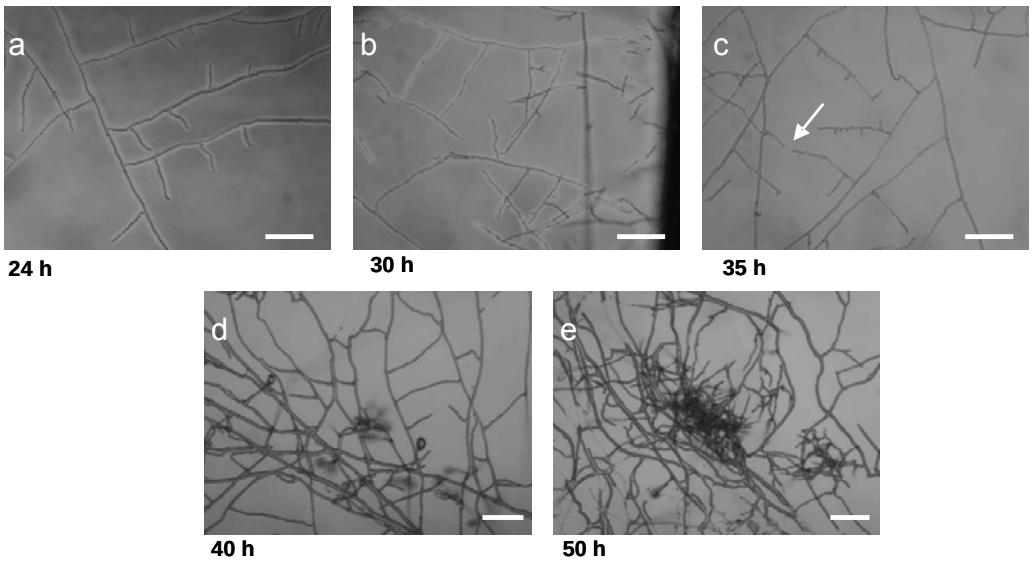

Figure 2. Under controlled culture conditions (see material and methods) single leader hyphae (**2a**) start to grow towards each other (**2b**) and possibly fuse (**2c, see arrow**). In **2d**, a hyphal lattice is seen in which primary hyphal knots may be formed (**2e**). All pictures were taken from the same window at different places. Times of incubation of the cultures upon window creation are given below each photo. Scale bar measures 50 µm (a-c) and 20 µm (d, e).

Primary hyphal knots preferentially form in the window close to the edges of agar (Fig. 3a, c). Usually it is not one, but a number of primary hyphal knots that form in the mycelium at certain distances to each other (Fig. 3b, d). In most cases, more than three to four primary hyphal knots were observed at a localized position (Fig. 4). We calculated

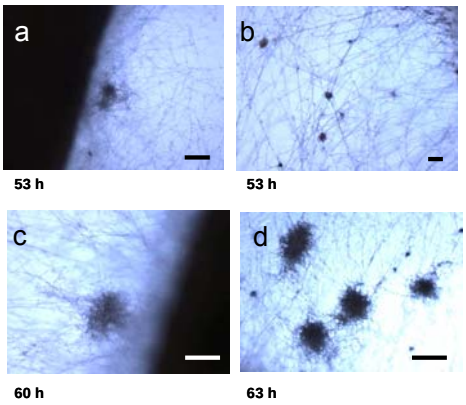

Figure 3. Primary hyphal knots within a window of homokaryon AmutBmut. All primary hyphal knots were from the same window. Times of photographing upon cutting the window are given below of each photo. Note that black shade in picture a and c, is agar medium that marks the outer borders of the window. Scale bar measures 100 µm.

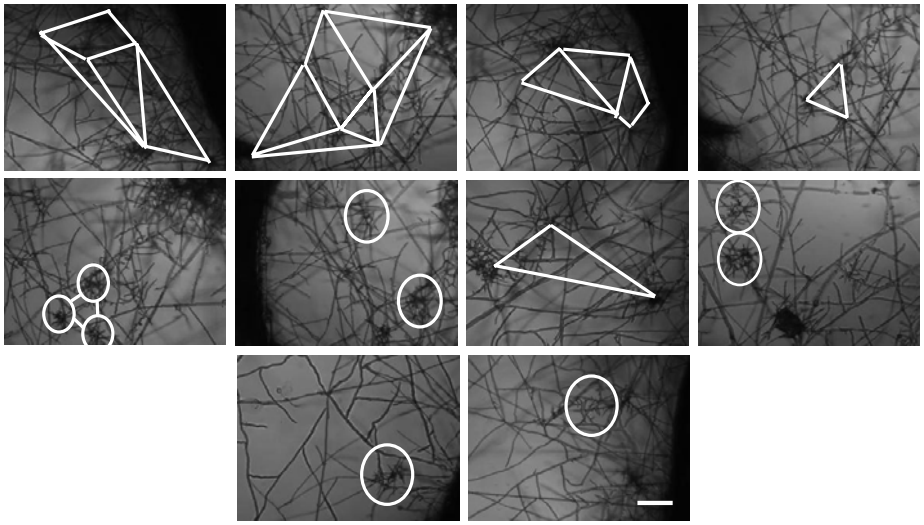

Figure 4. Formation of primary hyphal knots in the culture plates. The white lines showing the distances between primary hyphal knots within a localized area in the window. Individual primary hyphal knots were marked with circles. Scale bar measures 50 µm.

the distances between these different primary hyphal knots (Fig. 4) which are within the range of 58.6 to 568.0 µm in distance to each other (in average 225.2±109.9 µm, calculated from 105 distances measured from center to center of neighboring hyphal knots; in total, 74 hyphal knots were considered).

2.4.2 Technical difficulties during observations

Light is one of the important environmental factors that regulate the *C. cinerea* development. Blue light was found to be the repressor of primary hyphal knot formation in *C. cinerea*. Even a second exposure of the cultures to blue light changes

their developmental patterns (Elliot 1994, Lu 1974, Liu 2001). Therefore, to enable primary hyphal knot development in windows made in cultures of homokaryon Amut Bmut, a yellow filter was used in the light path of the microscope, and observations were made in a dark room throughout the experiment. However, to develop secondary hyphal knots from primary hyphal knots, later a light signal is necessary. During the initial set of experiments, we encountered difficulties of producing secondary hyphal knots within the mycelium due to lack of knowledge regarding the time and length of light induction needed. Therefore, we repeatedly ended up in documenting either oidia (Fig. 5, 7) or sclerotia (Fig. 6). With experience, the actual time point to give a short light pulse for secondary hyphal knot formation can be recognized (see above). The other major difficulty we faced repeatedly during the observations was desiccation of the hyphae within the observation windows due to the dryness on surface of the Petri plates. This was overcome by pouring more agar medium in the plates (ca. 6 mm in height). By keeping these conditions, we were successful in producing secondary hyphal knots repeatedly during the series of experiments. Condensation of water in the lid of the Petri dishes, dropping of water onto the mycelium and running of water into the window were avoided by inverting the plates upside down in between two time points of observation. Various shapes of windows were tested during this study. Observations were made with round windows, triangular windows, long thin rectangular windows and windows of more or less squared form. With round windows, it is difficult to find back the exact place of observation after the incubation periods. With long thin rectangular windows, hyphae from both sides grow towards each other and overlay each other causing a very dense hyphal mesh that made hyphal knots are very difficult to identify. With triangular windows, hyphal growth over the whole area was very irregular. Hyphae at the corners of the triangle will form into a dense mesh, whereas

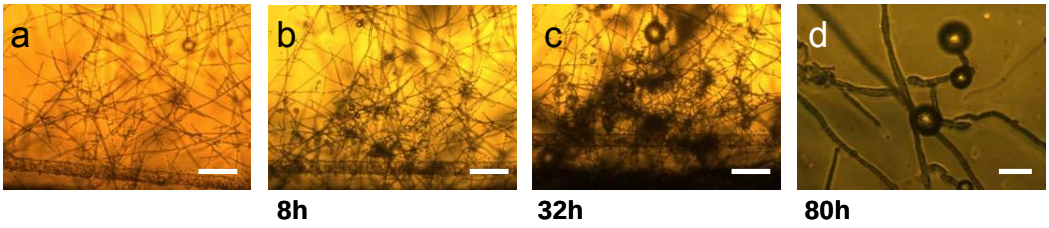

Figure 5. A time course of development of mycelial growth within a window. The same area is shown over the time: pure vegetative mycelium (**a**) and mycelium with starting primary hyphal knot formation (**b**). At this time point, cultures were transferred into a day/night light cycle. Later, liquid droplets were seen in the mycelium (**c**) that upon enlargement (**d**) were identified as oidiophores, i.e. specialized aerial hyphae that produce oidia at their tips (Polak et al. 1997, 2000). Scale bar measures 100 µm (a-c) and 20 µm (d).

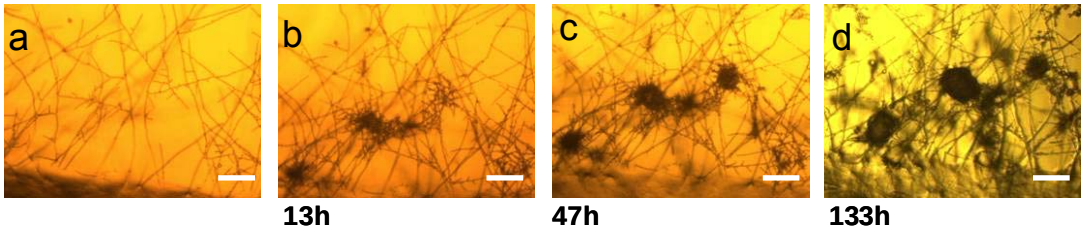

Figure 6. A time course of development of mycelial growth within a window. The same area is shown over the time: pure vegetative mycelium (**a**) and mycelium with starting primary hyphal knot formation (**b**). Upon extended incubation in dark conditions the primary hyphal knots form these round compact structures with a melanized dark outer cell layer (**c**, **d**). Scale bar measures 100 µm.

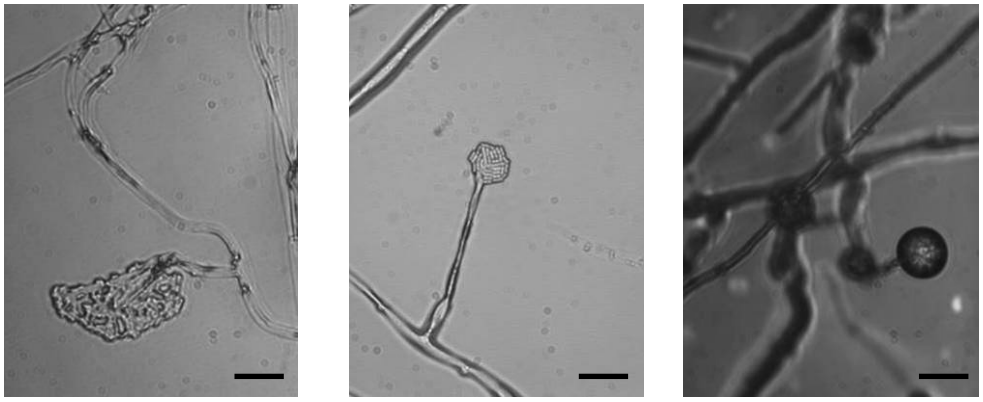

Figure 7. Asexual spore formation in the window due incubation over 30 mins of continuous light with subsequent incubation in dark for more than 12 hrs (scale bar measures 20 µm).

hyphae at the sides of the triangle need comparably long time to grow and develop hyphal interactions. Therefore, hyphae in the window have an uneven age making it difficult to define the correct time point for setting a light signal during the primary hyphal knot formation to induce formation of secondary hyphal knots. Therefore, we preferred windows of squared form, as the hyphae grow from all sides in a relatively comparable time and it is easy to observe and identify the previously observed hyphal structures under the microscope.

2.4.3 Time course of development in cultures kept in continuous dark

Observation windows were made in a dimension (approx. 1 cm x 1 cm) where the hyphae can be seen very clearly during a time course over several days of fungal development under the microscope. Within windows, we were able to better follow the growth of the mycelium and, within the mycelium, the development of the defined

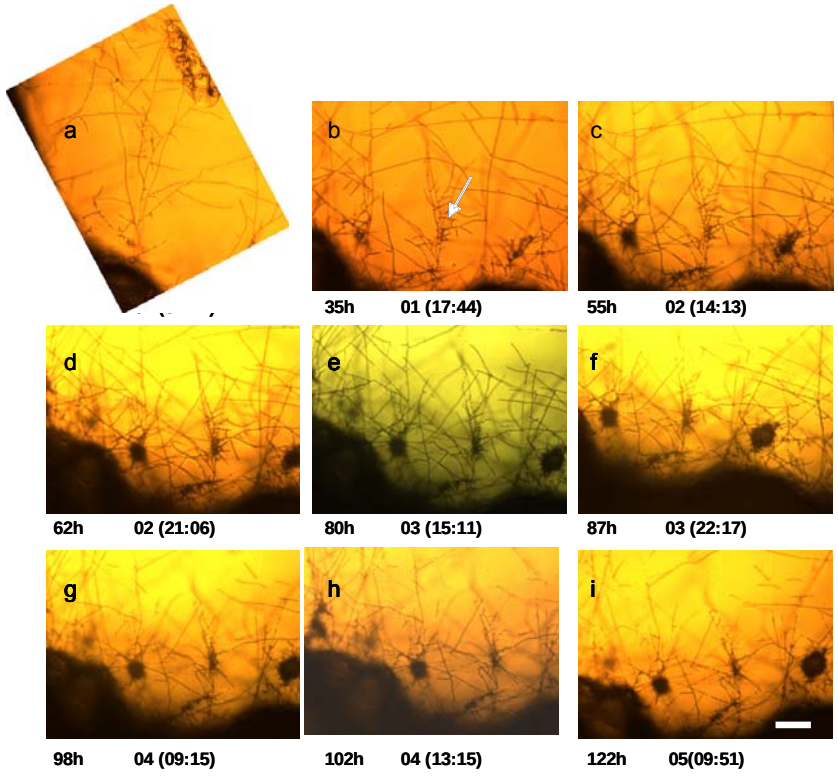

Figure 8. Hyphal growth at 28°C in continuous dark demonstrating development of primary hyphal knots and sclerotia at defined places in a mycelium over the time. In Fig. a, young mycelium with leading hyphae and first side branching is shown. 10 h later in Fig. b, early stages of primary hyphal knots are found in a similar mycelial region and this area was subsequently observed over the time. Note at 83 h, the environmental conditions lead to dryness of the culture, thereby arresting the developmental processes in the mycelium. Underneath each photo, the times of incubation after cutting the window are given together with a date and time referring to the actual hours of the days, the experiment was running. Arrow pointing the area where hyphal development was followed in this study and showed in zoomed version in Fig. 10. Scale bar measures 100 µm.

structures. Within the observation window shown in Fig. 8, the hyphae start slowly to develop into the window after 14-16 h of incubation at 28°C in the dark after the window was made. After 23-24 h of incubation (Fig. 8a), the hyphae grew faster. In the following period of time, the main focus of observation was laid on observing the hyphae at the periphery of the window (Fig. 8). On defined areas, some leader hyphae at this time point form numerous short side branches (Fig. 9a) referred to as trunk hyphae (Glass and Fleissner 2006). 35 hours later the place of intense branching became more obvious - for better visualization of the process of branching, the middle area of the photo shown in Fig. 8b is shown enlarged in Fig. 9b. Two neighboring structures left and right to the place shown enlarged in Fig. 9 were already somewhat further developed into young primary hyphal knots (at around 55 h incubation, Fig. 8b). In subsequent photos, these structures transformed into sclerotia (Fig. 8c).

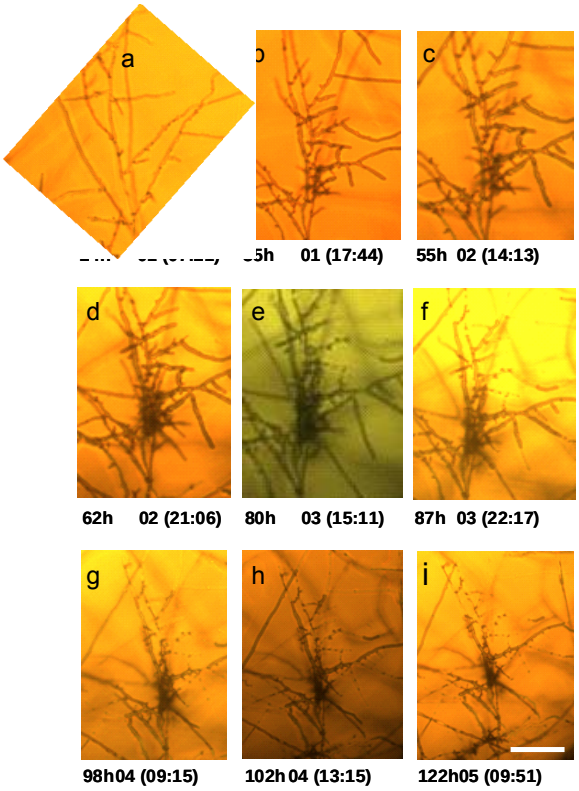

Figure 9. Hyphal growth at 28°C in continuous dark shown to demonstrate the development of primary hyphal knots and sclerotia at defined places in a mycelium over the time. In Fig. a, young mycelium with leading hyphae and first side branching is shown. 10 h later in Fig. b, early stages of primary hyphal knots are found in a similar mycelial region and this area was subsequently observed over the time. The pictures are enlargements of defined areas of mycelium shown in Fig. 8. Underneath each photo, the total time of incubation after cutting the window is given together with a date and time referring to the actual hours of the days, the experiment was running. Scale bar meassures 100 µm.

When the culture plate was incubated for more than 80 h in the dark, these resting stages matured and at this point, the development stopped (Fig. 8e). In parallel, the highly branched structure seen in the middle of the photo 8b continued the development. Intense side branching is observed (see Fig. 8c and 9c at 55 hours of incubation), and formation of a loose aggregate by these branches (see Fig. 8d and 9d at 62 hours incubation). Unfortunately, development of this structure did not continue by desiccation of the hyphae (Fig. 8 f-i, Fig. 9 f-i).

The entire development shown in Fig. 8 and Fig. 9 occurred at 28°C under complete dark conditions. The microscopic observations were done using the yellow filter to exclude from the white light in the microscope all those wave lengths that are active in induction of other developmental structures.

2.4.4 Light induction of secondary hyphal knots in observation windows of cultures of homokaryon AmutBmut

Upon receipt of an appropriate light signal and at ambient temperature (25-28^{o}C in a culture room), primary hyphal knots transform into tightly packed globular three-dimensional structure called secondary hyphal knots (Fig. 10a). Further on, these structures need to stay in defined fruiting conditions with day/night light rhythms (Granado et al. 1997) in order to develop into the complex fruiting structures called primordia (Fig. 10b), which later on develop into mature fruiting bodies. The transition from secondary hyphal knots to primordia formation takes around 24 h, after the appearance of single leader hyphae within the observation window developing in about 40 h through branching and hyphal aggregation into primary hyphal knots and formation of secondary hyphal knots from primary hyphal knots in about 24 h.

The transition between primary hyphal knots to secondary hyphal knots is one of the critical steps in this study. As mentioned earlier, when light hits the cultures before primary hyphal knot formation, mycelium starts producing asexual oidiophores (Fig. 5, 7) (Kertesz-Chaloupková et al. 1998, Kües et al. 1998, Polak et al. 1997). In contrast, with no light the primary hyphal knot within the mycelium turns into the resting structures called sclerotia (Fig. 6). To recognize the correct time point of light induction and the amount of light required, much experience is needed. Repeated experiments revealed that when young primary hyphal knots such as seen in Fig. 2e are present in the mycelium, presenting the cultures for a few minutes (approx. 2 min) to a white light source (normal day light) and transferring them into a day/night rhythms will lead to the formation of secondary hyphal knots. In this study for the day periods, cultures were kept at room temperature (ca. 20-22^{o}C) in a laboratory under natural light. After 24 h, secondary hyphal knots were seen in the windows, and 24 h later young primordia (Fig. 10).

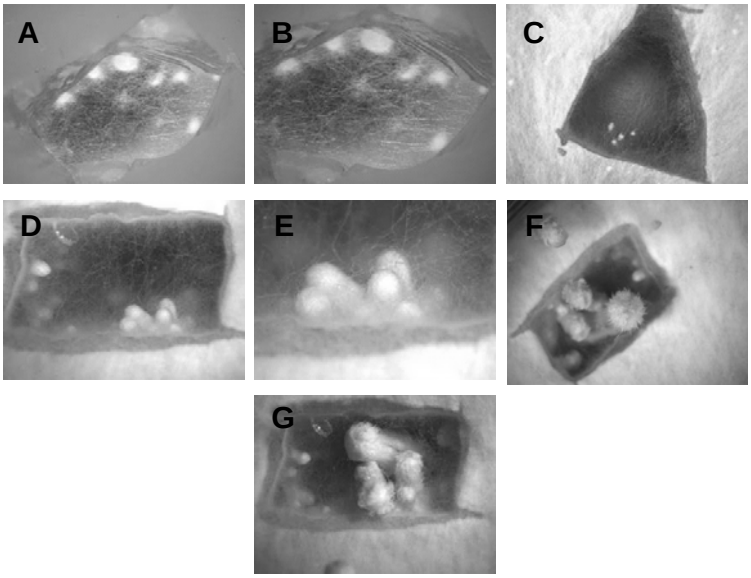

Figure 10. Secondary hyphal knots within a window in an agar culture of homokaryon AmutBmut. Note in 11 C, that there are also secondary hyphal knots in the mycelium on the agar. One day old primordia in a window are shown.

2.4.5 Application of reporter constructs in analysis of initial stages of fruiting

To study the spatio-temporal expression of galectins within the vegetative mycelium at the early stages of fruiting body development and within developing primordia, we constructed a reporter system using *C. cinerea* laccase gene *lcc*1 as a marker. We subcloned the promoters of the *C. cinerea* fruiting body-specific galectin genes c*gl*1 and *cgl*2 in front of laccase gene *lcc*1. The resulting plasmids were termed pYNS2 and pYSK36, respectively (Fig. 12, for further details please see chapter 1.3 in this thesis).

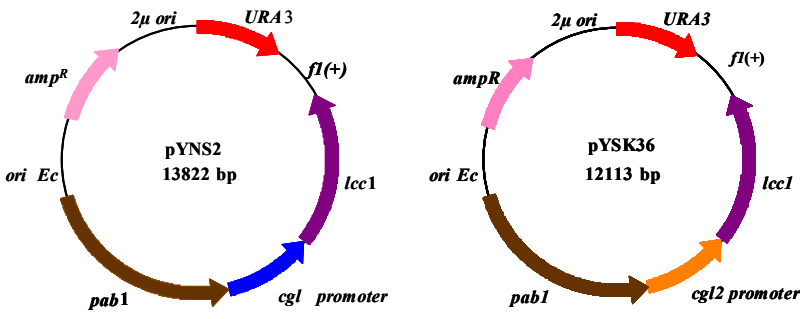

Figure 11. Promoters of galectin genes c*gl*1 *and cgl*2 were subcloned in front of laccase gene *lcc*1 to yield plasmids pYNS2 and pYNS36, respectively. These plasmids were transformed into the *pab-1* auxotrophic homokaryon AmutBmut for expression studies. amp^R = ampicillin resistance gene, *ori Ec* = replication origin for *Escherichia coli*, *f1(+)* = phage origin of replication, *2µm ori* = origin of replication for *Saccharomyces cerevisiae*, *URA3* – uracil selection marker in yeast, *pab1* = PABA (para-aminobenzoic acid) selection marker for *C. cinerea*, *cgl1* and *cgl2* promoter originate from the *C. cinerea* galectin genes *cgl1* and *cgl2*

These constructs were individually transformed into the *pab-1* auxotrophic homokaryon AmutBmut, making use of gene *pab1* to select for positive transformants (Granado et al. 1997). As a control, plasmid pPAB1-2 (Granado et al. 1997) was also transformed into the strain.

2.4.6 Plate assay with substrate ABTS for the detection of laccase activity

Positive transformants from regeneration media plates were pre-cultured on minimal media plates and then inoculated in the middle of YMG/T agar plates containing 0.5 mM ABTS (laccase substrate). When fully grown at 37°C in dark (i.e. when the mycelium reaches the edges of the Petri dishes), some plates were transferred into standard fruiting conditions at 28°C in a 12h dark/12h light regime and some were further kept at 37°C in the dark.

Under standard fruiting conditions, transformants with the laccase gene *lcc*1 under control of the *cgl*1 promoter showed coloration by ABTS conversion through the action of laccase (Kilaru et al. 2005) at the edges of the Petri dish where secondary hyphal knots were formed. The color change was observed only after the plates were shifted to the standard fruiting conditions at 28°C with day and night cycles (Fig. 12a). The color intensity at the outer edge of the plates increased over the following 3 days. After further 24 h, the color slowly diffused and disappeared fully after 12 h. This is in accordance with previous studies of Cgl1 expression using galectin antibodies in Western blot analysis (Boulianne et al. 2000). At 37°C in the dark, cultures of the same transformants did not stain the agar, even not upon prolonged incubation for further 3 days after the cultures were fully grown. Also this is in accordance to formerly established expression patterns of the *cgl1* gene (Boulianne et al. 2000).

Transformants with laccase gene *lcc*1 under control of the *cgl*2 promoter showed coloration in the agar, both at 37°C in the dark and at 28°C in the day and night rhythm. At 37°C in the dark, weakly coloration by laccase action started at the moment the mycelium reached the edge of the Petri dishes and thus fully covered the plates, suggesting that the *cgl*2 promoter is already active in this stage. Over the next 24 h, the color intensified, indicating higher laccase expression from the *cgl*2 promoter (Fig. 12b). This correlated with the Cgl2 expression data presented by Boulianne et al. (2000) and Bertossa et al. (2005). Upon transfer of plates to 28°C into fruiting conditions, the color ring at the edge of the Petri dishes intensified.

Furthermore, additional staining started from the inoculum at the middle of the plate two days after the transfer. The area of coloration spread within the agar from the inoculum to the edges of the Petri dishes over the next 24 h. At day 3 after transfer, the whole agar was stained dark brown (not shown).

Control transformants of plasmid pPAB1-2 showed no coloration during incubation at 37°C in dark, also not within the three days of further incubation at 37°C after the plates were fully grown. At 28°C in the day and night rhythm, control plates initially showed no color change (Fig. 12c). However, at the 4th day of incubation at fruiting conditions a color change was observed at the inoculum (diameter of area ca. 2 cm). In the next 24 h, staining spread over the whole agar and at the end, the whole agar was stained dark brown (not shown).

2.4.7 Localization of galectins during fruiting body development

Expression of laccase Lcc1 under control of the *cgl*1 promoter and the *cgl*2 promoter did not block further fruiting body development. Like control transformants, positive laccase transformants produced about 5 fruiting bodies per YMG/T agar plate. Since genes *cgl*1 and *cgl*2 are active also during the primordia formation up to maturation (Boulianne et al. 2000, Charlton et al. 1992), laccase activity was analyzed in primordia at the stage of karyogamy (compare Table 1 in chapter 4). First, each two primordia per transformant were taken into 1 ml of 120 mM sodium acetate buffer, pH 5.6 and grinded. By centrifugation, supernatant and cell debris were separated. 100 µl of 50mM ABTS was added to the supernatant. 1 ml of 120 mM sodium acetate was added to the cell debris and 100 µl of 50mM ABTS. In 24 h incubation at room temperature, neither the samples from laccase positive transformants nor from pPAB1-2 transformants gave a color reaction that would indicate laccase activity.

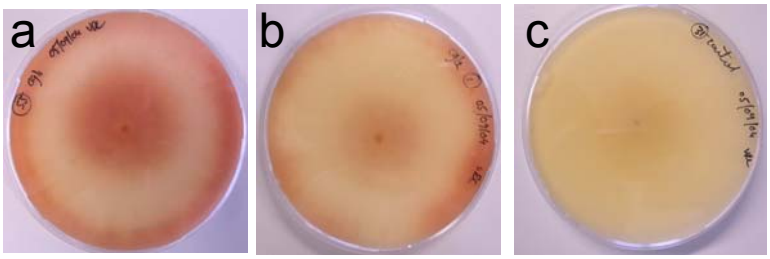

Figure 12. Plate assay to detect the laccase expression. a) Localized *lcc1* expression under control of the *cgl1* promoter. (b) Localized *lcc1* expression under control of the *cgl2* promoter. (c) No laccase expression in pPAB1-2 control transformant.

Table 1. Expression of *lcc*1 under the control of galectin gene promoters in the cultures of *C. cinerea* homokaryon AmutBmut transformants at different environmental conditions.

Construct in transformants	Number of transformants		Phenotype	
	Tested	Positive	37°C continuous dark	After transferring from 37°C to 28°C day/night
*cgl*1 promoter/*lcc*1 in pYNS2	58	22	No expression	Expression at the edges of the culture where secondary hyphal knots were seen
*cgl*2 promoter/*lcc*1 in pYSK36	17	7	Expression at the edges of the culture	Disappearance of staining at the outer edges; expression starts slowly at the inoculum and migrate towards the outer edge of the cultures
pPAB1-2 (Control)	38	38	No expression	No expression

In a second experiment, living material was analyzed for laccase activity. Fresh primordia (at day 5, undergoing karyogamy, minor stipe elongation) were sectioned and incubated in 800 µl of 120 mM sodium acetate buffer containing 50 mM ABTS (100 µl). Developing primordia from a *cgl*2 transformant with *lcc*1 under control of the *cgl*2 promoter showed a color change in the inner tissues of the stipe upon incubation at room temperature for two days. However, the buffer also showed a blue color indicating laccase activity (Fig. 13). Primordia of a transformants *cgl*1 and the surrounding buffer showed such coloration only after 3 days of incubation at room temperature (not shown). No coloration was observed in control samples of primordia from pPAB1-2 transformants (not shown).

Figure 13. Localization of laccase activity in primordia samples of a transformant with *lcc*1 under the control of *cgl*2 promoter.

Charlton et al. (1992) showed in Northern blot analysis the *cgl*1 and/or *cgl*2 genes are well expressed in primordia during the stage of karyogamy. According to this observation, we would have expected laccase activity directly upon harvest of the primordia. That it took 2, respectively 3 days of incubation before detecting a color reaction could either mean that laccase was before not expressed in the tissues, or that laccase was directly degraded upon expression. In the later case, a proteolytic activity has to be postulated that stops its reaction after some time allowing late functional expression of laccase.

2.5 Discussion

Fruiting body formation in *C. cinerea* is influenced by several environmental and genetic factors. Environmental factors like light, temperature, humidity and nutrients were well studied in *C. cinerea*. Apart from the mating type genes, genetic factors influencing the fruiting development are so far little known (Kües and Liu 2000, Kües et al. 2004, Wessels 1993, Kües 2000). Within the early stages of fruiting body development two fruiting specific galectin genes (*cgl*1 and *cgl*2) were found expressed in secondary and primary hyphal knots, respectively, and in young primordia in the extracellular matrix of cap and stipe (Boulianne et al. 2000). Within these regions in other basidiomycetes, expression of fungal specific hydrophobins was reported (Walser et al. 2003). P. J. Walser in his unpublished observations showed an interaction between galectins and hydrophobins in *in-vitro* conditions (see chapter 1). This gives rise to the hypothesis, that both galectins and hydrophobins might have an influential role in fruiting body development, either in combination or individually. For better understanding of expression patterns during early stages of fruiting body development a detailed description and clear visualization of these stages by photography is of great importance.

Previously, differences in the terminology of developmental stages of fruiting between different fungal species created unwanted confusion in understanding the cytological changes during fruiting (Walser et al. 2003). However, also in *C. cinerea*, different research groups follow different nomenclatures (Liu 2001). Probably this is one reason why the initial steps in fruiting are still poorly described (Matthews and Niederpruem 1972, 1973, Liu 2001, Göbel 2003). Another reason is the special technical demand and the request for particular personal skills in observation of very early developmental stages (Liu 2001, Göbel 2003). In this study, we applied both

cytological and molecular approaches to define the initial stages of fruiting body development in *C. cinerea* and the precise localization of fruiting body specific galectins, respectively.

Following the observation made by Liu (2001) and Göbel (2003), we further extended our microscopic studies on hyphal knots formation in *C. cinerea*, using a window technique developed first by Liu (2001). The observation windows were made in the fully grown mycelium agar cultures. A square shape of 1 cm x 1 cm was proven to be best for the observation of hyphal knots under the microscope. The plates were incubated in inverted position so that water condensation within the window can be prevented, which is important for that hyphal growth can be observed clearly under the microscope. The observation and documentation was done at every 3-4 hours time intervals. The entire development of hyphal growth and primary hyphal knot formation happens at 28^{o}C under complete dark conditions. The microscopic observations were done using the yellow filter to restrict UV white light, as the white light induces changes in *C. cinerea* development (Kües et al. 1998). White light exposure to cultures at around 40 h after window making and incubation leads to production of oidia (Liu 2001, this study).

The first sign of hyphae growing into the window is seen after 14-16 hours after a window was made, under incubation conditions of 28^{o}C in the dark. After 23-24 h of incubation the hyphae start to grow fast. Hyphae forming the subapical branching were repeatedly found forming the primary hyphal knots. Formation of primary hyphal knots occurs after 55 h incubation. The entire development happens at 28^{o}C under complete dark conditions. When the culture plates are incubated more than 70 h in dark, the three-dimensional hyphal structures develop into a resting stage called sclerotia, where they stop their development.

Influence of the external environmental factor like light within the time course of the hyphal knots development were followed. Formation of primary hyphal knots is restricted to outer edges of the window. In this study, for the first time light induced secondary hyphal knot formation was repeatedly achieved in observation windows in mycelial cultures of homokaryon AmutBmut. The secondary hyphal knots were regularly localised, at the outer edges of the windows where before the primary hyphal knots were seen. However, also in this study the direct transition from primary hyphal knot to secondary hyphal knot was not documented. But secondary hyphal knots were observed always within the regions of primary hyphal knot formation in

the observation window, which is in support of the hypothesis that secondary hyphal knots developed from primary hyphal knots.

In addition, there is indirect genetic evidence for this morphological change by mutants that are blocked in primary hyphal knot formation and sclerotia production and will never give rise to secondary hyphal knots (Moore 1981). Moreover when fruiting is induced in the *C. cinerea* monokaryon AT8 by introducing of foreign *A* and *B* mating type genes, there is also induction of primary hyphal knot formation (Kües et al. 2002). Thus in future, it would be valuable to elucidate the transition between primary hyphal knot to secondary hyphal knots with the window technique, knowing now the precise time point at which a light signal has to be given and the amount of light required.

Furthermore, taking the advantage of a well developed laccase reporter system (Kilaru et al. 2006), we studied the spatio-temporal expression from the galectin gene promoters using *C. cinerea* laccase (*lcc*1) gene as reporter. Transformants with the *cgl*1 promoter showed coloration after the light induction at the edges of mycelium cultures of homokaryon AmutBmut, where secondary hyphal knots were seen. The color change was observed only after the plates were shifted to fruiting conditions at 28^{o}C with day and night cycles. In contrast, transformants with the *cgl*2 promoter showed expression of laccase from the 5th day of growth in dark conditions at 37^{o}C. At later age, coloration is observed in the middle of the plate at the position of inoculum and over the time, the coloration spreads all over the plate. Transformants with pPAB1-2 that served as control did not show any staining till the end of experiment when color developed first at the inoculum in order to spread further over the whole plate. In conclusion, there is intrinsic laccase activity in AmutBmut but only at a senescent mycelial stage. Aging cultures of *C. cinerea* usually show browning underneath the mycelial mats (North et al. 1973) and phenoloxidase activities are possibly linked to such processes (Nyunoya and Ishikawa 1980).

Within developing primordia, expression of laccase is expected for both promoter-gene *lcc*1 constructs, since both *cgl*1 and *cgl*2 are expressed at that stage (Charlton et al. 1992, Boulianne et al. 2000). Galectin proteins are localized within outer stipe and outer cap tissues. Therefore, in our laccase expression test we would have expected blue coloration specifically in these tissues. However, at the point of primordia harvest laccase activity was not detected, but within primordia staining occurred within the inner stipe tissues finally after 2 to 3 days of incubation. Within primordia

at the stage of karyogamy, transcripts were detected for the natural laccase genes *lcc*1 to *lcc*4, *lcc*11 to *lcc*13 and *lcc*16 and *lcc*17, but enzymatic activity at this stage were not detected (M. Navarro-González, personal communication). M. Navarro-González, tested laccase activities from the very early stages of primordia formation till fruiting body maturation. However, she found enzymatic activity earliest at the developmental stage of meiosis (see table 1 in chapter 4) and in the mature fruiting bodies (observations by M. Navarro-González, personal communication). A similar pattern of phenol oxidase activity during fruiting body development of *C. cinerea* was reported by Vnechak and Schwalb (1989). Expression of laccase genes occurs with changing genes over the whole fruiting process (observations by M. Navarro-González, personal communication). Proteolytic degradation of produced enzyme could be an explanation for these contradictory results. Indeed in overexpression studies of laccases in *C. cinerea*, Kilaru et al. (2006) observed that for some genes laccase activity can be detected in agar possibly by protection of the enzymes against slower diffusion of proteases, but not in liquid cultures, where proteases are freely diffusible. In other cases, no laccase activity in *C. cinerea* was found albeit the amounts of laccase gene transcripts were present and the activity of the proteins could only be proven by subsequently expression of cDNA in the yeast *Saccharomyces cervisiae* (Kilaru et al. 2006).

In conclusion, lack of detection in primordia at the stage of karyogamy is not a proof for lack of expression from the *cgl*1 and *cgl*2 promoters. 2 days later in the aging primordia, localization of laccase activity in the transformants with *lcc*1 under control of the *cgl*2 promoter was observed in the inner tissues of stipe in developing primordia, and after 3 days of incubation in a buffer, staining of inner stipe tissues occurred in primordia of a transformant with the *cgl1* promoter. In nature, upon karyogamy the primordia rapidly develop further into a mature mushroom. Arresting at the stage of karyogamy as forced here by harvesting and incubation in a sodium acetate buffer occurs in nature for a large percentage of primordia that translocate nutrients to a single primordium in favor for its maturation into fruiting body (Moore 1989). Whether upon aging of such senescent primordia *cgl*1 and *cgl*2 gene expression occurs, has not been analyzed so far.

In conclusion from the results from this chapter, we showed that it is possible to follow over the time on a single structure the whole development of primary hyphal knots. The light requirements needed for reliable formation of primary hyphal knots

into secondary hyphal knots have been established but future microscopic work needs to definitely proof that the secondary hyphal knots are coming from primary hyphal knots. Furthermore, the study showed that laccase gene *lcc*1 under control of galectin gene promoters can be used a reporter gene to identify mycelial regions where primary hyphal knots, respectively secondary hyphal knots will be formed. In later stages of development, expression results with the constructs were not obtained and it is possible that protease activities block laccase activities during tissue formation and maturation. Thus, other promoter fusions with a gene for a different type of reporter might have to be developed. However, since at last the early stages of fruiting could clearly be documented with expression of *lcc*1, in the future other promoters might be tested for activity in early fruiting combining them with the gene *lcc*1. For example, with the availability of *C. cinerea* genomic sequence (http://www.broad.mit.edu/annotation/fungi/coprinus_ cinereus/) by repeated Blast searching using the known fungal galectin sequences (the approach as followed in chapter 3), we identified a potential third galectin gene (*cgl*3) (see chapter 1.3), whose functional features and expression patterns have yet to be revealed. Moreover, the fungus has multiple hydrophobin genes (see chapter 3) whose expression in vegetative mycelium and early stages of fruiting can be followed making use of the *lcc*1 reporter system.

2.6 Acknowledgements.

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CHAPTER 3

Coprinopsis cinerea (*Coprinus cinereus*) has multiple hydrophobin genes

3.1 Abstract

Coprinopsis cinerea has the largest hydrophobin gene family known so far in the basidiomycetes. Within the genome of *C. cinerea*, we found 32 potential hydrophobin genes by multiple Blast searches using known hydrophobin sequences from other fungi, and using different cut-off E-values ($1e^{-3}$ to $1e^{-0}$). Homologies are very low, so that some heterologous hydrophobins detected none of the *C. cinerea* genes and no heterologous hydrophobins found all the genes. Furthermore, two extra genes were detected in Blast searches with the deduced *C. cinerea* proteins, giving a total of 34 potential hydrophobin genes in the genome of *C. cinerea*, one of which (*coH*7) had a frameshift. Several of the multiple hydrophobin genes are distributed on different contigs in clusters. Clustered genes have high similarities at the protein level, suggesting that these genes arise from recent duplication events. The translated protein sequences contain eight cysteine residues at the conserved positions typical for the class I hydrophobins. Protein analysis using different technical approaches (ESI-LC and SELDI Mass Spectrometry) identified different proteins during growth and different stages of fruiting body development. Phylogenetic analysis of the hydrophobins from *C. cinerea* and heterologous basidiomycetes contradicts the earlier hypothesis on evolutionary divergence of hydrophobins based on their stage of expression.

Key words: Hydrophobins-Multi-gene family-*Coprinopsis*-Fruiting body development

3.2 Introduction

Hydrophobins are small-secreted proteins of about 100 to 140 amino acids that are only known in fungi. These slightly hydrophobic proteins are characterized by 8 conserved cysteines embedded in amino acid sequences of an overall low homology. Upon secretion into liquid medium, hydrophobins self-assemble at air-water interfaces, thereby forming amphipathic films. Upon attachment onto cellular surfaces, such films enable fungal structures to grow into the air and protect them from adverse environmental conditions. Hydrophobin films are very stable and it needs harsh acid treatment for their dissociation (for further details see reviews of Wessels 1999, Wösten 2001, Whiteford and Spanu 2002, Walser et al. 2003, Wösten and Wessels 2006).

Based on the biochemical and hydropathy patterns, hydrophobins are divided into two classes, I and II (Wessels 1994, 1997). Class I proteins form characteristic stable rodlet layers resembling amyloid structures (Wösten et al. 1993). In contrast, class II hydrophobins are easier to dissolve. They assemble to crystalline fibrils and highly ordered crystalline monolayer films (Torkkeli et al. 2002, Hakanpää et al. 2004). Proteins of both classes are widely distributed in ascomycetes (Whiteford and Spanu 2001), whilst in basidiomycetes up to now only class I hydrophobins have been reported (Walser et al. 2003). Class I hydrophobins from basidiomycetes are phylogenetically diverged from those of ascomycetes (Wösten 2001).

Hydrophobins play a major role in structural hyphal development and tissue differentiation. For example, in the best-studied basidiomycete *Schizophyllum commune* hydrophobin SC3 is primarily expressed in the vegetative mycelium of homokaryons and dikaryons where it covers individual aerial hyphae. In the fruiting bodies, SC3 and also SC1, SC4, and SC6 are expressed. They protect the tissues of fruiting bodies from water logging. Likewise, air channels in mushrooms of *Agaricus bisporus* are protected by ABH1/HYPB hydrophobins preventing them from collapsing and filling with water (further reading in Wösten 2001, Walser et al. 2003).

In *Coprinopsis cinerea* (*Coprinus cinereus*), a class I hydrophobin (CoH1) was reported from mycelial samples of monokaryon JV6. CoH1 and a potential other hydrophobin, CoH2 are encoded by genes (*coH*1 and *coH*2) localized in a close proximity to each other (Ásgeirsdóttir et al. 1997). In addition, hydrophobins have been isolated from mushroom primordia and shown *in-vitro* to interact with fruiting body-specific galectins (Walser et al. 2003). Galectins are extracellular matrix proteins belonging to the family of β-galactoside binding lectins (Cooper et al. 1997, Boulianne et

al. 2000, Walser et al. 2004). The galectins in *C. cinerea* are specifically expressed in outer mushroom stipe and cap tissues (Boulianne et al. 2000, Walser et al. 2005). In other basidiomycetes, such fruiting body tissues have been shown to produce specific hydrophobins (Wessels et al. 1995, Lugones et al. 1996, de Groot et al. 1999). Thus, it will be interesting to establish in the future whether the *in-vitro* interaction observed between *C. cinerea* galectins and hydrophobins is a biological relevant finding (Walser et al. 2003).

Structurally, the fruiting bodies and the developmental course of fruiting are best understood in *C. cinerea* (Moore 1998, Kües 2000, Walser et al. 2003) and various genes specifically acting in fruiting body development have been identified from mutant analysis in *C. cinerea* (Kamada 2002, Liu et al. 2005, Terashima et al. 2005). However, genes for mushroom hydrophobins have not yet been described for this species in contrast to various other species: *Agrocybe aegerita*, *A. bisporus*, *Dictyonema glabratum*, *Flamminula velutipes*, *Lentinula edodes*, *Pleurotus ostreatus* and *S. commune* (Walser et al. 2003). In this study, the genomic sequence of *C. cinerea* monokaryon Okayama 7 as released on 16th July 2003 by the Broad institute (http://www-genome.wi.mit.edu/annotation/fungi/coprinus_ cinereus/index.html) assisted in identification and analysis of further hydrophobin genes. In total, we identified from the genome a family of 34 different hydrophobin genes and showed by protein analysis that two were expressed in the mycelium and four different hydrophobins in fruiting body primordia.

3.3 Material and methods

3.3.1 Strains and cultural conditions.

C. cinerea strains JV6 (*A42 B42*) and Okayama 7 (*A43 B43*) are monokaryons, strain AmutBmut (*A43mut B43mut pab1*) a self-compatible homokaryon producing fruiting bodies due to defects in the mating type loci (May et al. 1991, Kertesz-Chaloupková et al 1998). Strains were routinely grown at 37°C on YMG/T medium (Granado et al. 1997). Dikaryons were produced by placing mycelial samples of two compatible strains in close vicinity on YGM/T agar plates (Walser et al. 2001). Fruiting conditions were as described by Granado et al. (1997). 2-day old primordia (Walser et al. 2003) and vegetative mycelium grown for 5 days in YMG/T liquid medium were harvested and freeze dried for hydrophobin isolation using the extraction procedure described by

Lugones et al. (1996). Mycelium of *S. commune* monokaryon 4-40 (*MAT*A43 *MAT*B43; CBS 341.81) was obtained for hydrophobin isolation from liquid cultures grown in constant light at 25°C in minimal medium (Dons et al. 1979).

3.3.2 Sequence analysis.

Gene bank accession numbers for class I hydrophobin sequences from basidiomycetes used in BLAST search (tblastn, with different cut-off levels) of the *C. cinerea* genomic sequence (http://www-genome.wi.mit.edu/annotation/fungi/coprinus_cinereus/ index.html) are found in the legend of Fig. 1. Accession numbers of ascomycetous hydrophobin sequences used in Blast searches are *Claviceps fusiformis* CFTH1: Q9UVIA, *Trichoderma reesei* HFBII: P79073, HFBI: P52754, *Aspergillus fumigatus* P41746, *Aspergillus nidulans* DEWA: EAA58809, *Gibberella moniliformis* HYD1: AA016867; HYD2: AA016868; HYD3: AA016869; HYD4: AA016870; HYD5: AAN76355, *Neurospora crassa* RODL: Q04571; Ccg-2: A46222, *Cladosporium fulvum* hcf2: CAD974454; hcf3: CAD92803; hcf4: CAD92804, *Aspergillus oryzae* hypA: BAC77247; hypB: BAC 77248, *Metarhizium anisopliae* ssgA: JC1429, *Magnaporthe grisea* MPG1: AAA20128, *Paracoccidioides brasiliensis* hyd1: AAM88289, *Ophiostoma ulmi* CU: Q06153. *C. cinerea* hydrophobin gene sequences were analyzed for intron positions using consensus sequences defined for this species (Seitz et al. 1996, Hoegger et al. 2004). Alignments of deduced protein sequences were generated with ClustalX (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html) and manual adjustments were made in Gene Doc ver. 2.6.002 (http://www.psc.edu/biomed/genedoc/). Phylogenetic analysis of amino acid sequences was performed with Mega ver. 2.1 (http: //www. megasoftware net/). Signal peptides were predicted with SignalP ver. 2.0 (http: www.cbs.dtu.dk/services/). Molecular weights of processed proteins were calculated with ExPasy (http://www. expasy.org/tools /protparam.html) program.

3.3.3 Protein methods.

Hydrophobins were isolated by using tri-fluroacetic acid (TFA) as described in Ásgeirsdóttir et al. 1(998) and about 20 µg per sample separated on 15% SDS-PAGE and stained with silver and Coomassie Blue for molecular weight determination and for peptide digestion, respectively (Ásgeirsdóttir et al. 1998). Protein glycosylation was tested on an acrylamide gel by periodic acid staining following the

protocol described by Packer *et al*. (1998). SC3 isolated from *S. commune* 4-40 served as positive control (known glycoprotein) (Ásgeirsdóttir et al. 1994). Total protein amount in the samples was determined by the modified micro Lowry method (Peterson 1977) using bovine serum albumin (BSA) as a standard. Coomassie Blue-stained protein bands were excised from gels and digested with chymotrypsin for 24 h in dark at room temperature, following the protocol from Bruker Daltonik GmbH (Proteomic protocols for mass spectrometry). Peptide sequencing was performed with ESI-LC-MS (Electro Spray Ionization-Liquid Chromatograph-Mass Spectrometry; Esquire-3000, Bruker Daltonics, Bremen, Germany) and intact protein masses were determined by SELDI - TOF (Surface-Enhanced Laser Desorption Ionization/Time of Flight) MS (Ciphergen Biosystems, Fremont, USA). All data were processed by Bruker Daltonics Data Analysis software and MS/MS spectra were exported in Mascot Generic Format (MGF). Mascot (version 2.0, Matrix Science) searches were performed at first against the MSDB protein database (Protein sequence database by the Proteomics Department at the Hammersmith Campus of Imperial College, London; http://www.matrixscience.com/help/seq_db_setup_msdb.html). In a second search, a custom-made database was used that consisted of all SNAP predicted and annotated protein sequences from the *C. cinerea* genome (Jason Stajich, Duke University, unpublished) combined with the Swiss-Prot database (http://www.expays.org/sprot/), and 34 sequences of *C. cinerea* hydrophobins annotated within this study (unpublished). All cysteine residues were searched as reduced but carbamidomethyl-cysteine and methionine residues were not allowed to be oxidized. Two cleavage sites (Tyr, Trp; Kamp 1986) were allowed for digestions with chymotrypsin (sequencing grade from Roche laboratories) in the searches and Mascot parameters were set to use monoisotopic masses with tolerance of 1.5 for precursor ions and 0.5 for fragment ions.

3.3.4 RNA isolation and cDNA synthesis.

Total RNA was isolated from the mycelial and fruiting samples as mentioned in material and methods of the paper of Boulianne et al. (2000). Samples were grinded to a fine powder and total RNA was extracted by using guanidinium isothiocyanate (Boulianne et al. 2000). cDNA was synthesized using RevertAid M-MuLV Reverse Transcriptase (Fermentas, Gerard and D'Alessio 1994) according to manufacturer's instructions. 2 µg of total RNA was used in each reaction and the obtained cDNA was used for transcript profiling. The synthesized cDNA was analyzed using ß-tubulin gene

specific primers (ß-tubF, 5'-ATGCGTGAAATCGTCCACCTC-3', ß-tubR, 5'-TCACACTGAGCGGTGAGAAC-3'). The cDNA was used for hydrophobin transcript analysis using gene specific primers (Table 1) individually on the above described samples from different strains and stages of fruiting body development in C. cinerea. The annealing temperature for all the PCR reactions were at 55oC for 30 sec. Aliquots of the PCR products were then analyzed on 2% agarose gels.

3.4 Results

3.4.1 Defining potential hydrophobin genes from the genome of *C. cinerea* Okayama 7

Using the published sequences of hydrophobins CoH1 and CoH2 from *C. cinerea* monokaryon JV6 (Ásgeirsdóttir et al. 1997) in tblastn searches (E-values $1e^{-3}$, $1e^{-2}$, $1e^{-1}$, $1e^{0}$), we detected in the Okayama 7 genome alleles of genes *coH*1 and *coH*2 and, in addition, copies of 24 extra genes. CoH1 detected 23 of these extra genes, CoH2 only 16 of them (Fig. 1B). The results indicate that, due to poor overall sequence conservation, hydrophobin genes are easily overlooked within a genome by searching only with a single or with a few known protein sequences.

To identify as many different hydrophobin genes as possible, we next searched the *C. cinerea* genome with all hydrophobin sequences so far available from basidiomycetes (see legend of Fig. 1A) using the E-value $1e^{-3}$ as a cut off value. Not all proteins of the other species detected sequences of *C. cinerea* (i.e. *U. maydis* Hum2, *L. edodes* Le.Hyd1; Fig. 1B). AF331452 of *P. ostreatus* var. *florida* identified only one gene (*coH*31; Fig. 1A). No one of the heterologous sequences detected all *C. cinerea* genes but in most cases several different genes were found (between 8 and 30 different genes) and not any two heterologous proteins found the same subset of genes (Fig. 1B). In addition to the 26 genes already known from the analysis with CoH1 and CoH2, six further hydrophobin genes appeared in the screen with heterologous sequences, giving a total number of 32 different *C. cinerea* hydrophobin genes (Fig. 1A). When we used sequences from class I hydrophobins and class II hydrophobins from ascomycetes (E-value: $1e^{-3}$), no *C. cinerea* hydrophobin gene was detected.

Table 1. Gene-specific primers for *C. cinerea* genes (forward: coding strand, reverse: non-coding strand; note that primers used for *coH1* are chimeric and come from production of an expression construct made for transformation of *coH1* into *S. commune*, R. Velagapudi unpublished. * In brackets: new primer sequences in confirming extra 5´introns in these genes by amplifying cDNAs of the expected sizes

Name	Primer sequence	Type
Sc3_*coH1*	TTCGCTATCCTTCCTTACAACCTGCTCGCC ATGCAGTTCAAGTTCTTGTC	forward
lcc1term_*coH1*	ACTGGCCCTCTGGTCAACTATAATA TTATTTAGAGGTTGATGTTGATGG	reverse
coH2	GGTTGCTGAACATTTCC	forward
coH2	CGCACAAACGAACTTCA	reverse
coH3	CGCTTCTCTCGCTGTT	forward
coH3	GCGCTCGACGAACTG	reverse
coH4	GTGCAATACCGGTCCA(*TTGGCTGCAGTCTCCG)	forward
coH4	CGTTAATATAATCACCCGA	reverse
coH5	CAAAGTTCTCTCTACCC	forward
coH5	CTCAAACATCCCCCAC	reverse
coH6	GTTCAGTGCTGCAATAGT	forward
coH6	CTGAGTTGGCAGGGAT	reverse
coH7	TCCACTGCGTTGCTAC	forward
coH7	CTCGAACTCAGAACGC	reverse
coH8	AGGAGATGACGACTGC(*GTCACCGCTATTCCTCG)	forward
coH8	GCCTCCTGGACAAGC	reverse
coH9	ATGAAAGCCACCACTTTC	forward
coH9	GCAGAGGAGTGGAAAG	reverse
coH10	TAGCAGCTATTCCAAGC	forward
coH10	ATGGTATGGGAGAGGC	reverse
coH11	GCTTTGACTGTTGCTAC	forward
coH11	TGAGTGACGGAAATGAAG	reverse
coH12	TCACCGTCTCCGTGA	forward
coH12	CGGAATGATGGGTCG	reverse
coH13	TCCTCTTCCCAGCAC	forward
coH13	GTCCACCTCATCCGG	reverse
coH14	CTCCTTGCAGTTGCTC	forward
coH14	GGCCACAATTCACAAAG	reverse
coH15	CACTATGCTGCTCCAAC(*GTTTTCCTCAACTTGGCC)	forward
coH15	CGAGCGAGGGAAAGA	reverse
coH16	GCCCAACTATAACCCAG	forward
coH16	GGCCAGAGTCGTGAA	reverse
coH17	GCTGCAATAAGGCTCAG	forward
coH17	GCAGGGTAGTCGTAAG	reverse

Name	Primer sequence	Type
coH18	GCACCAAGAGTGTGC(*AAGCTCGCTTTCATCGC)	forward
coH18	TAGTAGGGAATGTGGGT	reverse
coH19	AATTGCTCTCGTCGATC	forward
coH19	GGAACGGGAGTGAACA	reverse
coH20	GCAACGACGTTAAGGAG	forward
coH20	CTTGCGATAGGGTTTGT	reverse
coH21	ACTCGTCTGTCAAGAAG	forward
coH21	CACTGTGAGATGAGAGC	reverse
coH22	ACGAGCAATGCGAAG	forward
coH22	CCCACTTGATTACGGC	reverse
coH23	GGTGCTTAACATCGACC	forward
coH23	CTCACCCGCCAATAG	reverse
coH24	AATGGAGGTGTCGTCC	forward
coH24	CGCAGAGCGGGGTAT	reverse
coH25	GCAGCAACAATTTGAGC	forward
coH25	ATGCGTTGAAGGGTGC	reverse
coH26	GGTGGCACAGTTCAG	forward
coH26	GAGCAGCACATGGTC	reverse
coH27	GAGGTCGAGTACGAAC	forward
coH27	GATAATGTGAAAGCTCC	reverse
coH28	TCACCTCTGCTCTGC	forward
coH28	ACCTATCCCCTGGGT	reverse
coH29	Same as *coH28*	
coH29	GCCTGCAGGGTTATG	reverse
coH30	CTCTCGACTCTCATCC	forward
coH30	CATGAAGAAATATGGTGCA	reverse
coH31	ACCACCTCCCACTCT	forward
coH31	CGTATCCGCTGGATCA	reverse
coH32	TCCGCCTCTCACTCA	forward
coH32	CAACCTACCATCCCGA	reverse
coH33	CCAGAACAGCTTTCTCAA	forward
coH33	CTCCAGTGAAAACGCC	reverse
coH34	CACTCACCACCTCAC	forward
coH34	TCCCAACCATGAACTGC	reverse
β-tubulin	ATGCGTGAAATCGTCCACCTC	forward
β-tubulin	TCACACTGAGCGGTGAGAAC	reverse

In a further round of screening (E-value: $1e^{-3}$), we used the deduced protein sequences of all *C. cinerea* hydrophobin genes in searching the genome of the fungus. In consequence, two more genes appeared (*coH*8, *coH*10; Fig. 1B). *coH*8 was detected only by CoH9, whereas *coH10* was detected by 5 other hydrophobin sequences of *C. cinerea* (Fig. 1B). Interestingly in the reversal analysis, CoH8 detects *coH*8, *coH*9 and *coH*19 (Fig. 2). Moreover, CoH10 detects 22 different genes (Fig.1 A). Similar trends that identification of genes by protein products was often not reciprocal were observed also for other proteins and genes (Fig. 1B). The results indicate that the difference in reciprocal recognition are due to the very low overall similarity of hydrophobins being in the range of the standard cut-off value of identity in the Blast searches (E-value: $1e^{-3}$). When lowering the E-value to $1e^{-2}$, $1e^{-1}$ and $1e^{-0}$, respectively, no further hydrophobin genes were detected although in general the number of hits to the already found genes increased. With the heterologous hydrophobins, even at the level $1e^{0}$ a maximum of 32 different *C. cinerea* genes were depicted only in two cases (PoH1 and Vmh1-2 of *P. ostreatus*; see Fig. 3 in the appendix) and no single *C. cinerea* gene was found by any heterologous hydrophobin. At the E-value $1e^{-1}$, Le.hyd1 of *L. edodes* found just CoH27, and at $1e^{-0}$ a total of 11 genes were identified, whereas Hum2 of *U. maydis* only found CoH27 at the E-value of $1e^{-0}$ and no other genes (see attachment to this chapter). Gene *coH*8 was still not found by any of the heterologous proteins unlike gene *coH*10 that was found by HYD-Pt3 at the E-value $1e^{-2}$, by a total of 10 heterologous proteins at the E-value $1e^{-1}$ and by a total of 15 genes at a cut-off level of $1e^{-0}$. Also in the parallel analysis with all homologous proteins, no hydrophobin gene was found by all proteins, even not at the cut off level of $1e^{-0}$. At this low cut-off level, only *coH*9 and *coH*19 recognized gene *coH*8 and only four extra hydrophobins (*coH*2, *coH*4, *coH*7, *coH*10) found gene *coH*9 as compared to the analysis with the E-value $1e^{-3}$ (see Fig. 3 in attachment of this chapter).

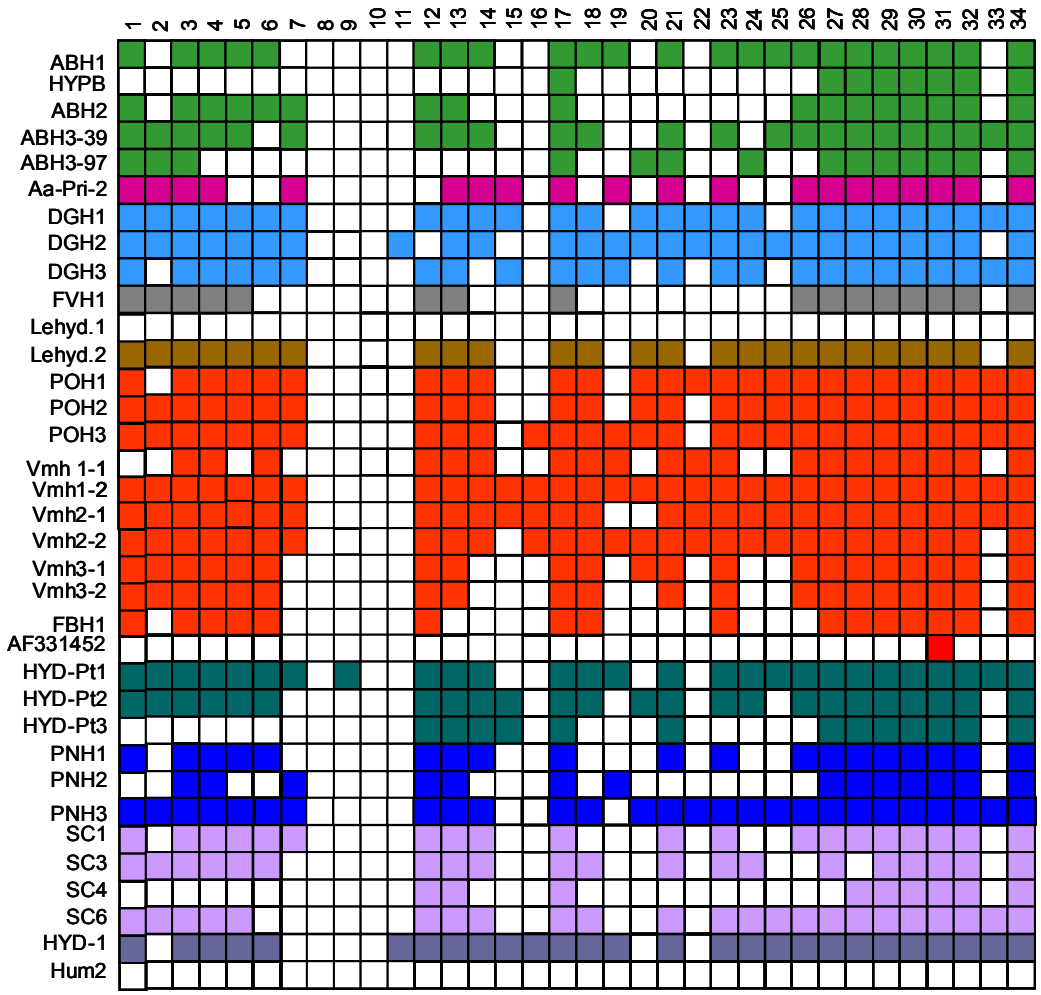

Figure 1(a). **Identification of *C. cinerea* hydrophobin genes** (top line: numbers indicate the respective *coH* genes) by hydrophobins from other basidiomycetes (shown left) by Blast searches (cut off level: $1e^{-3}$) of the *C. cinerea* genome (http://www-genome.wi.mit.edu/annotation/fungi/coprinus_cinereus/index.html). Gene bank accession numbers of the known basidio- and ascomycete hydrophobins: *A. bisporus* ABH1/HYPA: CAA03494, ABH2/HYPC: P49073, HYPB: CAA03496; ABH3-39: CAA74939.1, ABH3-97: CAA74940.1; *A. aegerita* Aa-pri2: AAD41222; *D. glabratum* DGH1: CAC86002, DGH2: CAC86005, DGH3: CAC86006; *F. velutipes* FVH1: BAB17622; *L. edodes* Le.hyd1: AAG00900; Le.hyd2: AAG00901; *P. ostreatus* POH1: CAA12391, POH2: CAA74987, PHO3: CAA76494, Vmh1-1: CAD12829, Vmh1-2: CAD12830, Vmh2-1: CAD12833, Vmh2-2: CAD12834, Vmh3-1: CAD12831, Vmh3-2: CAD12832, Fbh1: CAC95144, Af3311452: AAL57034; *Pisolithus tinctorius* HYD-Pt1: P52748, HYD-Pt2: P52749, HYD-Pt3: AAC95356; *Pholiota nameko* PNH1/H251: BAB84545, PNH2/H263: BAB84546, PNH3/H315: BAB84547; *S. commune* SC1: P04158, SC3: P16933, SC4: P16934, SC6: O74300; *Tricholoma terreum* Hyd1: AAL05426.1; for *U. maydis* Hum2 (see Bohlmann 1996). Colored boxes indicate hits in the Blast searches.

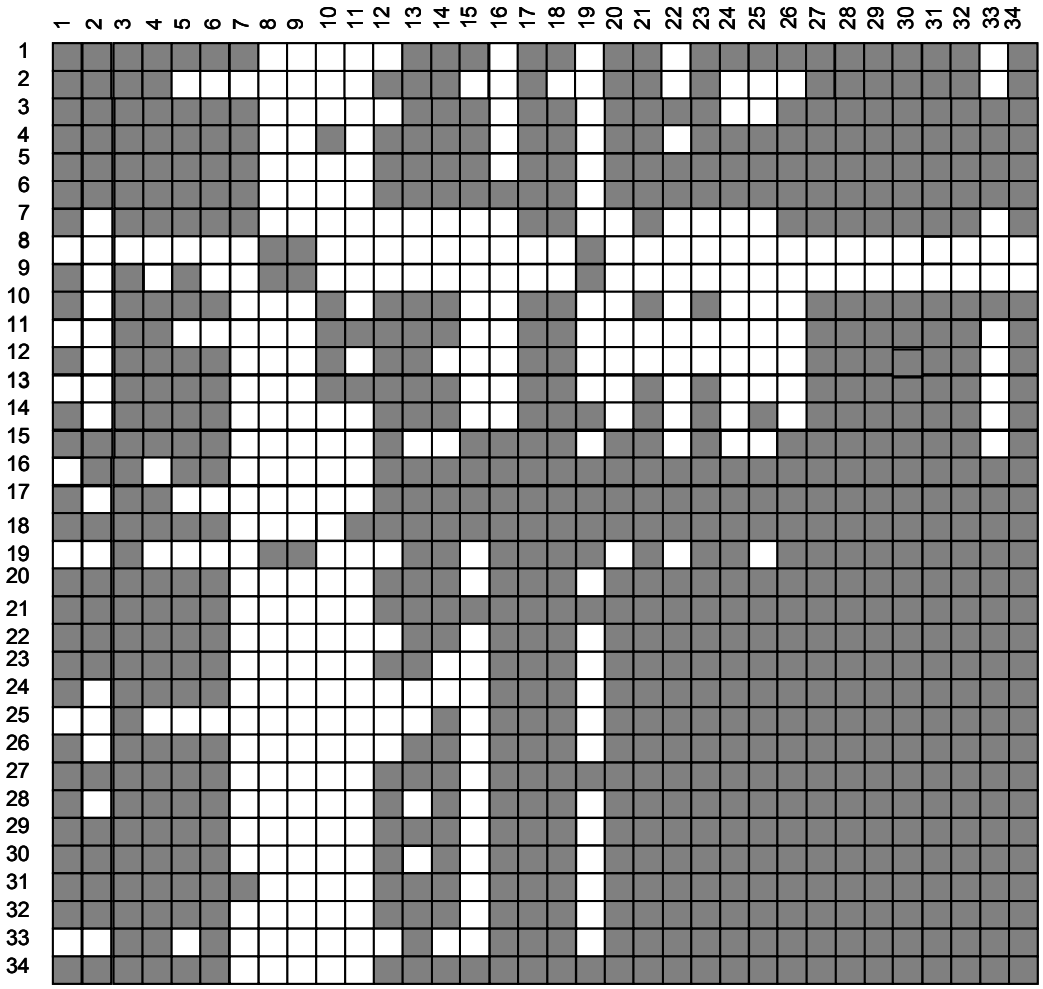

Figure 1(b). **Cross-identification of *C. cinerea* hydrophobin genes** by blasting (cut off level: $1e^{-3}$) all the deduced proteins back to the *C. cinerea* genome (http://www-genome.wi.mit.edu/annotation/fungi/coprinus_ cinereus/index.html) in order to check for missing hydrophobin genes and defining the final number of the hydrophobins. Numbers at the left indicate the respective CoH proteins used in Blast searches, numbers above the diagram those of the *coH* genes that were detected (marked in grey), respectively non-detected in the individual Blast searches.

3.4.2 Phylogenetic analysis of hydrophobin genes of *C. cinerea*

Hydrophobin genes of *C. cinerea* are distributed on contigs of the genome of monokaryon Okayama 7 in one cluster of 7 genes (including the already known genes *coH*1 and *coH*2), one cluster of 6 genes, 2 clusters of 3 genes and 4 clusters of 2 genes (including the cluster of *coH*8 and *coH*9). In seven contigs, single genes were found (Fig. 2). Those genes clustering closely together are in most cases also more closely related in protein sequence (Fig. 3), suggesting these genes were generated from each other by duplication events. The genes have between 1 to 5 introns (Fig. 3 and see also Fig. 2 in chapter 5).

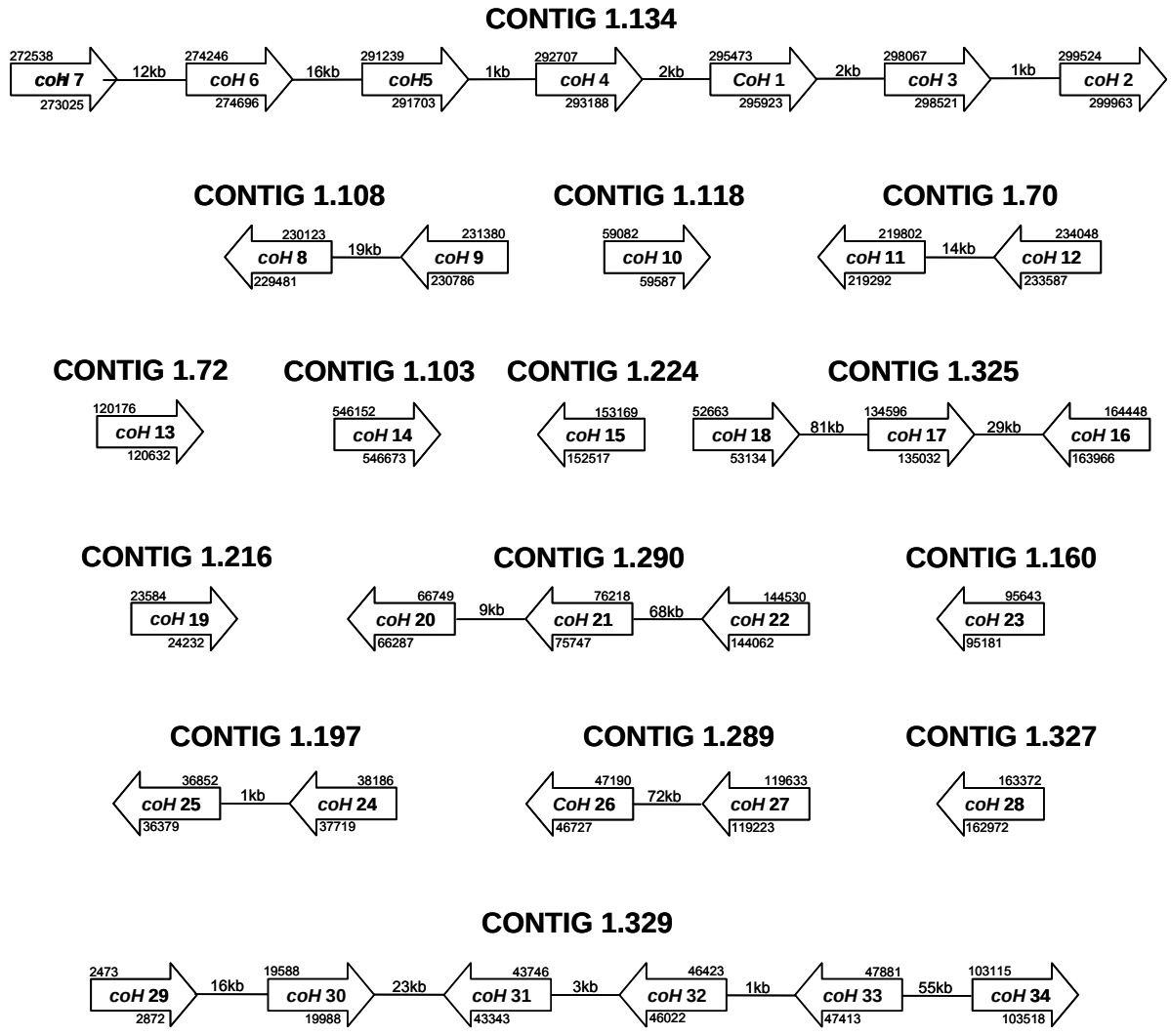

Figure 2. Localization of hydrophobin genes on different contigs in the genome of *C. cinerea* strain Okayama 7. The distance between the neighboring genes were given in between the genes and the start and stop positions of the genes on the top and below of the broad arrows representing the genes, respectively.

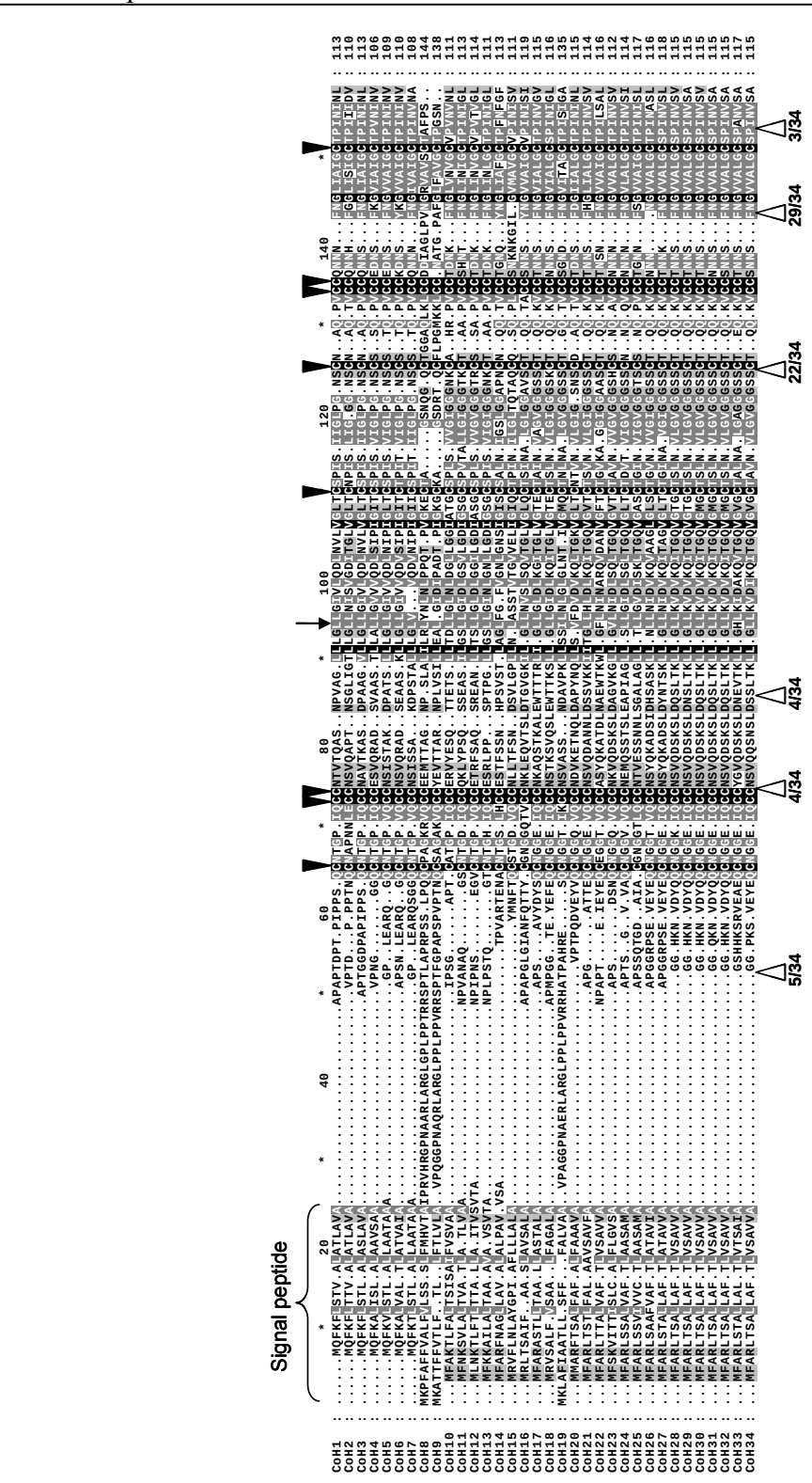

Figure 3. **Alignment of the deduced hydrophobin sequences from the *C. cinerea* genome**. (http://www.broad.mit.edu /annotation/ fungi/coprinus_cinereus/). Filled triangles indicate the eight cysteine residues at conserved positions, empty triangles show the intron positions in the corresponding genes and the numbers underneath the triangles indicate the number of genes that have an intron at the corresponding positions. The vertical arrow indicates the position of a frameshift in gene *CoH7*. The alignment was done by using Clustal X (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html) and the manual adjustments of the alignment were done using Gene doc software (http://www.psc.edu/biomed/genedoc/).

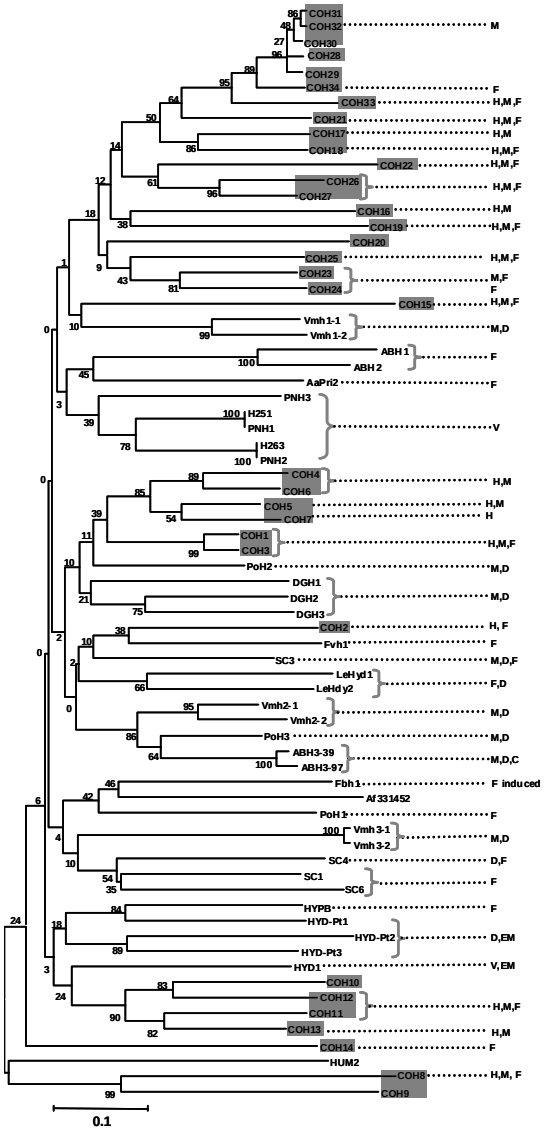

Figure 4. Phylogenetic analysis of all known hydrophobins from basidiomycetes, including the deduced hydrophobins from *C. cinerea* (CoH1-CoH34, this study). Clustal alignment was done using program Clustal X and the tree was constructed using the program MEGA version 2.1. Amino acid residues in front of the first cysteine were omitted in all proteins in order to obtain for sequence comparison the hydrophobin core regions as the N-terminal amino acid regions differ vary much in amino acid number and overall sequence (Wösten 2001). The dark grey color code refers to proteins deduced from *C. cinerea.* For source of proteins from other species see Figure 1(a). Stages of expression: C = mycelial cords, V = vegetative mycelium (unspecified); M = vegetative monokaryotic mycelium; D = vegetative dikaryotic mycelium; H = vegetative mycelium of self-compatible homokaryon; F = fruiting body; EM = ectomycorrhiza (for references on expression, see Walser et al. 2003)**.**

All but *coH7* translates into a complete hydrophobin sequence (Fig. 3). For phylogenetic analysis of hydrophobin genes, this frame shift was ignored and a complete protein assembled by deleting 2 base pairs (as indicated in Fig. 3). The deduced 34 hydrophobins contain the complete sequence of hydrophobin class I proteins with an N-terminal leader

peptide and the typical 8 cysteine residues being distributed at conserved positions in an otherwise variably conserved sequence (Fig. 3).

When comparing the *C. cinerea* hydrophobin sequences with those of other basidiomycetes (Fig. 4), the *C. cinerea* proteins group at six different places in the phylogenetic tree. Three of these clusters contain only *C. cinerea* proteins whilst the others contain hydrophobins from other species.

3.4.3 Isolation and identification of hydrophobins from *C. cinerea*

Hydrophobins were isolated from the vegetative mycelial samples of two monokaryons (JV6 and Okayama 7), the self-compatible homokaryon AmutBmut and of two dikaryons (JV6 x Okayama 7 and JV6 x AmutBmut). Furthermore, hydrophobins were isolated from primordia (day 2 stage, for definition see chapter 4) from the two dikaryons and homokaryon AmutBmut. Extracted hydrophobins were separated on 15% SDS-PAGE. Hydrophobin specific bands were identified in the range of 10-15 kDa, showing strain- as well as stage-specific expression patterns (Fig. 5). Mycelium of monokaryon JV6 had the highest number of different hydrophobin bands with one localized at 15 kDa, one at 14 kDa and three further in the range of 10 to 12 kDa. Mycelium of monokaryon Okayama 7 had a similar pattern as mycelium of the self-compatible homokaryon AmutBmut with a band at 14.5 kDa and another band at 10.5 kDa. Mycelium from the dikaryon AmutBmut x JV6 did not distinguish in its band pattern from mycelium of homokaryon AmutBmut whilst the dikaryon Okayama 7 x JV6 shared bands in size with both parental monokaryons and there was an extra band at 17 kDa. The three different samples from primordia totally distinguished from any mycelial sample. Proteins isolated from primordia samples of dikaryons and homokaryon AmutBmut were well separated, differently from most the samples from vegetative mycelium (Fig. 5).

Hydrophobin samples from all the different strains and stages were analyzed by SELDI-MS (Fig. 6). Also in SELDI-MS, strain- and stage-specific patterns occurred that correspond

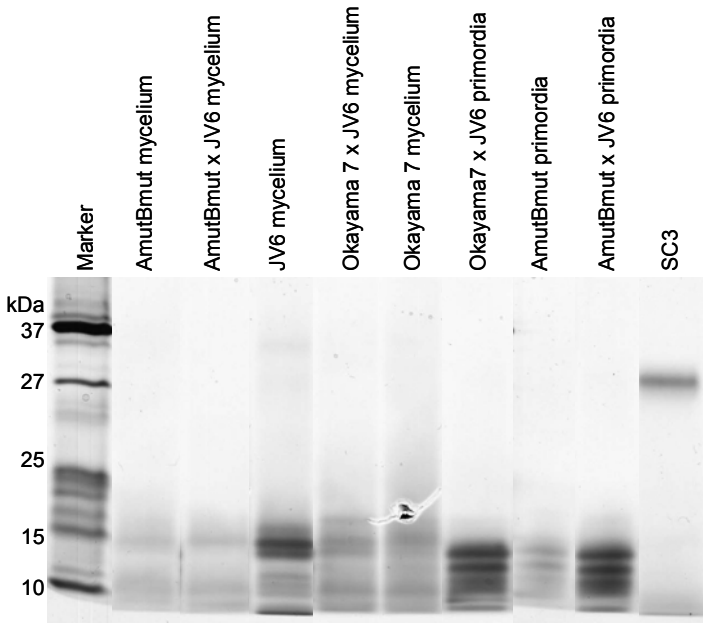

Figure 5. SDS-gel separating *C. cinerea* hydrophobins. Proteins were isolated from different mono-, di- and homokaryotic mycelium and primordia samples as indicated in the top lane. A size marker is given in the first lane of the gel and the SC3 from *S. commune* serves as positive control in the last lane. All the protein samples were reduced with ß-mercapto-ethanol and silver stained for molecular weight determination

well with the band patterns of the SDS-PAGE gels shown in Fig. 6. Four clear mass peaks and two extra peaks are seen in the diagram of mycelium from monokaryon JV6, four of which reoccurred in the mycelium of Okayama 7 x JV6. Other peaks in dikaryon Okayama 7 x JV6 are shared with the Okayama 7 mycelium as well as with the dikaryon AmutBmut x JV6 mycelium and AmutBmut mycelium (Fig. 6). In contrast, all the primordial samples had three peaks unique to this developmental stage. Theoretical sizes of the 34 *C. cinerea* hydrophobins range between 8593 and 15168 Da (Table 2). However, many of the hydrophobins have nearly identical sizes hindering individual protein identification (see Table 2). The masses given by distinctive peaks in the SELDI-MS were compared with the theoretically calculated masses of hydrophobins (Table 2). However, all masses obtained by SELDI-MS analysis differed from those of the theoretical values (Fig. 6). Hydrophobins from JV6 and AmutBmut mycelium, and primordia were tested for glycosylation. However, none of the *C. cinerea* samples stained positively with the Schiff's reagent unlike SC3 of *S. commune* used as a control (Fig. 7), a known glycoprotein (Lugones 1998). In accordance, sequence analysis of the *C. cinerea* hydrophobins did not reveal any N- or O- glycosylation sites (http://www.cbs.dtu.dk). Protein glycosylation is therefore not an explanation for the detected differences between deduced mass values and of by SELDI-MS.

Next, protein bands were excised from SDS-PAGE gels and in-gel digested with chymotrypsin (cuts C-terminal to F/Y/W/M/L, not before P; Kamp 1986) as described in material and methods. The resulting peptides were eluted from gel pieces and analyzed by ESI-LC-MS. Homology searches resulted in low and/or no significant hits from the known *C. cinerea* and other fungal hydrophobin sequences present in the NCBI GenBank database. Therefore, an offline database was created as described in material and methods. Homology analysis against this database resulted in significant hits for seven different hydrophobins from *C. cinerea* (Table 3). Four different hydrophobins (CoH25, CoH26, CoH27, and CoH33 were identified from mono/homokaryotic mycelial samples (JV6 and AmutBmut). In dikaryotic primordia samples (AmutBmut x JV6 and Okayama 7 x JV6), two proteins (CoH14 and CoH25) were identified, in addition in homokaryon AmutBmut primordia four different hydrophobins (CoH14, CoH23, CoH24, and CoH25) (Table 2). Theoretical sequence analysis showed that not all hydrophobins are cleaved by chymotrypisn or it cleaves the protein in such way that peptides are too large or very small and therefore difficult to analyze by MS analysis. According to report by Wang *et al*. (2004), large peptides are poorly ionized and small peptides are lost during the elution from gel pieces and during the injecting procedure. Other proteolytic enzymes were also tested on *C. cinerea* hydrophobins for obtaining better fragment sizes. However, the most commonly used enzyme, trypsin, is not efficient on hydrophobins as the deduced protein sequences of the *C. cinerea* hydrophobin genes contain no or poor cleavage sites for this specific peptidase (i.e. the C-terminal sides of K and R residues; Kamp 1986). Accordingly, cleavage of hydrophobin bands cut from gels with trypsin and subsequent elution of peptides from gel samples yielded no peptides that were identified by Mass Spectrometry. Another proteolytic enzyme, pepsin, cleaves very unspecifically at the C-terminal side of F, L, W, Y, A, E, and Q residues (Kamp 1986), giving very small peptides when successfully applied to hydrophobins (further details in the attachment of this chapter) which are likely poorly ionized (Wang et al. 2004). In mass spectrometry upon pepsin digestion of hydrophobins, no positive hits were therefore obtained.

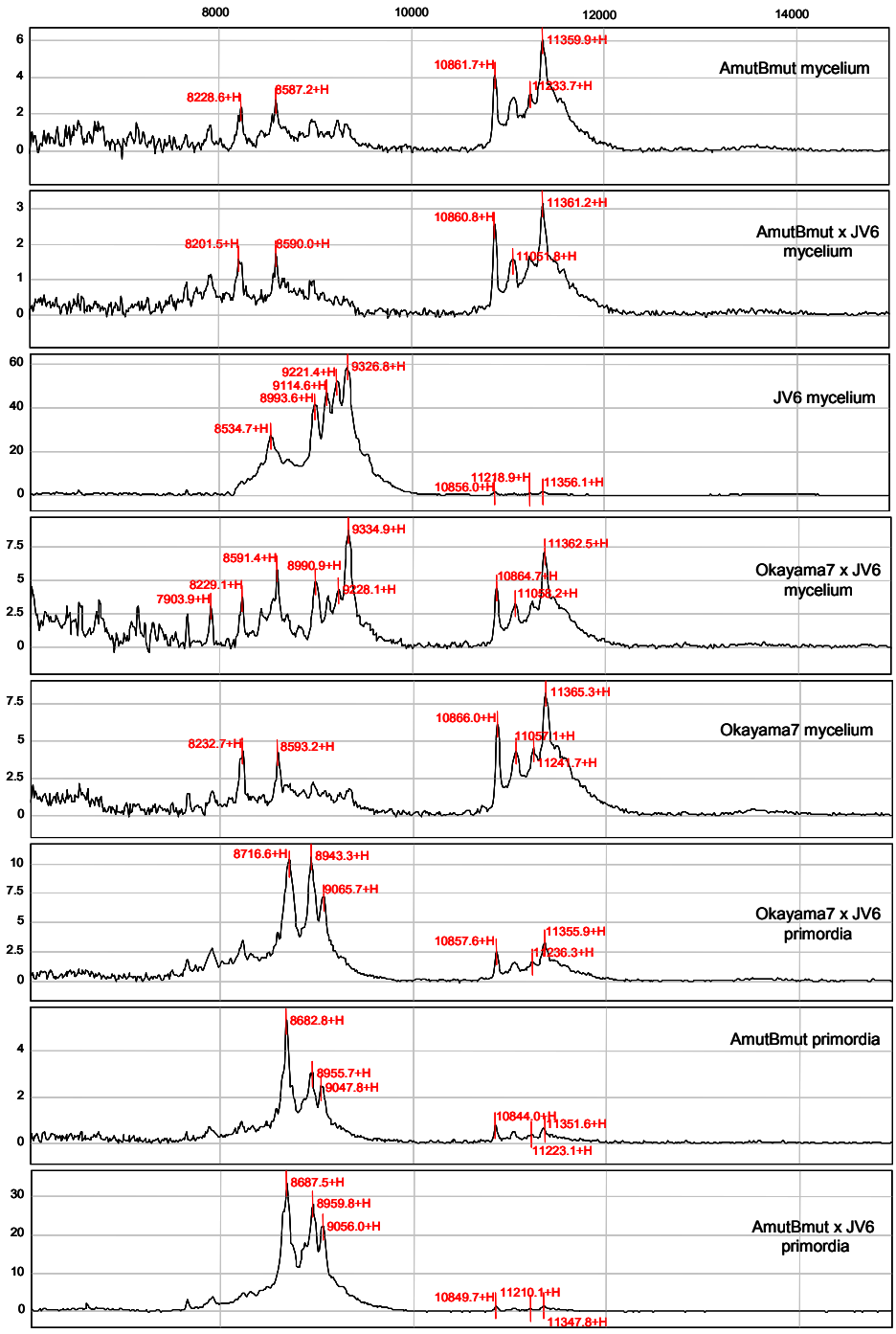

Figure 6. Peptide masses from the SELDI mass spectrometry. Hydrophobin samples from the different strains and stages of *C. cinerea* development were analyzed as indicated in the figure. SELDI obtained masses were compared with the theoretical masses calculated for all the *C. cinerea* hydrophobins. The theoretical masses are listed in Table 2.

Table 2. Calculated theoretical masses for the complete and mature hydrophobins using ExPASy software (http://www.expasy.org/)

Protein	Complete protein (Da)	Mature protein (Da)
CoH1	11484.4	9691.2
CoH2	11154.9	9347.6
CoH3	11302.2	9509.0
CoH4	10559.2	8872.2
CoH5	10998.8	9196.6
CoH6	11229.1	9485.9
CoH7	10870.5	9066.4
CoH8	15168.4	12595.7
CoH9	14290.8	12030.9
CoH10	11260.0	9089.4
CoH11	11140.8	9124.4
CoH12	11458.2	9153.4
CoH13	10961.8	9003.4
CoH14	11426.9	9242.2
CoH15	11669.8	9481.0
CoH16	11795.6	10020.5
CoH17	11605.3	9629.9
CoH18	11934.7	10157.6
CoH19	13525.6	11398.0
CoH20	12158.8	10069.3
CoH21	11605.3	9535.8
CoH22	12071.8	10008.2
CoH23	11219.8	9136.2
CoH24	11255.9	9244.4
CoH25	11270.9	9247.4
CoH26	11739.3	9685.8
CoH27	12109.8	10060.3
CoH28	11769.5	9706.0
CoH29	11728.4	9664.2
CoH30	11800.6	9737.0
CoH31	11776.5	9655.9
CoH32	11758.5	9694.9
CoH33	11857.4	9811.9
CoH34	11616.3	9495.7

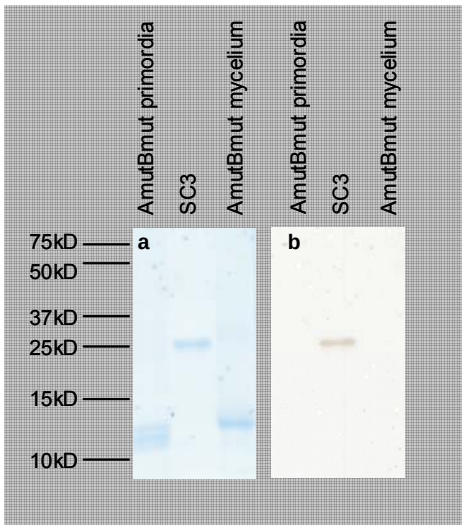

Figure 7. Analysis of glycosylation of hydrophobins. 15% SDS-PAGE showing hydrophobins from AmutBmut primorda and vegetative mycelium stained with Coomassie blue (a) and glycostaining (b). The well studied glycosylated hydrophobin (SC3) from *S. commune* mycelium served as positive control.

Table 3. Peptide sequences obtained from ESI-LC-MS analysis identified expressed hydrophobins in different developmental stages and strains from *C. cinerea*. The coefficient values (mean values from repeated base peak analysis; X conc.) for the peptide identification are given in the last column of the table.

Sample	Results	Peptide	X conc.
AmutBmut mycelium	CoH33	Y.GVQDSKSLDNEVTKLL.G	4.948
	CoH27	L.NIDVKQITAGVGL.T	3.994
		Y.QKADSLDYNTSKL.L	3.005
	CoH26	L.NIDVKQVAAGL.G	2.948
		Y.QKADSIDHSASKLL.N	2.656
Okayama 7 mycelium	CoH25	L.TLLGVDISKL.T	3.407
Okayama 7 x JV6 primordia	CoH25	L.TLLGVDISKL.T	3.490
	CoH23	L.DAGVKGLLGVL.N	2.962
	CoH14	F.SSNHPSVSTL.A	2.975
Amut Bmut primordia	CoH14	F.SSNHPSVSTLAGLF.G	2.905
	CoH25	L.TLLGVDISKL.T	3.353
	CoH23	L.DAGVKGLLGVL.N	2.823
		F.NGVVALGCTPINVSV.	2.802
	CoH24	L.ALGCTPINVSI	3.307
AmutBmut x JV6 primordia	CoH25	L.TLLGVDISKL.T	3.922
	CoH14	F.SSNHPSVSTL.A	2.552
S. commune mycelium	Sc3	F.NGLINIGCTPINIL.	3.143

3.4.4 Transcript analysis using gene specific primers

Since identification by protein analysis proved to be difficult, gene expression was performed by isolating total RNA and synthesizing cDNA from the different strains and developmental stages (see materials and methods). The synthesized cDNA was used for transcript profiling of the hydrophobin genes in PCR with gene specific primers (Table 1). Primers used for transcript profiling were designed for most genes in such way, that splicing of all introns was confirmed. For genes *coH*4, *coH*8, *coH*15 and *coH*19, however only 1-4 introns in the 5´regions of the genes were confirmed in the profiling analysis. Complete splicing of the transcripts were subsequently proven by four extra primers (Table 1) positioned directly at or close to the ATG-start codon (not shown). All expressed genes (in total 26, see below) gave PCR bands of expected sizes from correct splicing.

In total, three quarters of genes were found to produce transcripts in the different samples (Fig. 8). However, the individual transcript profiles differed from each other. Strong bands were obtained in RT-PCR for *coH*18, *coH*19, *coH*24, *coH*25, *coH*33 from caps of AmutBmut primordia and *coH*14, *coH*24, *coH*25 and *coH*33 from stipes of primordia (Fig. 8). Strikingly, amongst these were all the genes for which a protein product was found in the LC-MS analysis (Table 3). In vegetative mycelium, strain AmutBmut gave rise strong transcript patterns for genes *coH*1, *coH*3, *coH5*, *coH*4, *coH6*, *coH*8, *coH1*9, *coH*27, and *coH*33, strain Okayama 7 for *coH*1, *coH*3, *coH*4, *coH*5, *coH*6, *coH1*9, *coH*26, and *coH*27, and strain JV6 for *coH*1, *coH*3, *coH*4, *coH5*, *coH6*, *coH*8, *coH16*, *coH*17, *coH*18, *coH*19, *coH*26, and *coH*27 (Table 1 in the Appendix, Fig. 8 & 9). Whilst amongst the highly expressed genes in AmutBmut mycelium were those of which proteins were detected by ESI-LC-MS (CoH14 and CoH25, Table 3), there were no transcripts for *coH*25 in the Okayama 7 sample. Cultures used for the hydrophobin isolation and total RNA isolation were of the same age (harvested at the day 5 of incubation at 37^{o}C) of biomass production within different liquid cultures, so it was possible that *coH*25 expression occurred at an early growth phase but switched off at later growth whilst the very stable hydrophobins still remained in the cultures.

Of the many low expressed genes (Fig. 8), no protein product was detected in the ESI-LC-MS approach. It is possible that protein amounts were too low for detection. Nevertheless, our ESI-LC-MS analysis was not complete, since only some specific bands

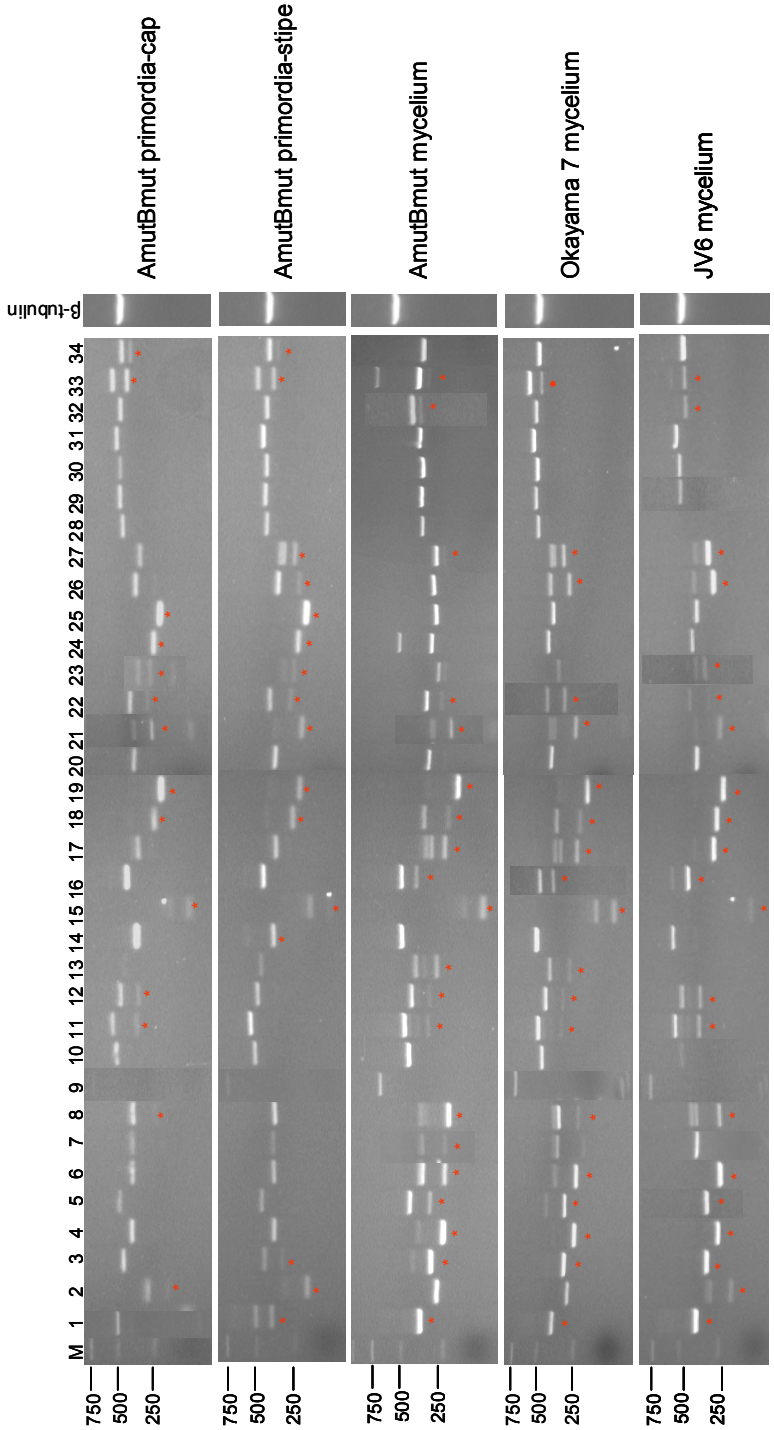

Figure 8. The asterisk (*) mark the cDNAs of expressed genes and the unmarked bands correspond to the genomic DNA sizes of the respective genes. The top lane shows the 34 different hydrophobin genes (*coH1-coH34*). Amplification of the β-tubulin cDNA served as quality control of the cDNA and the PCR reactions. Note that the size of the amplified fragment of β-tubulin cDNA is 1 kb and not shown to scale with the size marker (Fermentas) at the left.

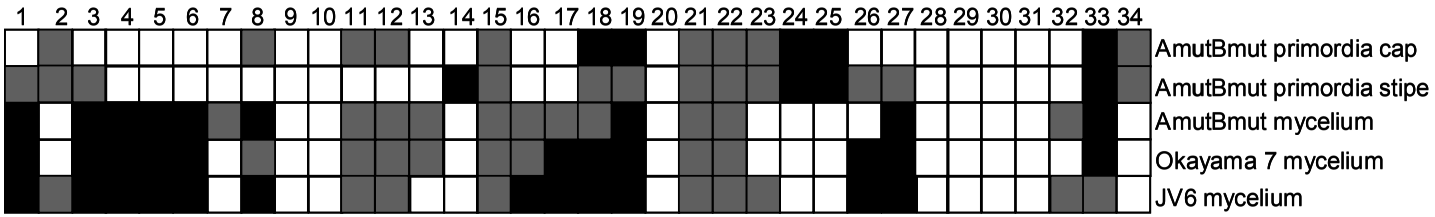

Figure 9. Expression pattern of the hydrophobin genes in the mycelium of monokaryotic strains of *C. cinerea* and in the mycelium and primordia tissues of homokaryon AmutBmut. The numbers at the top of the figure indicate the different *coH* genes. cDNAs of genes that in PCR gave strong bands (Fig. 8) are marked in black, cDNAs that gave weak bands (Fig. 8) in light grey and cases where no cDNA was amplified in white.

Table 4. The following table shows the expressed hydrophobin genes in different mono- and homokaryotic strains and samples of *C. cinerea* and the total numbers of genes transcribed in each tissue.

Stage	Strong expression	Weak expression	Total number of genes transcribed
AmutBmut primordia-cap	18, 19, 24, 25, 33	2,8,11,12,15,21,22,23,34	14
AmutBmut primordia-stipe	14,24,25,33	1,2,3,15,18,19,21,22,23, 26,27,34	16
AmutBmut mycelium	1,3,4,5,6,8,19,27, 33	7,11,12,13,15,16,17,18,21 ,22,32	20
Okayama 7 mycelium	1,3,4,5,6,17,18,19,26, 27,33	8,11,12,13,15,16,21,22	19
JV6 mycelium	1,3,4,5,6,8,16,17,18, 19,26,27	2,11,12,15,21,22,23,32,33	21

were eluted from the gel (Fig. 5). Further tests with the whole mixture of isolated chymotrypsin-digested hydrophobins to show expression of proteins from highly expressed genes failed by blocking the HPLC columns of the ESI-LC-MS, possibly due to coating of the column with undigested hydrophobins.

Comparing the transcribed genes within the different samples revealed that only the strongly expressed *coH*14 was found to be specific to the stipe of the primordia and *coH*34 expression was shared between cap and stipe (Fig. 8 and 9). *coH*24 and *coH*25 were found expressed strongly in both cap and stipe but not in the mycelium of the monokaryons (Okayama7 and JV6) and one homokaryon (AmutBmut). Most other transcripts in the mycelial samples were shared but *coH*32 transcripts were found only in the AmutBmut and JV6 mycelium (Fig. 8 and 9).

In conclusion, we found transcripts for a total of 27 genes. The expressed *C. cinerea* genes clustered at various places in the phylogenetic tree of the deduced protein sequences of all known basidiomycete genes (Fig. 4). There is no clear-cut division between proteins from vegetative and fruiting-specific tissues. In conclusion, the phylogenetic tree cannot specify what kind of functions hydrophobins from *C. cinerea* might have or at what developmental stage a protein might be expressed.

3.5 Discussion

By protein isolation and sequencing and/or cDNA analysis, four expressed hydrophobin genes have been described in *S. commune*, three in *A. bisporus* and *P. ostreatus*, and two in *L. edodes* (Walser et al. 2003). In *C. cinerea*, *coH*1 has been shown previously to be expressed in the monokaryotic mycelium of strain JV6 and evidence for another closely linked gene came from DNA sequencing (Ásgeirsdóttir et al. 1997). An unexpected result from this study was that in *C. cinerea* there are as many as 34 hydrophobin genes in a single organism and that many of them might be functional. This result prompted to analyze the available *Phanerochaete chrysosporium* genomic sequence (Martinez et al. 2004) in similar multiple searching strategy as performed here for *C. cinerea*. In *P. chrysosporium,* we found 20 different hydrophobin genes in total (Velagapudi and Peddireddi, unpublished observation, chapter 5). From the analysis in *C. cinerea* as well as in *P. chrysosporium*, it is however clear that hydrophobin genes are difficult to detect in the genome of a fungus because of the low homology at the amino acid level. Detection of all hydrophobin gene copies from other genomes of the fungi needs therefore careful analysis. All so

far known genes in basidiomycetes code for class I type of hydrophobins (Walser et al. 2003, this study). Class II hydrophobin genes were not detected, neither in *C. cinerea* nor in *P. chrysosporium* although our Blast search analysis (at an E value $1e^{-3}$) of the two genomes included also a total of 20 different class II hydrophobins from ascomycetes, none of which gave a positive hit in the two genomes (not shown). However, there were also no hits in Blast searching (at an E value $1e^{-3}$) the two genomes with 16 different know class I hydrophobins of ascomycetes (not shown).

The presence of many hydrophobin encoding genes in a single organism can make functional studies very difficult, for example, when knockout mutants have to be produced. However, knocking out *SC*3 in *S. commune* was enough to give the vegetative mycelium a new water-logging phenotype and knocking out *SC*4 lead to easy water filling in the fruiting bodies (van Wetter et al. 2000). The ascomycete *Fusarium verticillioides* has at least three class I and two class II hydrophobin genes. All five genes were individually knocked-out. In the knock-out mutants of the class II hydrophobin genes a mycelial phenotype was not found but knocking out the class I hydrophobin genes HYD1, HYD2 and HYD3 individually caused a reduction in chain formation of asexual spores (microconidia, Fuchs et al. 2004). In another ascomycete *Cladosporium fulvum*, a total of six genes have been described, four class I and two class II genes. Knocking-out of class I gene for hydrophobin HCf1 caused a phenotype with reduced hydrophobicity of conidia resulting in reduced dispersal by water droplets (Whiteford et al. 2004).

Presence of 34 different hydrophobin genes in *C. cinerea* raises several questions regarding their expression within different stages of fruiting body development. Protein analyses performed in this study have their limitations with respect to hydrophobins. SELDI-MS analysis of protein samples from different types of mycelium and of primordia gave strain and stage-specific mass peaks. Accordingly, we would have expected very different expression data from transcript analysis. However, the overall expression pattern between monokaryons Okayama 7 and JV6 was found to be very similar. Only in JV6, transcripts of gene *coH*17 and *coH*18 appear to be more strongly expressed as seen in Okayama 7 (Fig. 8). But this does not explain the dramatic difference of the SELDI-MS pattern between the two monokaryotic strains (Fig. 6). Likewise, the overall differences in SELDI patterns for primordia and vegetative mycelium of homokaryon AmutBmut (Fig. 6) do not explain major differences in patterns of transcripts (Fig. 8). From our experimental data and

from theoretical analysis of protein sequences it is evident that hydrophobins in *C. cinerea* are not glycosylated. To our knowledge, other types of post-translational modifications are not known for any fungal hydrophobins, but can not be ruled out.

Alleles of other types of genes in *C. cinerea* are known to have only restricted homology, e.g. laccase gene *lcc*1 with 95 to 98% DNA identity gives rise to proteins with three amino acid exchanges (Hoegger et al. 2004, Kilaru et al. 2006). Sequence data for alleles of hydrophobin genes are so far only available for Okayama 7 and for CoH1 and CoH2 from JV6. It is another possibility that some of the differences observed in SELDI analysis are due to amino acid changes in the respective proteins. On the other hand, the SELDI- analysis is very much corresponding to the different hydrophobin band pattern of the individual samples, suggesting that different types of hydrophobins are present in the mycelial and primordia samples. ESI-LC-MS analysis of chymotrypsin digested and gel-eluted peptides identified a total of seven different hydrophobins, expressed either in mycelium of monokaryon (CoH25), homokaryon (CoH26, CoH27 and CoH33), dikaryon primordia (CoH14 and CoH25), and homokaryon primordia (CoH14, CoH23, CoH24 and CoH25). The protein CoH14 is found in three different primordia samples but not in mycelial samples, in agreement with the transcript analysis. Thus, CoH14 is likely a fruiting-specific gene.

Our LC-MS analysis is not complete due to reoccurring difficulties in protein digestion and peptide elution from gels. Digests performed in-solution never gave positive results in LC-MS due to poor solubility of hydrophobins and poor proteolytic digestion, also not when we used isolated SC3 from *S. commune*. Similar difficulties with SC3 were encountered by Wang et al. (2004) concluding that LC-MS is not a very suitable method to analyze such difficult proteins as hydrophobins.

From all our experimental work, the transcript analysis gives currently the clearest picture. From transcript analysis, we know that at least 26 of the 34 genes are active. There appear to be some differences in expression levels of common genes, very few genes are stage-specific. Early phylogenetic studies suggested a clear-cut division between hydrophobins expressed in vegetative mycelium and hydrophobins specific to the fruiting bodies (Wessels 2000). A more recent study using all the known and deduced hydrophobin sequences from basidiomycetes suggested already that fruiting and mycelial specific hydrophobins are not clearly separated from each other (Fig. 7, Walser et al. 2003). This analysis of *C. cinerea* further supports this conclusion (Fig. 4).

3.6 Acknowledgements.

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3.8 Appendix

Table 1 Theoretical fragment lengths of the 34 hydrophobin genes and the β-tubulin gene of *C. cinerea* amplified with the primers listed in Table 1 in chapter 3. The expected fragment lengths are given for both genomic DNA and cDNA.

Gene name	Expected size of genomic DNA	Expected size of cDNA
coH1	452	391
coH2	305	198
coH3	455	341
*coH4**	395 (481)	279 (338)
coH5	477	402
coH6	391	273
coH7	389	296
*coH8**	388 (679)	260 (541)
coH9	700	423
coH10	486	337
coH11	514	421
coH12	454	316
coH13	417	295
coH14	528	415
*coH15**	182 (823)	116 (249)
coH16	516	393
coH17	349	246
coH18	371	261
*coH19**	324 (668)	237 (546)
coH20	370	255
coH21	354	227
coH22	411	293
coH23	327	203
coH24	393	270
coH25	363	262
coH26	376	263
coH27	350	297
coH28	455	342
coH29	473	354
coH30	468	348
coH31	495	439
coH32	466	410
coH33	540	420
coH34	447	392
β-tub (control)	1609	1064

* In brackets: values of genomic and cDNA obtained in analysis of confirming extra 5´introns in these genes by amplifying cDNAs of the expected sizes

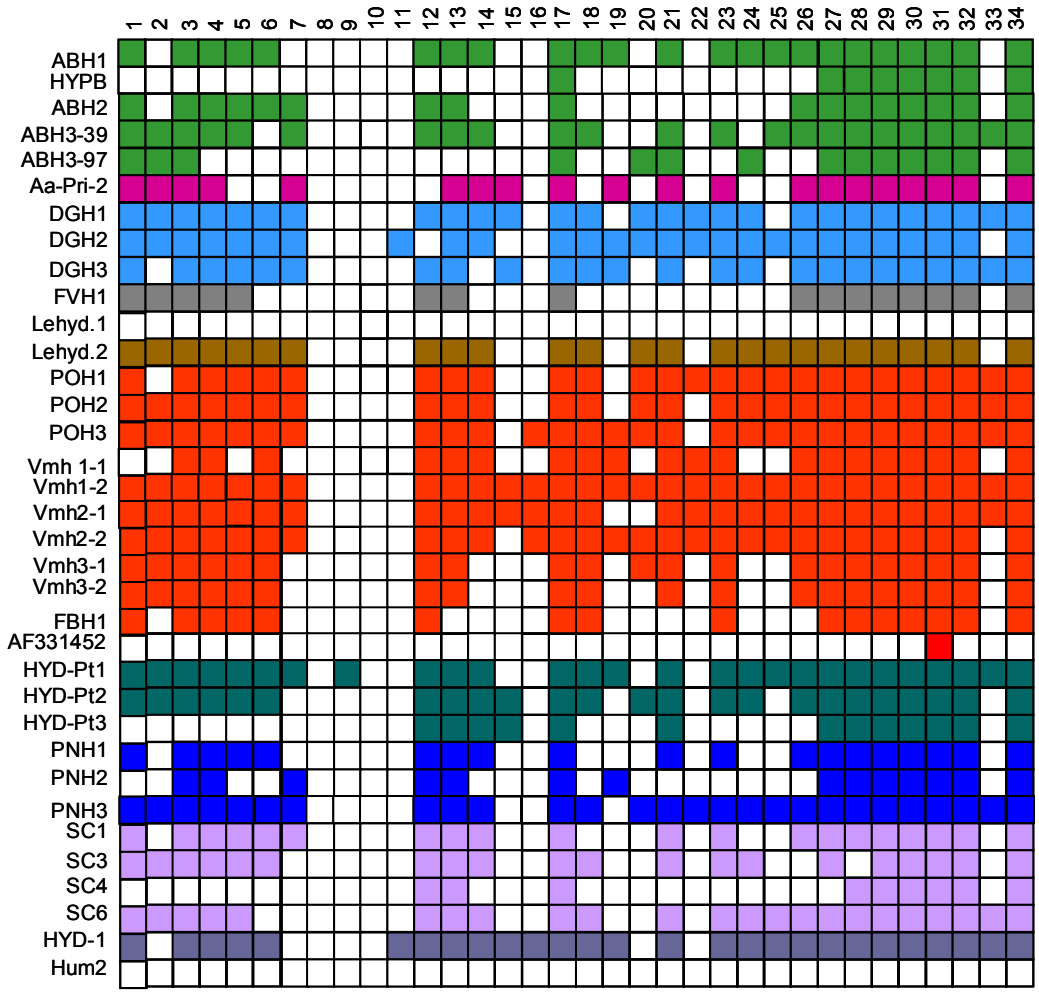


Figure 1 Identification of *C. cinerea* hydrophobin genes (top line: numbers indicate the respective *coH* genes) by hydrophobins from other basidiomycetes (names shown left; further explanations in the legend of Fig. 1a). The Blast search analysis was done at an expect value (E) of $1e^{-2}$. Colored boxes indicate hits in the Blast searches.

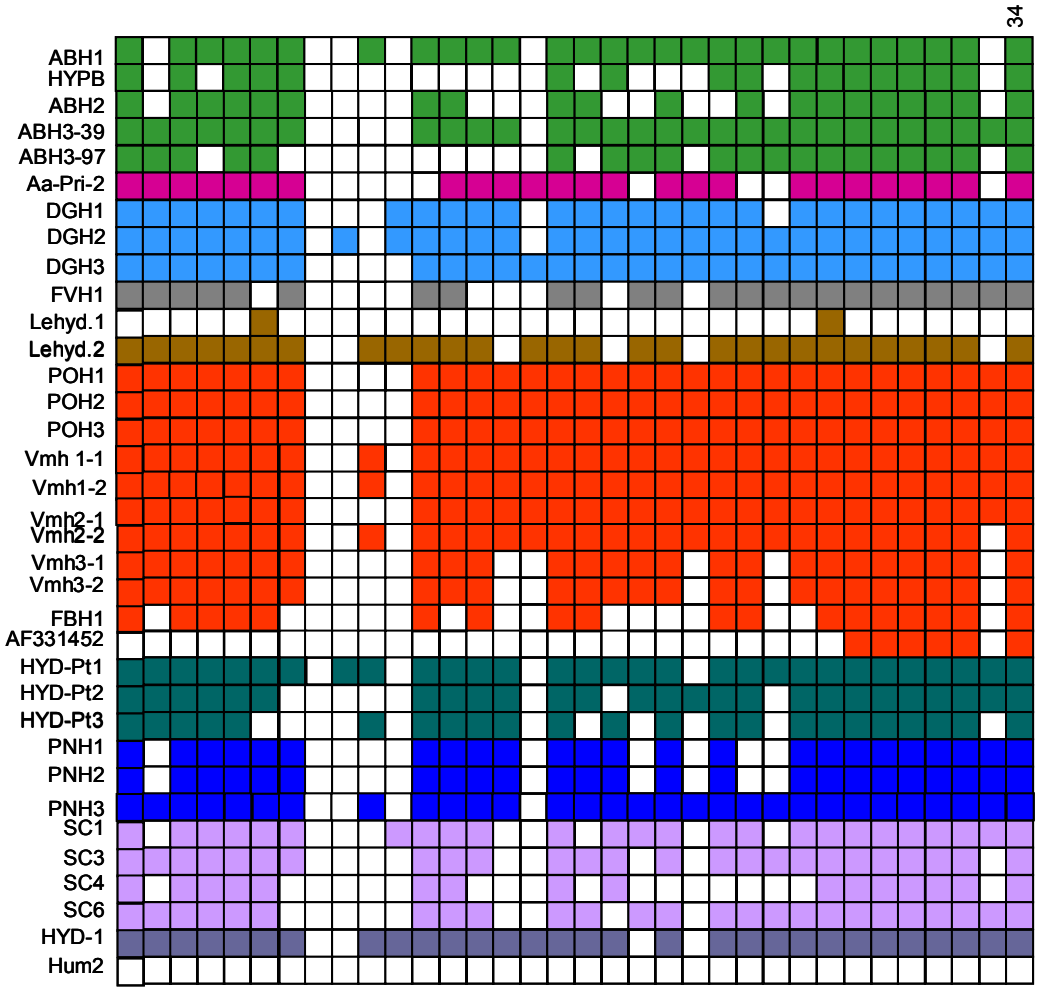


Figure 2 Identification of *C. cinerea* hydrophobin genes (top line: numbers indicate the respective *coH* genes) by hydrophobins from other basidiomycetes (names shown left; further explanations in the legend of Fig. 1a). The Blast search analysis was done at an expect value (E) of $1e^{-1}$. Colored boxes indicate hits in the Blast searches.

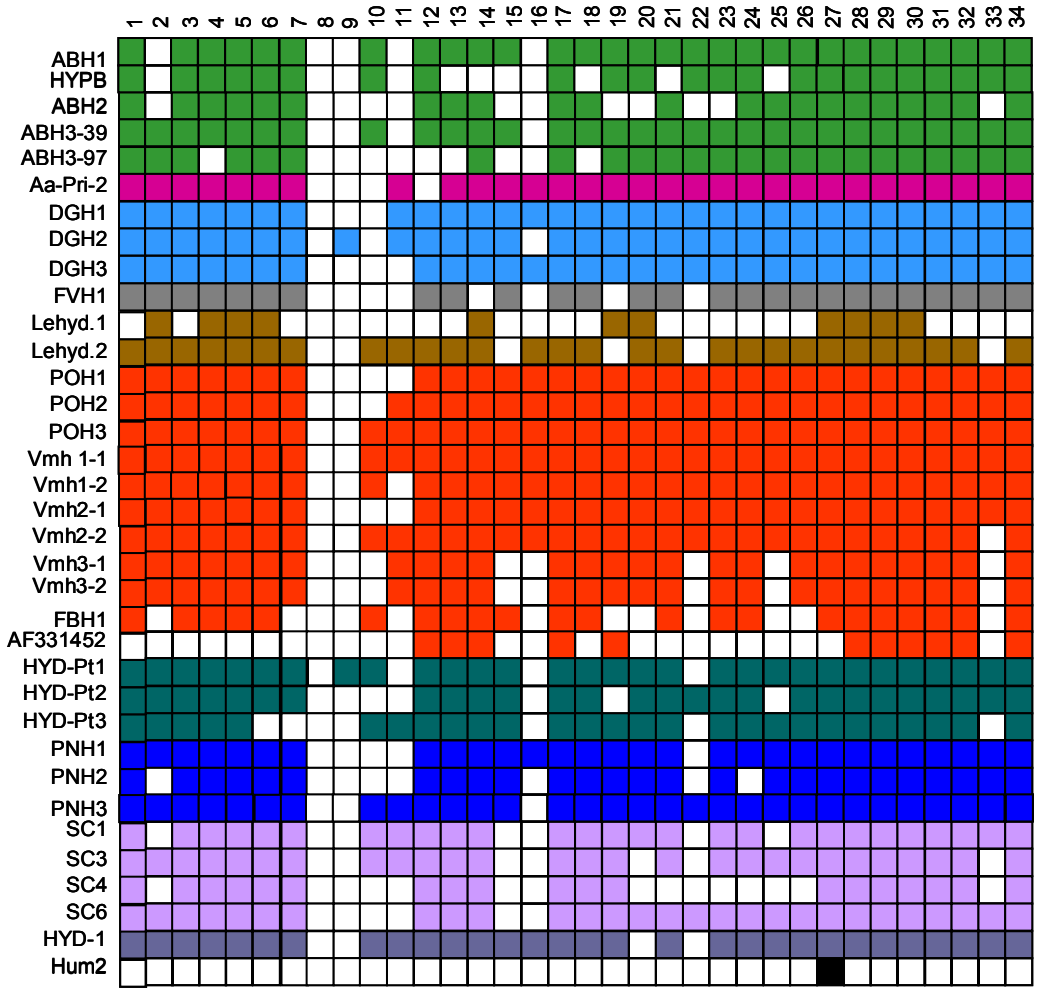


Figure 3 Identification of *C. cinerea* hydrophobin genes (top line: numbers indicate the respective *coH* genes) by hydrophobins from other basidiomycetes (names shown left; further explanations in the legend of Fig. 1a). The Blast search analysis was done at an expect value (E) of $1e^{-0}$. Colored boxes indicate hits in the Blast searches.

Table 2. Compilation of the results shown in Fig. 1a of chapter 3 and in Figs. 1 to 3 of the appendix: Identification of *C. cinerea* hydrophobin genes by the 34 hydrophobin sequences deduced from the *C. cinerea* genome in reciprocal analysis by Blast searches, with lowering the stringency of the cut-off value (E-value) from $1e^{-3}$ to $1e^{-2}$, $1e^{-1}$, and $1e^{-0}$, respectively. The numbers of identified *Coprinopsis* hydrophobin genes per hydrophobin are given in this table

Protein	Number of hydrophobin genes detected at E-value			
	$1e^{-3}$	$1e^{-2}$	$1e^{-1}$	$1e^{-0}$
CoH1	25	28	28	28
CoH2	18	21	23	25
CoH3	25	27	28	32
CoH4	28	29	31	31
CoH5	28	31	31	32
CoH6	30	31	32	32
CoH7	17	21	23	26
CoH8	3	3	3	3
CoH9	6	6	8	10
CoH10	21	24	27	27
CoH11	16	17	24	28
CoH12	17	19	25	28
CoH13	20	22	24	28
CoH14	21	21	24	25
CoH15	22	25	27	27
CoH16	27	27	28	28
CoH17	26	26	28	30
CoH18	30	31	32	32
CoH19	21	28	29	31
CoH20	27	28	29	31
CoH21	29	30	30	32
CoH22	26	28	28	28
CoH23	26	28	28	31
CoH24	23	28	28	28
CoH25	20	29	29	30
CoH26	25	27	28	28
CoH27	28	30	30	31
CoH28	25	30	30	30
CoH29	27	29	29	30
CoH30	26	28	28	31
CoH31	28	30	30	31
CoH32	27	29	29	31
CoH33	24	26	27	29
CoH34	29	30	30	32

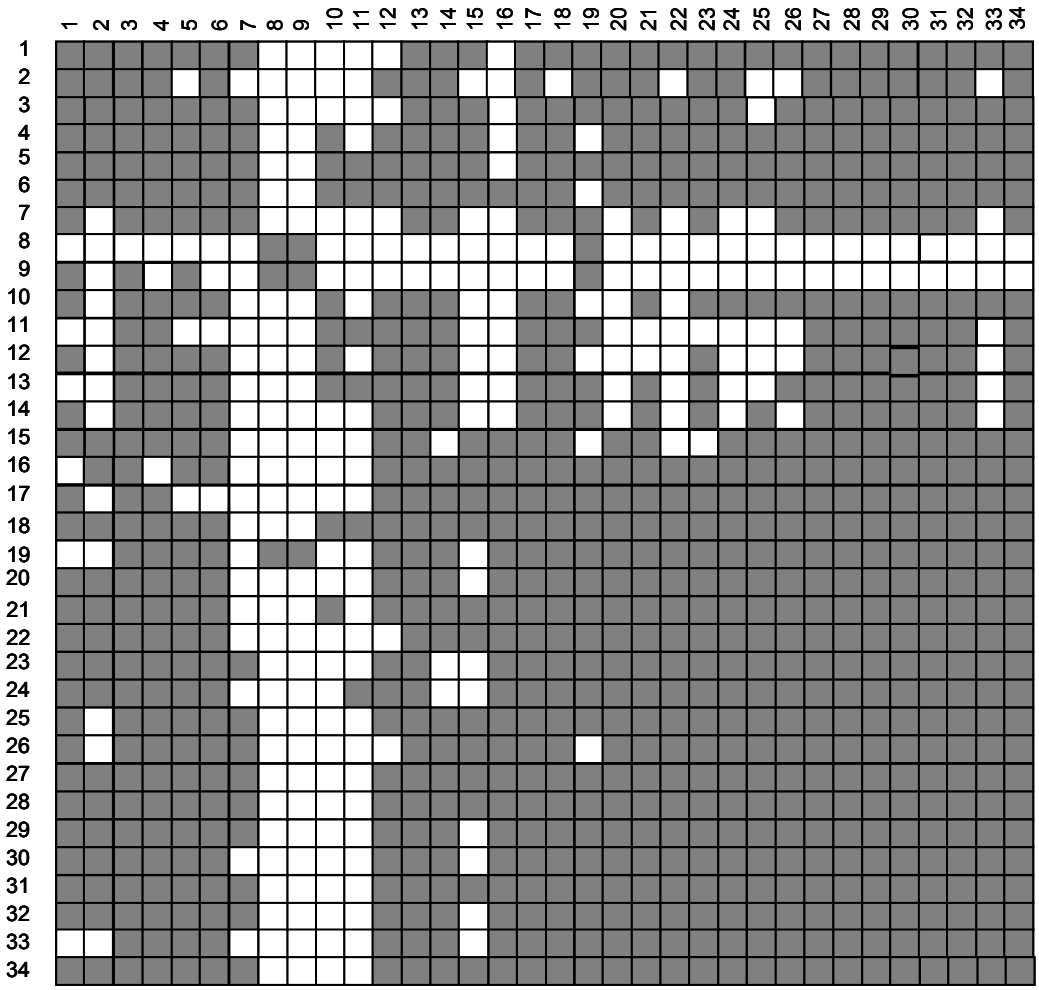


Figure 4 Cross-identification of *C. cinerea* hydrophobin genes by Blast searching all the hydrophobin genes deduced from the *C. cinerea* genome back to the *C. cinerea* genome. Numbers at the left indicate the respective CoH proteins used in Blast searches, numbers above the diagram those of the *coH* genes that were detected (marked in grey), respectively non-detected in the individual Blast searches. The Blast search analysis was done at an expect value (E) of $1e^{-2}$.

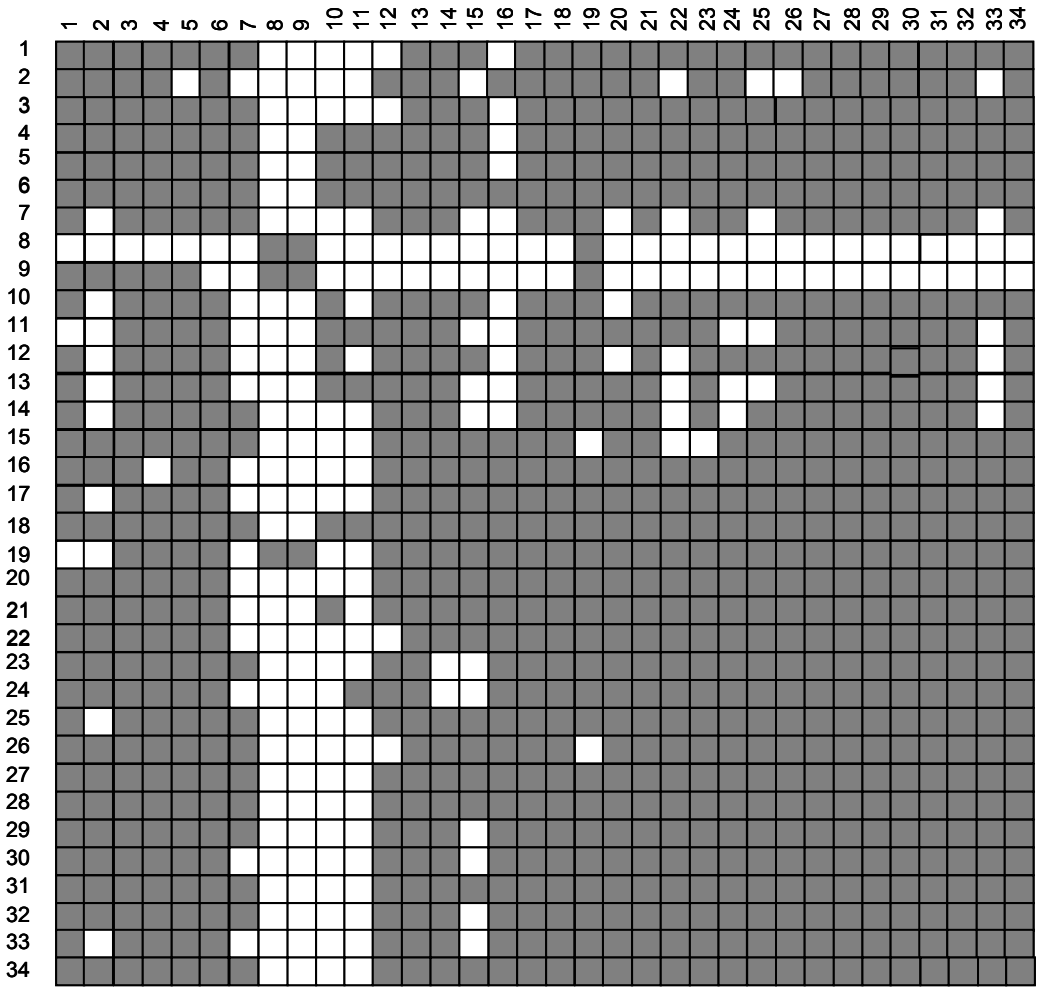


Figure 5 Cross-identification of *C. cinerea* hydrophobin genes by Blast searching all the hydrophobin genes deduced from the *C. cinerea* genome back in *C. cinerea* genome. Numbers at the left indicate the respective CoH proteins used in Blast searches, numbers above the diagram those of the *coH* genes that were detected (marked in grey), respectively non-detected in the individual Blast searches. The Blast search analysis was done at an expect value (E) of $1e^{-1}$.

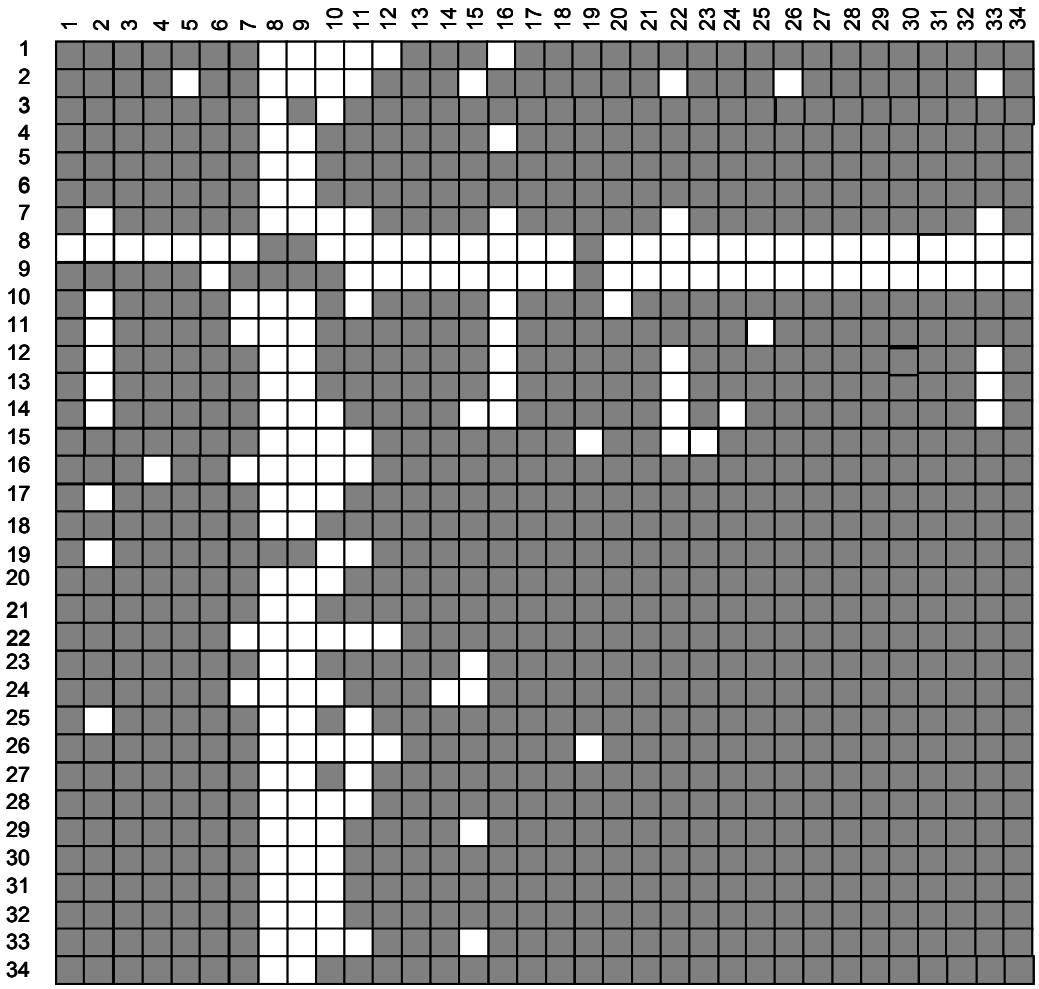


Figure 6 Cross-identification of *C. cinerea* hydrophobin genes by Blast searching all the hydrophobin genes deduced from the *C. cinerea* genome back in *C. cinerea* genome. Numbers at the left indicate the respective CoH proteins used in Blast searches, numbers above the diagram those of the *coH* genes that were detected (marked in grey), respectively non-detected in the individual Blast searches. The Blast search analysis was done at an expect value (E) of $1e^{-0}$.

Table 3. Compilation of the results shown in Fig. 1b of chapter 3 and in Figs. 4 to 6 of the appendix: Identification of *C. cinerea* hydrophobin genes by the heterologous basidiomycete hydrophobins in Blast searches, with lowering the stringency of the cut-off value (E-value) from $1e^{-3}$ to $1e^{-2}$, $1e^{-1}$, and $1e^{-0}$, respectively. The numbers of identified *Coprinopsis* hydrophobin genes per heterologous hydrophobin are given in this table

Protein	Number of hydrophobin genes detected at E-value			
	$1e^{-3}$	$1e^{-2}$	$1e^{-1}$	$1e^{-0}$
ABH1	23	23	28	30
HYPB	8	12	17	23
ABH2	17	17	20	23
ABH3-39	23	28	29	30
ABH3-97	14	17	21	24
Aa-Pri-2	20	21	25	30
DGH1	27	28	29	31
DGH2	27	30	31	31
DGH3	24	27	30	30
FVH1	16	24	24	26
Lehyd.1	0	0	2	11
Lehyd.2	25	27	28	28
POH1	26	27	30	30
POH2	26	30	30	31
POH3	28	29	30	32
Vmh1-1	20	30	31	32
Vmh1-2	30	30	31	31
Vmh2-1	28	29	30	30
Vmh2-2	28	28	30	31
Vmh3-1	21	22	25	26
Vmh3-2	20	22	25	26
FBH1	15	18	18	22
AF331452	1	4	6	11
HYD-Pt1	27	28	30	30
HYD-Pt2	24	24	26	27
HYD-Pt3	13	17	23	27
PNH1	19	24	24	29
PNH2	14	17	24	26
PNH3	27	28	30	31
SC1	20	21	25	27
SC3	20	22	25	27
SC4	9	13	16	19
SC6	23	23	25	28
HYD-1	27	27	30	30
Hum2	0	0	0	1

Table 4 Potential protein cleavage sites for hydrophobins with proteolytic enzymes (chymotrypsin and pepsin) were analyzed using peptide mass analyzer (http://www.expasy.org/tools/peptide-mass.html), and the resulting peptide sequences with the corresponding masses were given in the table respectively.

Protein	Peptide sequence	Mass	Peptide sequence	Mass
CoH1	TCSPISIIGLPGNSCNAQPV CCQNNNF	2794.2	IGCTPININL	1057.5
	IAIGCTPININL	1241.6	TCSPISIIGL	1003.5
			CCNTVTQ	768.3
			CNTGPIQ	732.3
			PGNSCNA	662.2
			PVCCQ	549.2
			NNNF	508.2
CoH2	IGGGNSCNAQTVCCQNNHF	1966.7	ISIGCTPIIIDV	1243.6
	ECCNSVQAPTNSGL	1422.5	NISVGDITGL	988.5
	ISIGCTPIIIDV	1243.6	IGGGNSCNA	792.3
	NISVGDITGL	988.5	TCNPISL	747.3
	TCNPISL	747.3	CCNSVQ	653.2
	TTVAL	504.3	PTNSGL	588.2
			TVCCQ	553.2
			NNHF	531.2
CoH3	TCSPISIIGLPGNSCNAQPV CCQNNSF	2767.2	IGCTPININL	1057.5
	IAIGCTPININL	1241.6	TCSPISIIGL	1003.5
			CNTGPIQ	732.3
			PGNSCNA	662.2
			PIPPSQ	638.3
			PVCCQ	549.2
CoH4	SIPIGITCSPISVIGLPGNS CSSQPVCCEDNSF	3324.5	SIPIGITCSPISVIGL	1569.8
	KGVIAIGCTPVNINV	1497.8	IGCTPVNINV	1029.5
	GVVVQDL	729.4	PGNSCSSQ	779.2
			CNTGPIQ	732.3
			PVCCE	550.2
			GVVVQ	501.3
CoH5	EARQGQCNTGPVQCCNSISAKDPATS	2879.3	IGCTPININV	1043.5
	TCSPISVIGLPGNSCSTQPV CCEDNSF	2758.1	TCSPISVIGL	989.5
	NGVVAIGCTPININV	1483.7	CCNSISTA	798.3
	GIVVQDL	743.4	PGNSCSTQ	793.3

			NIPIGL		626.3
			PVCCE		550.2
			GIVVQ		515.3
CoH6	GIVVQDVSIPIGITCTPITV IGLPGNSCSTQPVCCKDNSY	4092.0	DVSIPIGITCTPITVIGL	1812.0	
	EARQGQCNTGPVQCCNSVQR ADSEAASKL	3050.3	IGCTPININV	1043.5	
	KGVVAIGCTPININV	1497.8	PVCCKDNSY	1028.4	
			PGNSCSTQ	793.3	
			CNTGPVQ	718.3	
			CCNSVQ	653.2	
			GIVVQ	515.3	
CoH7	VVQDVNIPIGIICSPITIIG LPGNSCSTQPVCCQNNNF	3970.9	DVNIPIGIICSPITIIGL	1851.0	
	EARQSGGQCNTGPVQCCNSI SSAKDPSTAL	3009.3	IGCTPINVNA	1001.50	
	NGIVAIGCTPINVNA	1455.7	PGNSCSTQ	793.3	
			CCNSISSA	784.2	
			CNTGPVQ	718.3	
			KDPSTA	618.3	
			PVCCQ	549.2	
			NNNF	508.2	
CoH8	NLPPQTPVGKECTAGSNQGQ CTGGAQL	2656.2	PPTRRSPTL	1024.5	
	APRPSSLPQQCPAGKRVQCCEEM	2515.1	PVNGRVA	712.4	
	CCDDIAGLPVNGRVAVSCTA FPS	2295.0	PRPSSL	656.3	
	GPLPPTRRSPTL	1291.7	CCDDIA	639.2	
	TTAGNPSL	760.3	TPVGKE	630.3	
			GKRVQ	587.3	
CoH9	GIDIPADTPIGKGCKAGSDR TQCF	2450.1	PPVRRSPTF	1056.5	
	GPAPSPVPTNQCSAGAKVQC CY	2177.9	DTPIGKGCKA	989.5	
	ARGLPPLPPVRRSPTF	1761.0	PSPVPTNQ	839.4	
	AVPQGGPNAQRL	1207.6	VGCTPCSN	780.3	
	EVTTARNPL	1000.5	PGMKKL	673.4	
	CCNATGPAF	883.3	GSDRTQ	663.3	
	AVGCTPCSN	851.3	GIDIPA	585.3	

CoH10	VSVAAIPSGAPTCATGPIQC CERVY	2493.1	HRPVCCTDNKF	1319.5
	SVVGIGGGNKCAHRPVCCTD NKF	2362.1	VNVGCVPVNVNL	1226.6
	VNVGCVPVNVNL	1226.6	SVVGIGGGNKCA	1061.5
	ESQTTETSL	995.4	TGCSPL	577.2
	GGIATGCSPL	875.4	TGPIQ	515.2
CoH11	VAANPVANAQGSCNTGDIQC CQKL	2405.1	INIGCTPINIGL	1227.6
	GIGGGTKCTAAPVCCSHNTF	1923.8	GDIGSGCSPVTA	1063.4
	INIGCTPINIGL	1227.6	PVCCSHNTF	1007.4
	YPSQSSEASIL	1181.5	GSCNTGDIQ	894.3
	GDIGSGCSPVTAL	1176.5	GIGGGTKCTA	864.4
CoH12	SVVGVGGGTRCSSAPVCCTD NKF	2244.0	SVVGVGGGTRCSSA	1236.6
	INVGCVPVTVGL	1170.6	INVGCVPVTVGL	1170.6
	SAQSREANL	975.4	PVCCTDNKF	1026.4
	GDIASGCSPL	919.4	GVCNTGPVQ	874.4
			NPIPNSE	770.3
			ITVSVTA	690.4
			SGCSPL	563.2
CoH13	GDIGSGCSPISVIGIGGGNK CTAAPVCCTDNKF	3141.4	GDIGSGCSPISVIGIGGGNK CTA	2062.9
	GCTPINIGL	887.4	PVCCTDNKF	1026.4
			GTCNTGHIQ	930.4
			GCTPINIGL	887.4
			PPSPTPGL	765.4
CoH14	GGAPNCNQQTVCCTGNQY	1857.7	SSNHPSVSTL	1028.5
	SSNHPSVSTL	1028.5	GNSIGISCSA	908.4
	GNSIGISCSAL	1021.4	TVCCTGNQ	825.3
	HCCESTF	826.2	CNTGSL	594.2
	GCTPF	524.2	PNCNQ	575.2
	NIGSL	503.2	GCTPF	524.2
			NIGSL	503.2
CoH15	TQCSTGDVQCCNL	1371.5	CCSNKNKGIL	1079.5
	TQTAQCQSQPL	1204.5	VGCVPINISV	1000.5
	IGIQCTPINIL	1184.6	SSTVTGVVE	878.4
	CCSNKNKGIL	1079.5	CTPINIL	773.4
	AVGCVPINISV	1071.5	CSTGDVQ	709.2

	ASSTVTGVVEL	1062.5	SNDSVL	634.3
	SNDSVL	634.3		
	GPIAF	504.2		
CoH16	GGAVSCTQQTACCSNNSY	1793.6	IGCVPINISI	1028.5
	NGVVAIGCVPINISI	1468.8	DTGVGKIL	802.4
	CGNGGQTVCCNKL	1296.5	CCSNNSY	790.2
	QCTSINAL	849.4	TVCCNKL	780.3
	DTGVGKIL	802.4	CTSINA	608.2
	EQVTSL	676.3	VSCTQ	537.2
	SQVTGL	604.3	CGNGGQ	535.1
	AAPAPGL	596.3		
	GIANF	521.2		
	QTTY	512.2		
CoH17	VGTECTAINVAGVGGGSSCT QQKVCCTNNSF	3035.3	KVCCTNNSF	1015.4
	SQCNGGEIQCCNKAQSTKAL	2082.9	GCTPINVGV	859.4
	GCTPINVGV	859.4	GVGGGSSCTQ	852.3
	AAPSAVY	678.3	TTTRL	591.3
	TTRL	591.3	KGITGL	588.3
	KGITGL	588.3	CCNKA	538.2
	NGVIAL	586.3		
CoH18	EQCNGGEIQCCNSTKSVQSL	2127.9	KVCCNNNSF	1028.4
	GIGGGSKCTQQKVCCNNNSF	2044.8	CCNSTKSVQ	969.4
	GCSPINIGL	873.4	GIGGGSKCTQ	907.4
	VGTECTSL	809.3	GCSPINIGL	873.4
	KQITGL	659.4	PMPGGTE	688.2
	NGVIAL	586.3	TTKSL	549.3
	TTKSL	549.3		
CoH19	ARGLPPLPPVRRHATPAHRE SQCNGGTIKCCNIASPIRGC LPFPGNSVASSNDAVPKL	6028.0	CNGGTIKCCNIA	1196.5
	GVGGGSSCTGQTVCCSGNDF	1835.6	TVCCSGNDF	945.3
	NGVITAGCTPISIGA	1373.7	GVGGGSSCTGQ	909.3
	NTIVGM	634.3	PPVRRHA	832.4
	QCTNL	578.2	GCTPISIGA	818.4
			NTIVGMQ	762.3

				SPIRGCL		745.4
				NGVITA		574.3
				PGNSVA		544.2
CoH20	VQCNGGQVQCCNDVKETNQL		2179.9	TVCCTDNSF		989.3
	GIGSNSCDAQTVCCTDNSF		1921.7	GCTPININL		944.4
	GCTPININL		944.4	TCNTVNVL		863.4
	TCNTVNVL		863.4	GIGSNSCDA		823.3
	DAPYNQL		820.3	CCNDVKE		810.3
	DVDVKQL		816.4	DVDVKQ		703.3
	DGIIAL		601.3	TGKVGL		574.3
	TGKVGL		574.3			
CoH21	AAPGATTEQCNGGEVQCCNS VQDANNL		2694.1	DSSVKKIITGL		1160.6
	GIGGGSSCTQQKVCCTNNSF		1990.8	VGVTCTSVNVL		1091.5
	KQITGQVGVTCTSVNVL		1746.9	KVCCTNNSF		1015.4
	DSSVKKIITGL		1160.6	GCTPINVSV		889.4
	GCTPINVSV		889.4	GIGGGSSCTQ		866.3
				CCNSVQ		653.2
CoH22	TCTGVKAGGIGGAASCTQQK L		1950.9	CCTNSNF		788.2
	VSAVVANPAPTEIEY		1559.7	IGCTPIL		716.4
	EQCEGGTVQCCASY		1477.5	TCTGVKA		679.3
	NINARQVDANVGF		1417.7			
	NGVVAIGCTPIL		1156.6			
	CCTNSNF		788.2			
	QKATDL		675.3			
	NAEW		519.2			
CoH23	TGQVGVTCTAVNVVGVGGGS HCSNQAVCCNNNNF		3310.4	VNVVGVGGGSHCSNQ		1413.6
				VCCNNNNF		927.3
	GCTPINVSV		889.4	GCTPINVSV		889.4
	DAGVKGL		659.3	CCNKVQ		694.3
	NGVVAL		572.3	VGVTCTA		650.3
				PSDSNQ		647.2
				DSKSL		549.2
CoH24	TCTDVTVIGVGGGSSCNNQQ VCCNNNNF		2848.1	TCTDVTVIGVGGGSSCNNQ		1811.7

	AAPTSGVVAQCNGGVVQCCN EM	2137.9	VCCNNNNF	927.3
	GCTPINVSI	903.4	GCTPINVSI	903.4
	EAPIAGL	670.3	CNGGVVQ	676.3
	QSSTSL	622.3	PTSGVVA	630.3
	TGQVGL	574.3		
CoH25	TGQVGASCTGINVIGVGGGT SCSNQPVCCTGNNF	3202.4	SCTGINVIGVGGGTSCSNQ	1753.7
	AAPSSQTGDAIACGNGGTL	1690.7	IGCTPINISL	1030.5
	SGVVAIGCTPINISL	1443.7	PVCCTGNNF	954.3
	QCCNTVESSNNL	1311.5	GVDISKL	731.4
	GVDISKL	731.4	CCNTVE	668.2
			CGNGGTL	621.2
			SSNNL	534.2
CoH26	SCTGVNVVGIGGGSSCTQQK VCCNNNNGVVAL	3082.4	SCTGVNVVGIGGGSSCTQ	1625.7
	ATAVIAAPGGRPSEVEY	1687.8	KVCCNNNNGVVA	1234.5
	EQCNGGTVQCCNSY	1505.5	DSIDHSA	744.3
	QKADSIDHSASKL	1399.7	NIDVKQ	716.3
	NIDVKQVAAGL	1127.6	PGGRPSE	699.3
	GCTPINASL	875.4	CNGGTVQ	678.2
			GCTPINA	675.3
			CCNSY	589.1
CoH27	TCTGINAVGIGGGSSCTQQK VCCTNNKF	2791.2	KVCCTNNKF	1056.4
	EQCNGGEIQCCNSY	1547.5	VGIGGGSSCTQ	965.4
	NIDVKQITAGVGL	1327.7	GCSPINVSL	889.4
	GCSPINVSL	889.4	NIDVKQ	716.3
	QKADSL	661.3	TCTGINA	679.3
	NGVVAL	572.3	CCNSY	589.1
	NTSKL	562.3	NTSKL	562.3
CoH28	QQCNGGKIQCCNSVQDSKSL	2139.9	KVCCTNNSF	1015.4
	GVGGGSSCTQQKVCCTNNSF	1976.8	GGHKNVDY	889.4
	KVDVKQITGQVGTGCTSL	1833.9	GCSPINVSV	875.4
	GCSPINVSV		GVGGGSSCTQ	852.3
	NGVVAL	875.4	VGTGCTSL	737.3

		572.3	CNGGKIQ	719.3
			KVDVKQ	716.4
			CCNSVQ	653.2
			DSKSL	549.2
CoH29	QQCNGGEIQCCNSVQDSKSL	2140.9	KVCCTNNSF	1015.4
	GVGGGSSCTQQKVCCTNNSF	1976.8	GVGGGSSCTQ	852.3
	KVDVKQITGQVGTGCTSL	1833.9	GCSPINVSA	847.3
	GCSPINVSA	847.3	VGTGCTSL	737.3
	NGVVAL	572.3	KVDVKQ	716.4
			CCNSVQ	653.2
			DSKSL	549.2
CoH30	QQCNGGEIQCCNSVQDSKSL	2140.9	KVCCTNNSF	1015.4
	GVGGGSSCTQQKVCCTNNSF	1976.8	GCSPINVSV	875.4
			GVGGGSSCTQ	852.3
	KVDVKQITGQVGM	1402.7	VGMGCTSL	767.3
	GCSPINVSV	875.4	KVDVKQ	716.4
	NGVVAL	572.3	CCNSVQ	653.2
			DSKSL	549.2
CoH31	QQCNGGEIQCCNSVQDSKSL	2140.9	KVCCNNNSF	1028.4
	GVGGGSSCTQQKVCCNNNSF	1989.8	GVGGGSSCTQ	852.3
	KVDVKQITGQVGM	1402.	GCSPINVSA	847.3
	GCSPINVSA	847.3	VGMGCTSL	767.3
	NGVVAL	572.3	KVDVKQ	716.4
			CCNSVQ	653.2
			DSKSL	549.2
CoH32	QQCNGGEIQCCNSVQDSKSL	2140.9	KVCCSNNSF	1001.4
	GVGGGSSCTQQKVCCSNNSF	1962.	GGHKNVDY	889.4
	KVDVKQITGQVGM		GVGGGSSCTQ	852.3
	GCSPINVSA	1402.7	GCSPINVSA	847.3
	NGVVAL	847.3	VGMGCTSL	767.3
		572.3	KVDVKQ	716.4
			CCNSVQ	653.2
			DSKSL	549.2
CoH33	GAGGGSSCTEQKVCCTNNSF	1949.7	GSHHKSRVE	1036.5

	KIDAKQVTGQVGVGCTAL	1787.9	KVCCTNNSF	1015.4
	GVQDSKSL	833.4	GGGSSCTE	697.2
	DNEVTKL	818.4	VGVGCTA	606.2
	GCSPANVSA	805.3	DSKSL	549.2
	NGVVAL	572.3		
CoH34	KVDIKQITGQVGVGCTAVNV L	2142.1	KVCCSNNSF	1001.4
	EQCNGGEIQCCNSVQQSNSL	2140.8	GVGGGSSCTQ	852.3
	GVGGGSSCTQQKVCCSNNSF	1962.8	GCSPINVSA	847.3
	GCSPINVSA	847.3	KVDIKQ	730.4
	NGVVAL	572.3	CCNSVQ	653.2
			VGVGCTA	606.2

CHAPTER 4

Transcript profiles of hydrophobin genesduring development of *Coprinopsis cinerea*

4.1 Abstract

Coprinopsis start its life cycle with single sterile monokaryotic hyphae germinating from basidiospores and developing into branched mycelium by colonizing moist substrate surfaces. Fertile dikaryon formation occurs upon fusion of two compatible sterile monokaryons with different mating types. Upon nutrient depletion and under defined environmental conditions, the fertile dikaryotic mycelium may form fruiting bodies. In the genome of *C. cinerea*, there are 34 different genes for hydrophobins, small fungal-specific proteins found in many asco- and basidiomycetes. Here, expression of all 34 genes is followed during fungal development. In this analysis, a total of 29 different genes were found to be expressed in *C. cinerea*. RT-PCR analysis showed that most of the hydrophobin genes are expressed during fruiting body development. Very few genes are stage-specific and expressed only in mono-, or in dikaryotic mycelium or in tissues of fruiting bodies. Comparative transcript profiling of a wild type monokaryon and clones of this monokaryon transformed either with compatible *A*, compatible *B*, or compatible *A* and compatible *B* mating type genes revealed that there is no significant influence by the mating type pathways on expression of hydrophobin genes.

Keywords: Multi-gene family-Stage-specific expression-Transcripts analysis-Hydrophobins

4.2 Introduction

Fruiting body development in the higher basidiomycetes is a very complex process that is influenced by different environmental and genetic factors. Environmental factors such as nutrients, light, humidity and temperature play significant roles in mushroom development. Few genetic factors influencing fruiting development are so far known. The main regulators of development are the mating type genes (Wessels 1993, Kües 2000, Kües and Liu 2000, Kües et al. 2004).

In the model basidiomycete *Coprinopsis cinerea*, the effect of the genes of the two mating type loci were analyzed with respect to their regulation of sexual development (Kües et al. 1998, 2002). The *A* mating type genes encoding two types of homeodomain transcription factors were shown to induce fruiting body development but development arrests after tissue formation in the fruiting body primordia (Tymon et al. 1992, Kües et al. 1998). The *B* mating type genes encoding pheromone receptors enhance *A* mating type induced initiation of fruiting body development and induce maturation of fruiting bodies at the stage of karyogamy, when the tissues in the primordia are fully developed (Kües et al. 2002). In *Schizophyllum commune*, several genes were shown to be expressed in different tissues of fruiting bodies (Dons et al. 1984, Mulder and Wessels 1986). Of these, differential regulation of genes for hydrophobins is well documented and hydrophobins were shown to be important for the mushroom development (Wessels 1997, Wösten 2001, Whiteford and Spanu 2002, Walser et al. 2003). Hydrophobins are small fungal-specific extracellular proteins secreted through hyphae into the surrounding aqueous environment as monomers. These monomers aggregate and form amphipathic films upon hydrophobic and hydrophilic interfaces (Wösten et al. 1993). Such films coat surfaces of aerial mycelium and the fruiting bodies, helping the fungi in developing complex aerial structures under adverse environmental conditions (Wösten 2001, Walser et al. 2003).

In *S. commune,* the hydrophobin genes *SC*1, *SC*3*, SC*4 and *SC*6 were shown to be expressed in different tissues of fruiting bodies. These genes were shown to be regulated by mating type genes. *SC*3 is found expressed in mycelium of mono- and dikaryons, and *SC*1 and *SC*6 only in the mycelium of dikaryons, whilst expression of *SC*4 is restricted only to fruiting structures (Wessels 1991, 1995, Schuurs et al. 1998, Ásgeirsdóttir et al. 1995). Different hydrophobins from other basidiomycetes were also shown to be stage-specific in their expression. For example, *ABH*1 from *Agaricus bisporus* is highly expressed in fruiting bodies whereas *ABH*2 was found to be mycelium-specific. No transcripts for gene *ABH2* were seen in fruiting bodies (Lugones et al. 1996, de Groot et al.

1999). Likewise, *PoH*1 from *Pleurotus ostreatus* was found to be fruiting body-specific and *PoH*2 mycelium-specific (Ásgeirsdóttir et al. 1997, Peñas et al. 1998). In *C. cinerea,* one hydrophobin, CoH1, was detected in monokaryotic vegetative mycelium. Transcripts for *coH*1 were found in mono- and dikaryotic vegetative mycelium and also in primordia and young fruiting bodies. No transcripts or poor amounts of transcripts were reported in the later stages of fruiting body development (Ásgeirsdóttir et al. 1997).

Within the genome of *C. cinerea*, we found 34 potential hydrophobin genes with some genes localized in close proximity to each other in clusters, and others at individual positions within the genome (see chapter 3 for more details). In this study, we report a transcript profile analysis of the hydrophobin genes during different developmental stages of the fungal life cycle. The life cycle of *C. cinerea* starts with single monokaryotic hyphae germinating from basidiospores that develop into branched mycelium by colonizing a moist substrate surface. Dikaryons are formed upon fusion of two compatible monokaryons with different mating types. Upon nutrient depletion and under defined environmental conditions, such dikaryotic mycelium may form fruiting bodies (Kües 2000). Mono- and dikaryotic mycelia and different stages in fruiting body development were analyzed for expression of hydrophobins. Transcripts for 29 hydrophobin genes were found in *C. cinerea*. However, very few of these genes appear to be stage-specific in their expression. Transformants of a monokaryon with compatible *A*, with compatible *B* and with both compatible *A* and compatible *B* mating type genes were analyzed. Mating type genes have no or not much significant influence on hydrophobin gene regulation in *C. cinerea*.

4.3 Materials and Methods

4.3.1 C. cinerea strains and culture conditions.

Monokaryons of different genetic backgrounds were used in this study: JV6 (*A42, B42*), Okayama 7 (*A43, B43*), FA2222 (*A5*, *B6, acu-1, trp-1.1,1.6*), 218 (*A3*, *B1, trp-1.1,1.6*) (Kertesz-Chaloupková et al. 1998), the co-isogenic strains PS001-1 (*A42, B42*) and PS002-1 (*A3, B1*) (Srivilai et al. *in preparation*) and the self-compatible homokaryon AmutBmut (*A43mut*, *B43mut, pab-1*) (Swamy et al. 1984, May et al. 1991). Mating type genes influence on hydrophobins was analyzed using *trp3*$^{+}$ transformed clones of monokaryon AT8 (*A43*, *B43, trp-3, ade-3*) that were previously transformed with plasmid pDB3 and a compatible *A* mating type gene (*A*on), a compatible *B* mating type gene (*B*on) and compatible *A* and compatible *B* mating type genes (*A*on, *B*on), respectively, and, as a

control, a tryptophan-prototrophic control transformant AT8/pDB3 (Kües et al. 1998, 2002). Dikaryons were created by crossing two compatible strains (AmutBmut x JV6, Okayama7 x JV6) and (PS001-1 x PS002-1) as described by Walser et al. (2001). For aerial mycelium production, all cultures were grown at 37^{o}C on agar plates with standard *Coprinopsis* YMG/T medium (Granado et al. 1997) covered with sterile cellophane, until the growing mycelial front reached the edges of the Petri dishes. Mycelia were harvested from the cellophanes, transferred directly into liquid nitrogen and freeze dried. For testing early stages in fruiting (Table 1), plates of homokaryon AmutBmut were cultivated one day further at 37^{o}C to obtain a high number of primary hyphal knots. Secondary hyphal knots were induced by shifting cultures fully grown to the edge of the Petri-dishes into a standard fruiting regime (25^{o}C, light, 90% humidity in a 12 hours light and 12 hours dark regime - in this 24 h scheme, the moment when the light is switched on is arbitrarily set zero and the moment when light is switched off is arbitrarily set 12 hours; Granado et al. 1997). From cultures of different age, plain vegetative mycelium, mycelium with primary hyphal knots and mycelium with secondary hyphal knots were harvested, transferred into liquid nitrogen and freeze dried. Further on, fruiting stages were harvested from horse dung cultures. Homokaryon AmutBmut was grown on horse dung for 4 days at 37^{o}C and then transferred into fruiting conditions as described by Granado et al. (1997). Developing primordia and fruiting bodies were collected as described in Table 1. From primordia stage 2 (Table 1), cap and stipe tissues were separated before freezing in liquid nitrogen and all the samples were freeze dried. Samples were stored at -80^{o}C for further use.

4.3.2 DNA methods.

Genomic DNA was extracted as described by Zolan and Pukkila (1986) from all the wildtype monokaryotic strains mentioned above and in addition from the untransformed *trp3*-auxotrophic monokaryon AT8 (Kertesz-Chaloupová et al. 1998). Hydrophobin gene specific primers (Table 2) were tested on the genomic DNAs of all the strain used in this study.

4.3.2 RNA isolation and cDNA synthesis

Total RNA was isolated from the mycelial and the fruiting samples. Samples were ground to fine powdered material and total RNA was extracted by using guanidinium isothiocyanate (Boulianne et al. 2000). cDNAs were synthesized using RevertAid M-MuLV Reverse Transcriptase (Fermentas) according to the manufacturer's instructions

Table 1. Developmental stages of *C. cinerea* from vegetative mycelium to fruiting body maturation observed on homokaryon AmutBmut (Velagapudi, unpublished).

Sample number	Sample	Description of stage	Time point of harvest
Agar plate cultures			
0	Vegetative mycelium	Plain mycelium with no highly ordered structures	Cultures grown till the edge of plate (day 0, 1 h after light on)
1	Primary hyphal knot stage	Primary hyphal knots within outer ring of mycelium about 1 cm width	Edges of the cultures were taken for analysis (day 1, 1 h after light on)
2	Secondary hyphal knot stage	Mycelium with secondary hyphal knots	Cultures from edges were harvested (day 2, 1 h after light on)
Horse dung cultures			
3	Primordia of 2-3 mm (stage 1 primordia)	Primordia 3 days old forming secondary gills (1 day after secondary hyphal knot formation)	Day 3 (1 h after light on)
4	Primordia of 4-6 mm (stage 2 primordia)	Primordia 4 days old minor stipe elongation	Day 4 (1 h after light on)
5	Primordia of 9-10 mm (stage 3 primordia)	Primordia at day 5, minor stipe elongation undergoing karyogamy	Day 5 (1 h after light on)
6	Primordia of ~15 mm (stage 4 primordia)	Primordia with minor stipe elongation undergoing meiosis I	Day 5 (6 h after light on)
7	Primordia of ~17 mm (stage 5 primordia)	Young slowly elongating fruiting body, undergoing meiosis II	Day 5 (10 h after light on)
8	Young fruiting body of ~ 25 mm	Elongating fruiting body producing basidiospores	Day 6 morning (1 h after light on)
9	Young fruiting body of ~ 30 mm	Rapid stipe elongation, basidiospore production-basidiospore pigmentation	Day 6 afternoon (6 h after light on)
10	Young fruiting body of ~ 45 cm	Basidiospore maturation	Day 6 evening (10 h after light on)
11	Mature fruiting body of ~ 80-120 mm	Mature fruiting body spore releasing	Day 6, (2 h after light switch off)
12	Senescent fruiting body of ~ 80-120 mm	Autolysed fruiting body	Day 7, (6 h after light switch off)

(Gerard and D'Alessio 1993). 2 µg of total RNA was used in each reaction and obtained cDNA was used for transcript profiling. The quality of the synthesized cDNAs was analyzed using ß-tubulin gene specific primers (ß-tubF, 5'-ATGCGTGAAATCGTCCACCTC-3', ß-tubR, 5'-TCACACTGAGCGGTGAGAAC-3'). The cDNAs were used for hydrophobin transcript analysis using gene specific primers (Table 2). 25 µl PCR reaction mixture (20 mM Tris/HCl pH 8.3, 1.5 mM $MgCl_2$, 0.2 mM each dNTP and 1 U *Taq* polymerase), with gene specific primers at a final concentration of 0.4 µM were used as standard throughout this study. 10 µl aliquots of the PCR products were then analyzed on 2% agarose gels run for 60 min at 80V constant.

4.4 Results

4.4.1 Defining PCR conditions for transcript detection

To analyze the transcript profiles of the multiple hydrophobin genes in *C. cinerea*, gene specific primers were designed (Table 2) based on the hydrophobin gene sequences deduced from the sequenced genome of *C. cinerea* strain Okayama 7 (see chapter 3). For some genes, primers were designed based on promoter and terminator regions within the 20 bases upstream and downstream to the start and stop codons, respectively, in order to attain the highest specificity. In other cases, primers were designed from the internal gene sequence to obtain different fragment sizes (Table 2) in order to easily differentiate amplified cDNAs from each other on agarose gels. Attempts were made with combinations of each 4 to 5 different primer sets to apply the multiplex PCR technique in studying the hydrophobin gene transcript profiles. Tests on genomic DNA from homokaryon AmutBmut resulted in amplification of gene specific bands. However, too many unspecific extra bands appeared, making the pattern analysis very difficult. When primers were applied on cDNAs, similar difficulties were encountered. Therefore, all further analysis was done with primer pairs for individual hydrophobin genes. Annealing conditions for the primer pairs were tested with genomic DNA from AmutBmut and a temperature of 55^{o}C was found suitable for all primers, simplifying the PCR analysis with masses of samples. All primers were tested against genomic DNA of a number of monokaryons. All primer pairs (with few exceptions) gave fragments of expected sizes with genomic DNA from strains Okayama 7, JV6, AT8, PS001-1 and PS002-1. Amplification of genomic DNA was not observed for *coH*28 in JV6, *coH*30, *coH*32 and *coH*34 in AT8/pDB3, and in PS001-1 and PS002-1, even after repeated attempts. In contrast, in repeated

Table 1. Gene-specific primers for *C. cinerea* genes (forward: coding strand, reverse: non-coding strand; note that primers used for *coH1* are chimeric and come from production of an expression construct made for transformation of *coH1* into *S. commune*, R. Velagapudi unpublished. * In brackets: new primer sequences and values of genomic and cDNA obtained in analysis of confirming extra 5´introns in these genes by amplifying cDNAs of the expected sizes

Gene	Forward primer	Primer sequence	Expected size of genomic DNA	Expected size of cDNA
coH1	ttcgctatccttccttacaacctgctcgcc ATGCAGTTCAAGTTCTTGTC	actggccctctggtcaactataata ttatTTAGAGGTTGATGTTGATGG	452	391
coH2	GGTTGCTGAACATTTCC	CGCACAAACGAACTTCA	305	198
coH3	CGCTTCTCTCGCTGTT	GCGCTCGACGAACTG	455	341
coH4	GTGCAATACCGGTCCA (TTGGCTGCAGTCTCCG)	CGTTAATATAATCACCCGA	395 (481)	279 (338)
coH5	CAAAGTTCTCTCTACCC	CTCAAACATCCCCCAC	477	402
coH6	GTTCAGTGCTGCAATAGT	CTGAGTTGGCAGGGAT	391	273
coH7	TCCACTGCGTTGCTAC	CTCGAACTCAGAACGC	389	296
coH8	AGGAGATGACGACTGC (GTCACCGCTATTCCTCG)	GCCTCCTGGACAAGC	388 (679)	260 (541)
coH9	ATGAAAGCCACCACTTTC	GCAGAGGAGTGGAAAG	700	423
coH10	TAGCAGCTATTCCAAGC	ATGGTATGGGAGAGGC	486	337
coH11	GCTTTGACTGTTGCTAC	TGAGTGACGGAAATGAAG	514	421
coH12	TCACCGTCTCCGTGA	CGGAATGATGGGTCG	454	316
coH13	TCCTCTTCCCAGCAC	GTCCACCTCATCCGG	417	295
coH14	CTCCTTGCAGTTGCTC	GGCCACAATTCACAAAG	528	415
coH15	CACTATGCTGCTCCAAC (GTTTTCCTCAACTTGGCC)	CGAGCGAGGGAAAGA	182 (823)	116 (249)
coH16	GCCCAACTATAACCCAG	GGCCAGAGTCGTGAA	516	393
coH17	GCTGCAATAAGGCTCAG	GCAGGGTAGTCGTAAG	349	246
coH18	GCACCAAGAGTGTGC	TAGTAGGGAATGTGGGT	371	261
coH19	AATTGCTCTCGTCGATC (AAGCTCGCTTTCATCGC)	GGAACGGGAGTGAACA	324 (668)	237 (546)
coH20	GCAACGACGTTAAGGAG	CTTGCGATAGGGTTTGT	370	255
coH21	ACTCGTCTGTCAAGAAG	CACTGTGAGATGAGAGC	354	227
coH22	ACGAGCAATGCGAAG	CCCACTTGATTACGGC	411	293
coH23	GGTGCTTAACATCGACC	CTCACCCGCCAATAG	327	203
coH24	AATGGAGGTGTCGTCC	CGCAGAGCGGGGTAT	393	270
coH25	GCAGCAACAATTTGAGC	ATGCGTTGAAGGGTGC	363	262

experiments no bands were amplified with primer pairs for *coH*7, *coH*9, *coH*28, *coH*31, *coH*32, *coH*33, and *coH*34, respectively with DNA from monokaryon 218, and with primer pairs for *coH*4, *coH*7, *coH*15, *coH*18, *coH*24, *coH*28, *coH*30, *coH*31, *coH*32, *coH*33, and *coH*34, respectively with DNA from monokaryon FA2222, suggesting that these strains are divergent in the sequence of these hydrophobin genes.

For transcript analyses (Fig. 2 to 4), mycelial samples from monokaryons and dikaryons were collected and from transformants of monokaryon AT8 carrying foreign *A* and *B* mating type genes. Furthermore, three different stages of the mycelium of homokaryon AmutBmut were collected, the plain vegetative mycelium, mycelium with primary hyphal knots (phenotype: pkn) and mycelium with secondary hyphal knots (phenotype: skn). In addition, various stages in fruiting body development were collected from homokaryon AmutBmut, most of which were divided into cap and stipe samples (Fig. 1). Total RNA of all samples was isolated, cDNA synthesized and used in PCR with gene-specific primer pairs. Minor genomic DNA present in the cDNA samples served as internal control for successful PCR and as an indicator of levels of gene transcription.

4.4.2 Expression of hydrophobin genes in sterile monokaryons

Mycelial samples of different monokaryons (JV6, Okayama 7, AT8/pDB3, PS001-1 and PS002-1) grown on cellophane layered agar (YMG/T) plates were tested for hydrophobin gene expression. In total, 25 different genes were found to be expressed in the different monokaryons. 14 of these (*coH*1, *coH*3, *coH*4, *coH*5, *coH*6, *coH*8*, coH1*5, *coH*16, *coH19*, *coH*21, *coH*22, *coH*26, *coH*27 and *coH*33) were expressed in all strains. Other genes were found expressed in variable patterns between the five different monokaryons. 3 genes (*coH*11*, coH*17, and *coH*18) were expressed in four of the 5 strains, 1 (*coH*23) in 3 of the strains, and 2 (*coH*10, and *coH*12) in 2 of the strains and 5 (*coH*2, *coH*7, *coH*13, *coH*20, and *coH3*2) only in one strain (Fig. 2, and 5). Amongst those genes, *coH*1, *coH*3, *coH*4, *coH*5, *coH*6, *coH*8, *coH*16, *coH*19, *coH*26, *coH*27, and *coH*33 were highly expressed in at least 3 strains as RT-PCR gave strong bands in these monokaryons (AT8/pDB3, Okayama 7 and JV6) suggesting high transcript levels (Fig. 2). Genes *coH*7 and *coH*10 were found to be specific to the co-isogenic strains PS001-1 and PS002-1 as their transcripts were not found in other monokaryons (Fig. 2, 5).

Figure 1. **Fruiting body development in *Coprinopsis cinerea* strain homokaryon AmutBmut**. The mycelial cultures were harvested from YMG/T agar medium plates and fruiting body developmental stages were harvested from cultures grown on horse dung. Detailed description of the developmental stages and harvest timing were described in Table 1. Inserts in panel 1 and 2 show primary and secondary hyphal knots. Starting from sample 4, cap (C) and stipe (S) tissues were collected and analyzed independently. Total RNA was isolated from all the samples shown above and the corresponding cDNA was used for hydrophobin transcript profiling using hydrophobin gene specific primers (Table 2).

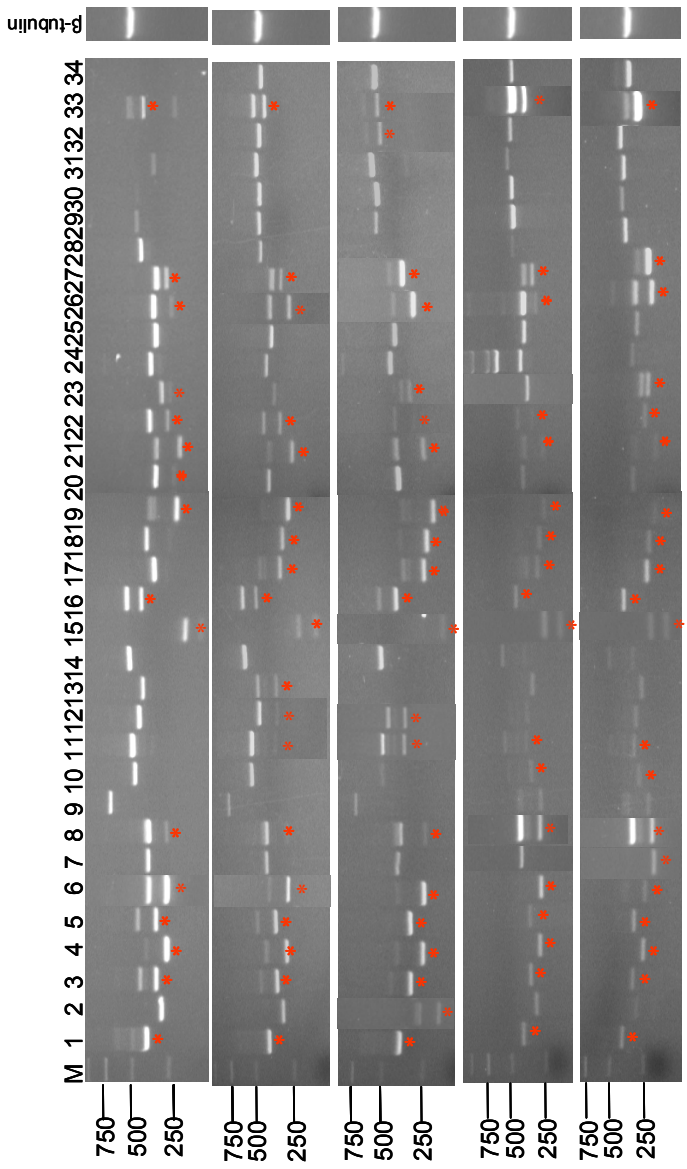


Figure 2. **RT-PCR analyses of hydrophobin genes in different sterile monokaryons**. Monokaryotic mycelial samples from AT8/pDB3, Okayama 7, JV6, PS001-1 and PS002-1 were harvested from cellophane layered agar plates. Total RNA was isolated and the synthesized cDNA was used for expression profiling with hydrophobin gene specific primer pairs (Table 2). Aliquots of PCR products were analyzed on 2% agarose gels. The top lane shows the 34 different hydrophobin genes (*coH1-coH34*). The asterisk (*) mark the cDNAs of expressed genes and the unmarked bands correspond to genomic DNA sizes of the respective genes. Amplification of β-tubulin cDNA served as quality control of the cDNA and the PCR reactions. Note that the size of the amplified fragment of β-tubulin cDNA is 1 kb and not shown to scale with the size marker (Fermentas) at the left.

4.4.3 Expression of hydrophobin genes in fertile dikaryons and homokaryon AmutBmut

Transcripts for 22 different hydrophobin genes were found in mycelium of dikaryotic strains (AmutBmut x JV6, Okayama 7 x JV6 and PS001-1 x PS002-1). 16 genes were expressed in all three dikaryons, 3 in two dikaryotic strains and 3 genes only in one strain (Fig. 3, Fig. 5). Strong bands in RT-PCR suggest high expression for 14 different genes (*coH*1, *coH*3, *coH*4, *coH*5, *coH*6, *coH*16, *coH*17, *coH*18, *coH*19, *coH*21, *coH*22, *coH*26, *coH*27 and *coH*33) (Fig. 3). Comparative analysis between mono- and dikaryotic samples revealed that the expression pattern for most genes is shared between the two types of mycelia. Only one gene (*coH*34) was found to be specific to dikaryotic mycelium (AmutBmut x JV6, and PS0011 x PS0021) (Fig. 3, Fig. 5). 20 different genes were found transcribed in mycelium of homokaryon AmutBmut, 9 of which were highly expressed. Gene *coH*3 gave also a strong band in RT-PCR with AmutBmut cDNA and there was also a transcript for *coH*32 found elsewhere only in JV6 (monokaryon) and PS001-1 x PS002-1 (dikaryon). Overall, the expression of the self-compatible homokaryon AmutBmut resembles mostly that of monokaryon Okayama 7 (Fig. 3, Fig. 5).

4.4.4 Influence of mating type genes on hydrophobin regulation

Mating type genes are the most important regulatory genes in the sexual development of *C. cinerea* (Kües 2000, Casselton and Olesnicky 1998). Transformants of monokaryon AT8 with compatible *A*, with compatible *B* and with compatible *A* and compatible *B* genes were available for transcript profile analysis (Kües et al. 2002). Transcripts for 18 different hydrophobin genes were found in different mating type specific transformants, whereas in the mating type-untransformed monokaryon AT8/pDB3 mycelium, 16 different genes were found expressed (Fig. 4, 5). Compared to mating type-untransformed strain AT8/pBD3, within the limits of detection, we found very few changes in the transcript pattern of the mating type transformants. In most instances, bands detected in RT-PCR analysis that were unique to one or two samples were very faint, such as the amplified *coH*20 cDNA band in the AT8/pDB samples of the monokaryon control (Fig. 4). In the *A*on transformant, a stronger band for *coH*13 was found, whereas a faint band was found in *B*on transformants, and a faint band for *coH*7 is found unique to the *A*on*B*on mating transformants (Fig. 5). A faint band for *coH*23 was present in the samples of the *A*on and *B*on mating type transformants of monokaryon AT8, but it was not in the case of the dikaryon-like *A*on*B*on transformant (Fig. 4, 5).

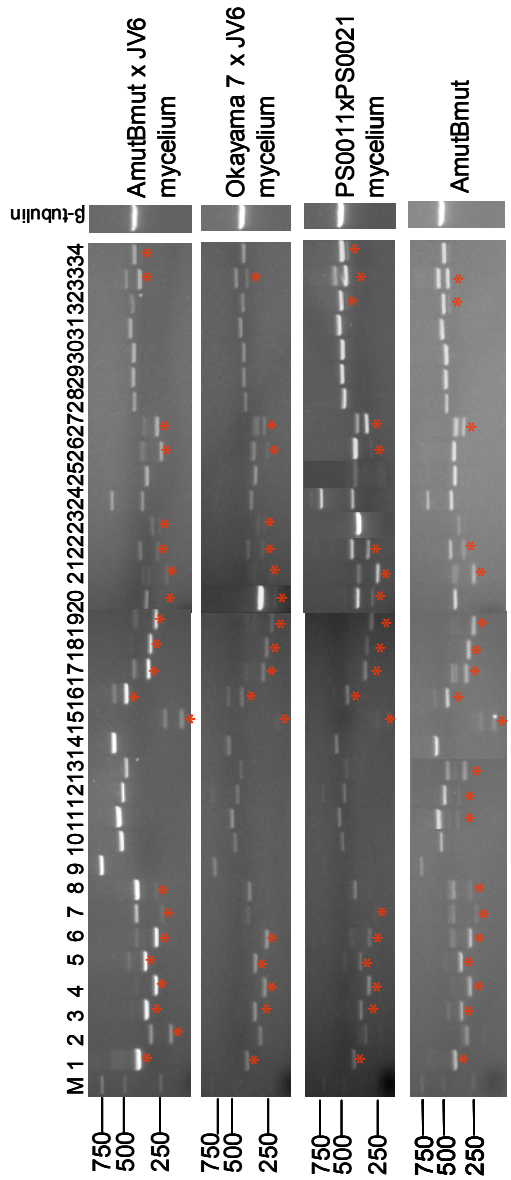


Figure 3. **Transcript profiling of hydrophobin genes in fertile dikaryons and homokaryon AmutBmut**. Dikaryotic mycelial samples from AmutBmut x JV6, Okayama 7 x JV6, PS001-1 x PS002-1 and mycelium from homokaryon AmutBmut were analyzed for the hydrophobin gene expression. The top lane shows the 34 different hydrophobin genes (*coH1*-*coH34*). The asterisk (*) mark the cDNAs of expressed genes and the unmarked bands correspond to genomic DNA sizes of the respective genes. Amplification of β-tubulin cDNA served as quality control of the cDNA and the PCR reactions. Note that the size of the amplified fragment of β-tubulin cDNA is 1 kb and not shown to scale with the size marker (Fermentas) at the left.

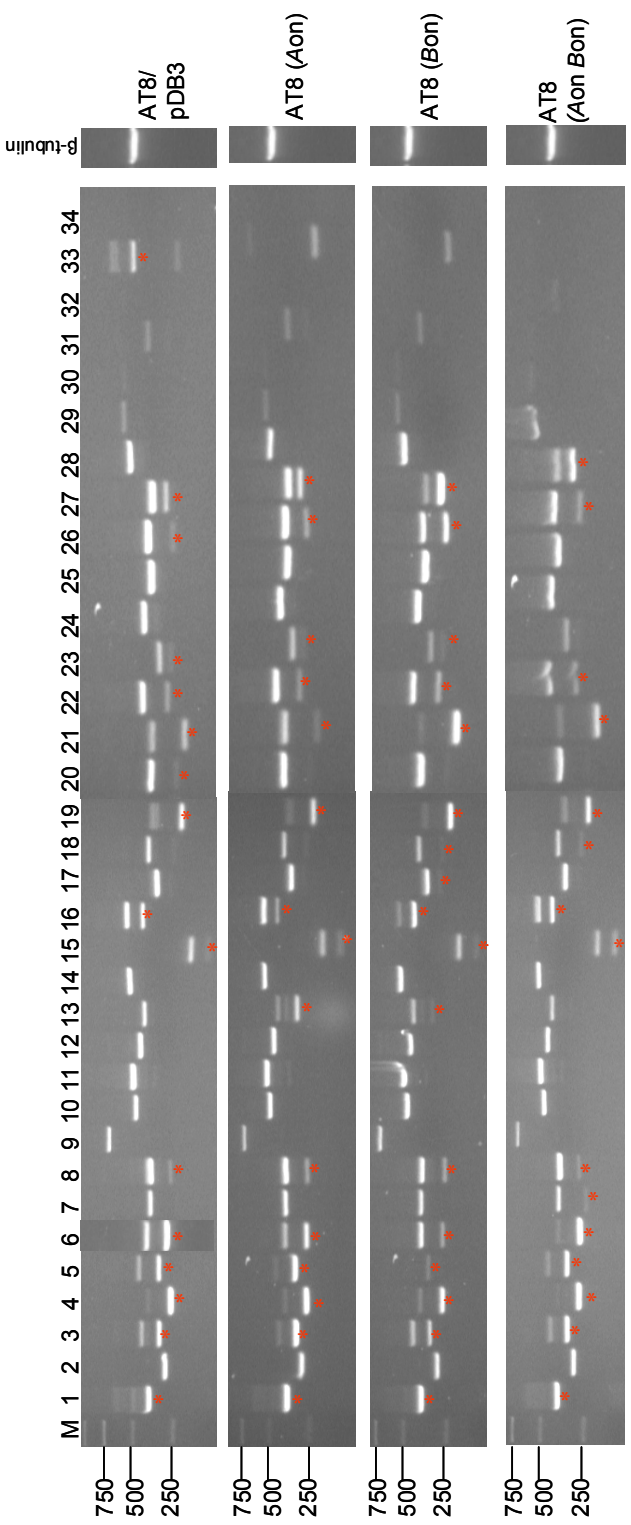


Figure 4. **Influence of mating type genes in hydrophobin gene regulation**. Transformants in monokaryon AT8 with compatible *A* (Aon), compatible *B* (Bon) and compatible *A* and compatible *B* (Aon Bon) mating types were tested for hydrophobin expression in the plain vegetative mycelial samples using gene specific primer pairs. Mating-type untransformed monokaryon AT8/pDB3 served as a control. The asterisk (*) mark the cDNAs of expressed genes and the unmarked bands correspond to genomic DNA sizes of the respective genes. Amplification of β-tubulin cDNA served as quality control of the cDNA and the PCR reactions. Note that the size of the amplified fragment of β-tubulin cDNA is 1 kb and not shown to scale with the size marker (Fermentas) at the left.

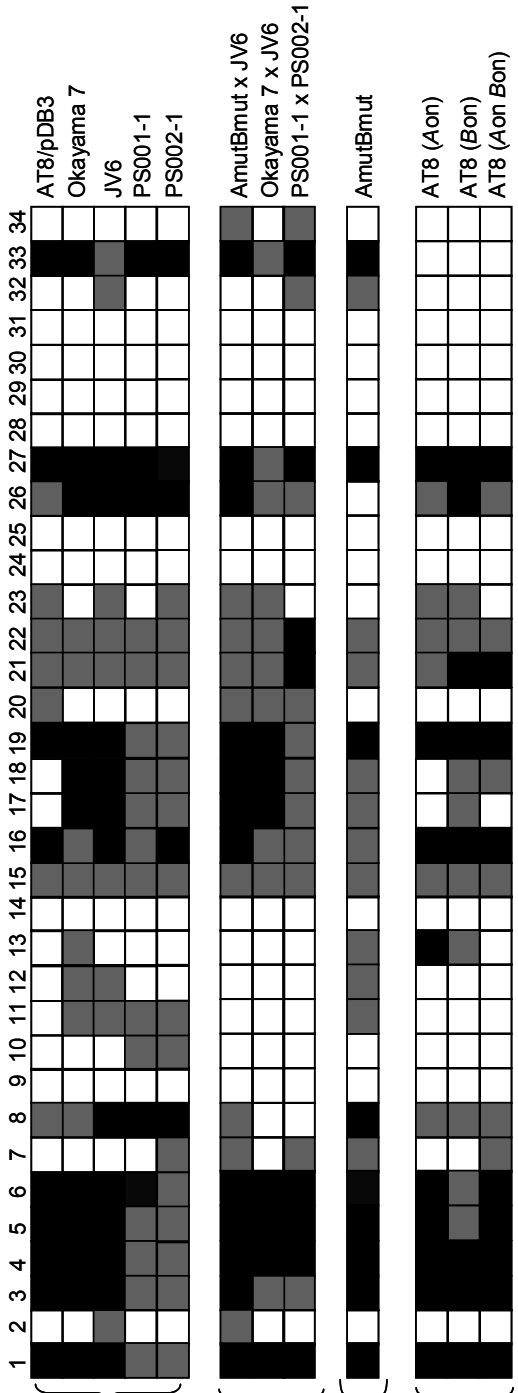


Figure 5. **Comparison of expression patterns of the hydrophobin genes in different strains of *C. cinerea*.** Expression pattern of the hydrophobin genes in the mycelium samples of different strains of *C. cinerea*. The numbers at the top of the figure indicate the different *coH* genes. cDNAs of genes that in PCR gave strong bands (Fig. 2, 3 and 4) are marked in black, cDNAs that gave weak bands in light grey and cases where no cDNA was amplified in white.

4.4.5 Genes expressed in the self-compatible homokaryon AmutBmut during fruiting body development

Different stages during fruiting body development of homokaryon AmutBmut were collected (Fig. 1 and Table 1). A total of 27 different hydrophobin genes formed transcripts during fruiting body development (Fig. 6, Fig. 7), 25 of which had transcripts also in monokaryons and/or in dikaryons (Fig. 2, Fig. 3). Transcripts for *coH*14, *coH*24 and *coH*25 were found with strong bands in RT-PCR starting with primary hyphal knot (*coH*25), secondary hyphal knot (*coH*2, *coH*24) and primordial formation (*coH*14), respectively. For these three genes, transcription was not detected in mycelial samples of homokaryon AmutBmut or any of the other strains, indicating that these genes are highly specific to fruiting body tissues. Transcripts were not found by RT-PCR for genes *coH*9, *coH*10, *coH*28, *coH*29, *coH*30, *coH*31, and *coH*32. Of these, *coH*10, and *coH*32 have transcripts in mycelial samples of monokaryons and/or dikaryons whereas for remaining genes transcripts were never found.

Differences were seen at levels of expression and place of expression. *coH*1 being strongly expressed in the various types of mycelia is also strongly expressed in stipe tissues till the end of rapid stipe elongation (stage 8, Fig. 6). In contrast, *coH*2 from the same cluster of genes was not found expressed in most mycelial samples (with exception from monokaryon JV6 and dikaryon AmutBmut x JV6, Fig. 3) but in a comparable pattern to *coH*1 in stipe tissues. However, both genes are poorly expressed in cap tissues. Other genes from the same cluster of genes (*coH*3-*coH*7; see chapter 3) with high level of expression in various mycelia are not or only faintly expressed during fruiting. Only gene *coH*5 is seen strongly expressed in the stipe at the stage of karyogamy (stage 6) and poorly in cap tissue during meiosis. *coH*6 that was strongly expressed in cap tissue during rapid stipe elongation, when *coH*1 and *coH*2 reduced their transcription. Compared to the plain AmutBmut mycelium, mycelium with primary and secondary hyphal knots expressed a few additional genes. In addition to *coH*24, *coH*2 was first found strongly expressed in the secondary hyphal knot (skn) stage (Figs. 6, 7). 5 different genes (*coH*11, *coH*12, *coH*13, *coH*25, and *coH*26) were found expressed in primary and secondary hyphal knot stages, but the transcripts for *coH*25 and *coH*26 was not found in the plain vegetative mycelium of homokaryon AmutBmut (Fig. 6). Interesting genes in fruiting are *coH*11 and *coH*12, also found clustered in the genome of *C. cinerea* (see chapter 3). In two monokaryons, few transcripts were seen for these two genes, and in fruiting, transcripts were found both in cap and stipe where the genes are obviously poorly transcribed (Figs. 2, 6, 7). *coH*13

expression is restricted to the primary hyphal knot stage apart from its faint expression in monokaryon, Okayama 7 (Figs. 2, 6).

For gene *coH*14 being a solitary gene in the *C. cinerea* genome (chapter 3), transcripts were only found in the primordia and fruiting body tissues, indicating that this gene is highly specific to the pathway of fruiting body development (Fig. 6). The gene cluster *coH*16, *coH*17 and *coH*18 in the *C. cinerea* genome (see chapter 3) showed different patterns in their stages of expression and in the level of expression. *coH*16, *coH*17, and *coH*18 in most instances share a common expression pattern in most of the mono- and dikaryotic mycelia and in the fruiting body tissues with mostly high expression levels, whereas *coH*17 is highly expressed in mono- and dikaryotic mycelia and within fruiting tissues, the gene is poorly transcribed at a few specific stages (Figs. 6, 7). Another cluster of genes with *coH*24 and *coH*25 (chapter 3) are of interest with respect to their differential expression. No transcripts were found for these genes in mono-, di- and homokaryotic vegetative mycelial samples, whereas in RT-PCR of fruiting tissues *coH*24 and *coH*25 gave stronger bands in almost every stage of fruiting body development (Figs. 6, 7). Therefore, along with *coH*14 and with restriction also *coH*24 and *coH*25 were found to be fruiting specific (from stage 1 to 10, Table 1, Figs. 6, 7). In the last interesting cluster of genes comprising in total 6 genes (*coH*29 – *coH*34, see chapter 3), expression for gene *coH*32 was found only in the plain vegetative mycelium of monokaryon JV6, homokaryon AmutBmut and in dikaryon mycelium of PS001-1 x PS002-1 (Figs. 3, 6, 7), whereas *coH*33 was found highly transcribed in almost all mono- di- and homokaryotic mycelial samples and in all stages of fruiting body development of AmutBmut. Transcripts for *coH*34 were only found in the cap and stipe tissues of fruiting bodies and in mycelial samples of two dikaryons (AmutBmut x JV6, and PS001-1 x PS002-1) and no expression was seen in monokaryon mycelial samples (Figs. 2, 3, 4, 5, 6, 7).

In conclusion, in total ten different genes (*coH14, coH15, coH16, coH18, coH19, coH21, coH24*, *coH25, coH33*, and *coH34*) were found highly expressed during most stages of fruiting body development of *C. cinerea* homokaryon AmutBmut (Fig. 6, Fig. 7). In comparison with mono- and dikaryon mycelial samples, four genes (*coH14, coH24, coH25* and *coH34*) are more or solely active during the fruiting body development (Fig. 6, 7). Only for two gene, *coH10* and *coH32*, transcripts were detected by RT-PCR in samples of mono- and dikaryotic mycelium, (JV6, AmutBmut and PS001-1xPS002-1) (Fig. 5) but none were found during fruiting.

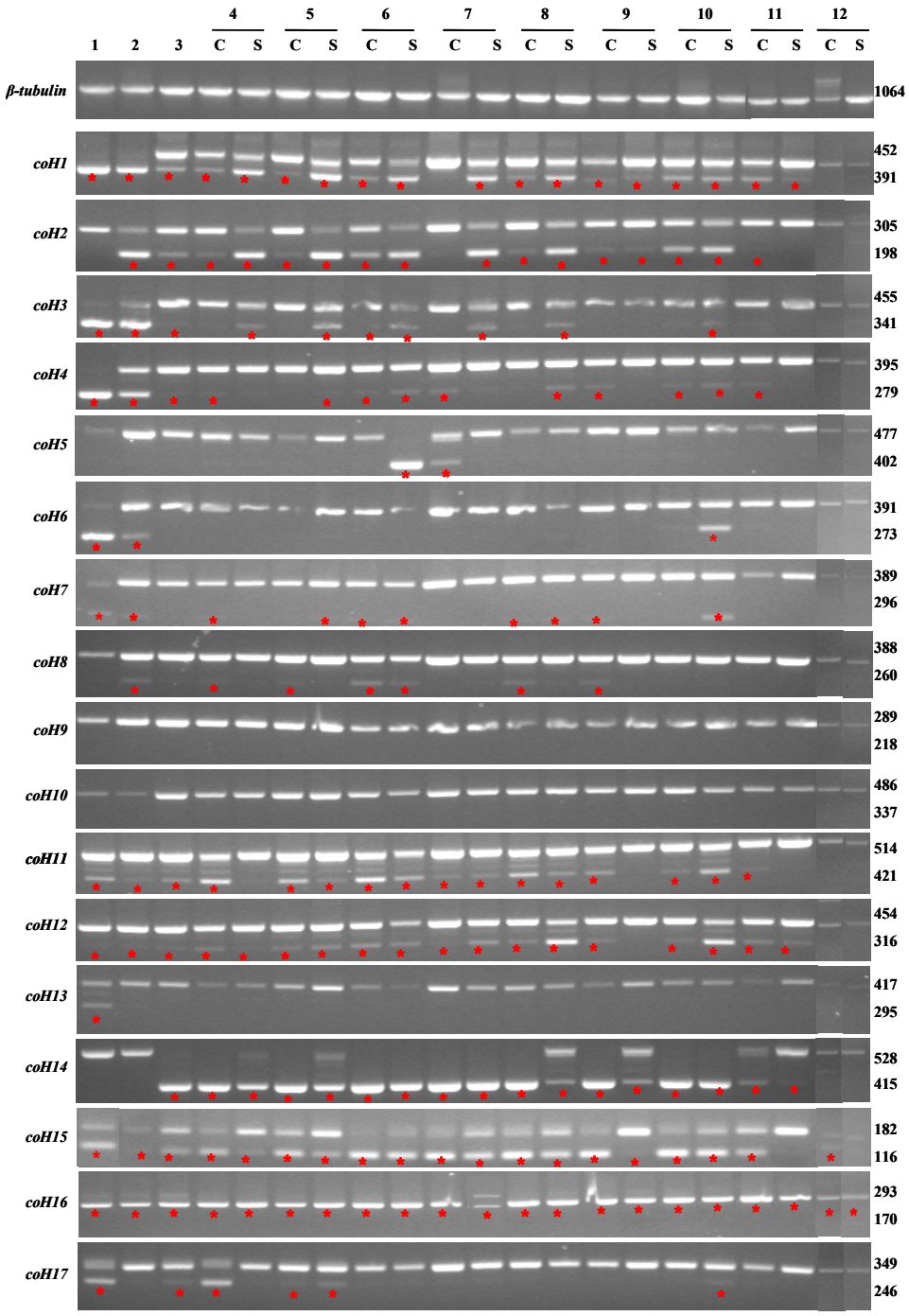
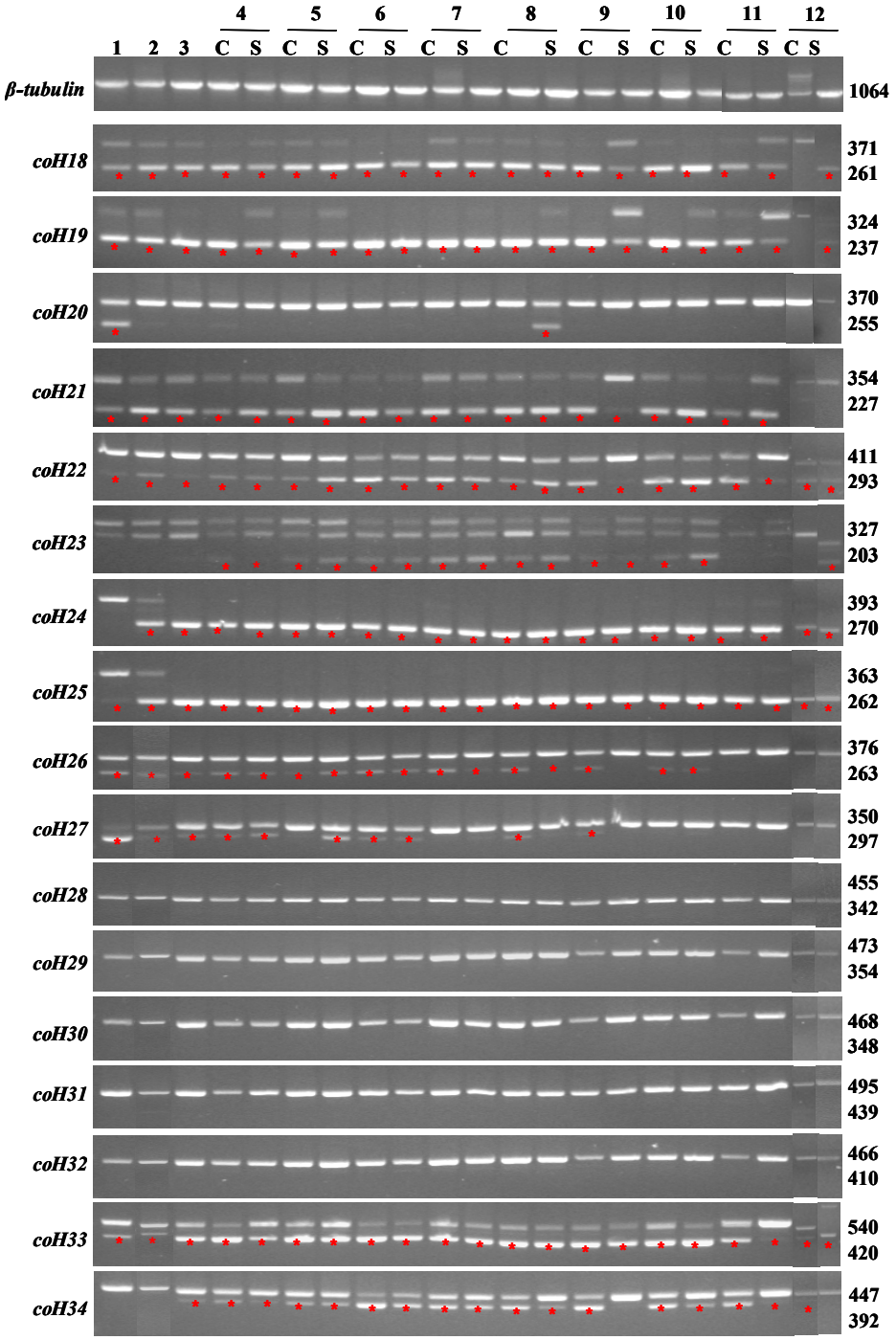


Figure 6. **Expression profiles of hydrophobin genes in different stages of fruiting body development in homokaryon AmutBmut**. The top lane shows different developmental stages from mycelium to mature fruiting bodies. Specific tissues labeled with "initials" as C for cap and S for stipe (see Table 1 and Fig. 1). The left column shows the hydrophobin genes (for corresponding primers, see Table 2) and the right column shows the expected genomic and cDNA sizes (Table 2). The asterisks (*) indicate expressed cDNA and the unmarked bands correspond to genomic DNA fragments.

Figure 6 (continued)



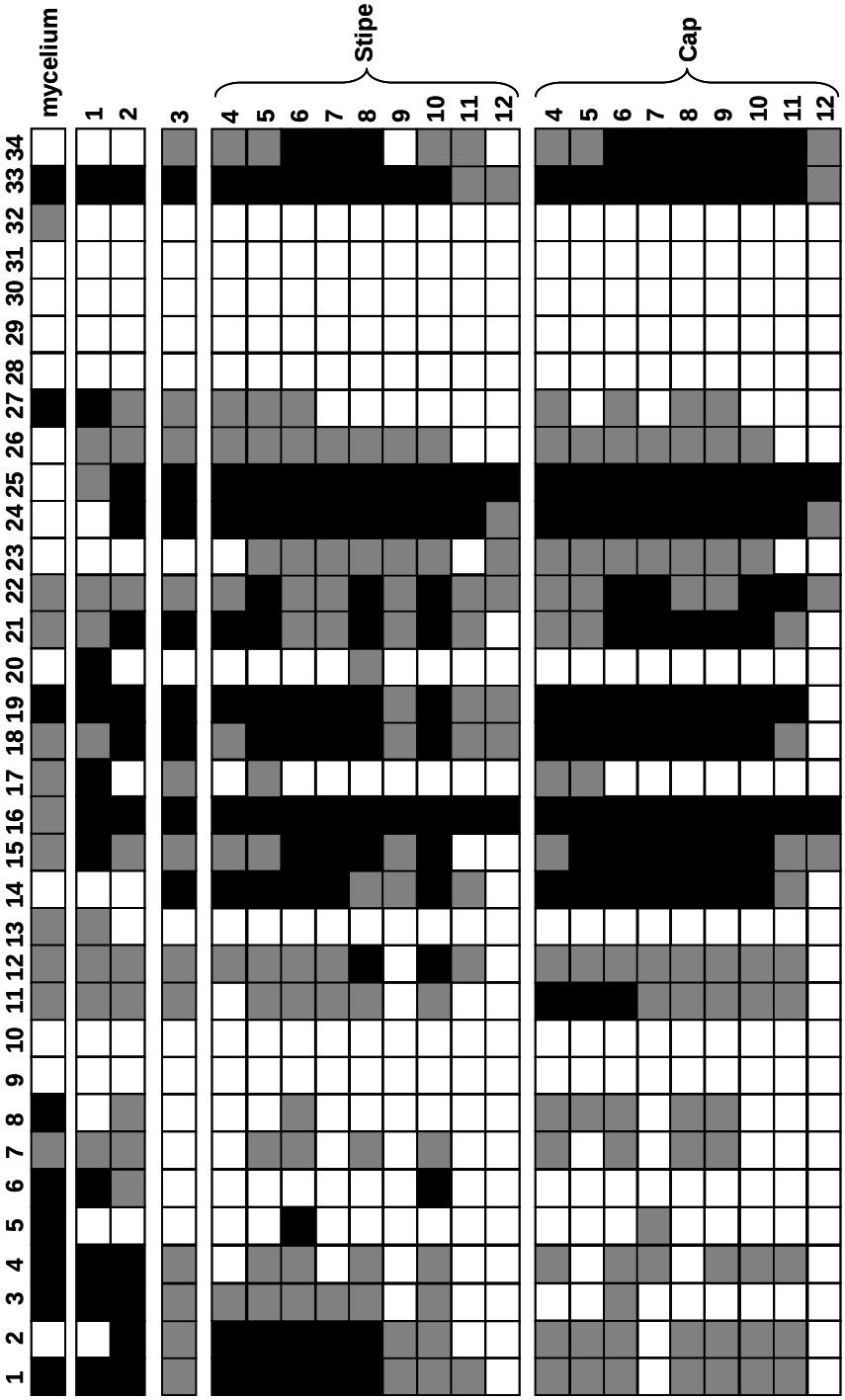


Figure 7. **Comparative analysis of the expressed hydrophobin genes in different tissues of fruiting body development of homokaryon AmutBmut**. The top lane indicates the *coH* genes, the column at the right refers to different stages of AmutBmut development (compare with Table 1 and Fig. 1). cDNAs of genes that in PCR gave strong bands (Fig. 8) are marked in black, cDNAs that gave weak bands (Fig. 6) in light grey and cases where no cDNA was amplified in white.

Finally, it can be noted that in the stage of senescence, most of the genes were not anymore active. Exceptions with low level of expression were *coH*18, *coH*19, *coH*22, *coH*24, *coH*25 and *coH*33 in stipe tissues and *coH*15, *coH*22, *coH*24, *coH25*, *coH*33 and *coH*34 in cap tissues (see Fig. 6, Fig. 7).

4.5 Discussion

From the genome of *C. cinerea*, in total 34 different hydrophobin genes were deduced by repeated Blast searching using all the known hydrophobin sequences from the NCBI GenBank database (see chapter 3). Here, transcript analysis was performed for all the deduced hydrophobin genes by primers specific to individual hydrophobin genes (Table 2). Different mycelial stages (monokaryon, dikaryon and a self-compatible homokaryon) and different stages from fruiting body development from homokaryon AmutBmut were analysed for transcripts for all hydrophobin genes.

Overall analysis of transcribed genes showed no dramatic difference with respect to their stage of expression (Table 3, Fig. 6). 25 different hydrophobin genes were found expressed in the five different sterile monokaryons (Fig. 2) and transcripts for 22 different hydrophobin genes were found in three different fertile dikaryon mycelial samples (Fig. 3). Within primordia and fruiting body tissues, transcripts for 26 different hydrophobin genes were detected (Fig. 6). In total, transcripts for 29 different hydrophobin genes were found in different strains and stages of *Coprinopsis* development (Fig. 7, 8). Between all the different stages, 18 transcribed genes are shared (Fig. 7). Genes *coH*10 and *coH*13 are specific to monokaryons (Fig. 5, 7), whereas genes *coH*14, *coH*24, and *coH*25 were found to be specific for fruiting body tissues (Fig. 6, 7). Whilst the comparison in Fig. 8a considered all expressed genes, in Fig. 8b the patterns for the highly expressed genes are shown. In total, 25genes (++ or +++ in Table 3) are left for such analysis of highly expressed hydrophobin genes. Ten genes were specifically highly expressed in fruiting body tissues, seven of these in cap and stipe (*coH*14, *coH*15, *coH*22, *coH*23, *coH*24, *coH*25, and *coH*34), one just in the cap (*coH*11) and two just in the stipe (*coH*2 and *coH*12), respectively. Four genes are highly expressed in both mono- and dikaryons (*coH*3, *coH*4, *coH*5 and *coH*27), one other gene only in the dikaryon (*coH*17) and 4 other genes only in monokaryons (*coH*6, *coH*8, *coH*13 and *coH*26). One gene (*coH*1) is highly expressed in the two types of mycelium and also in fruiting body stipes, two other genes (*coH*16 and *coH*21) in the monokaryon and in caps and stipes of the fruiting body and one

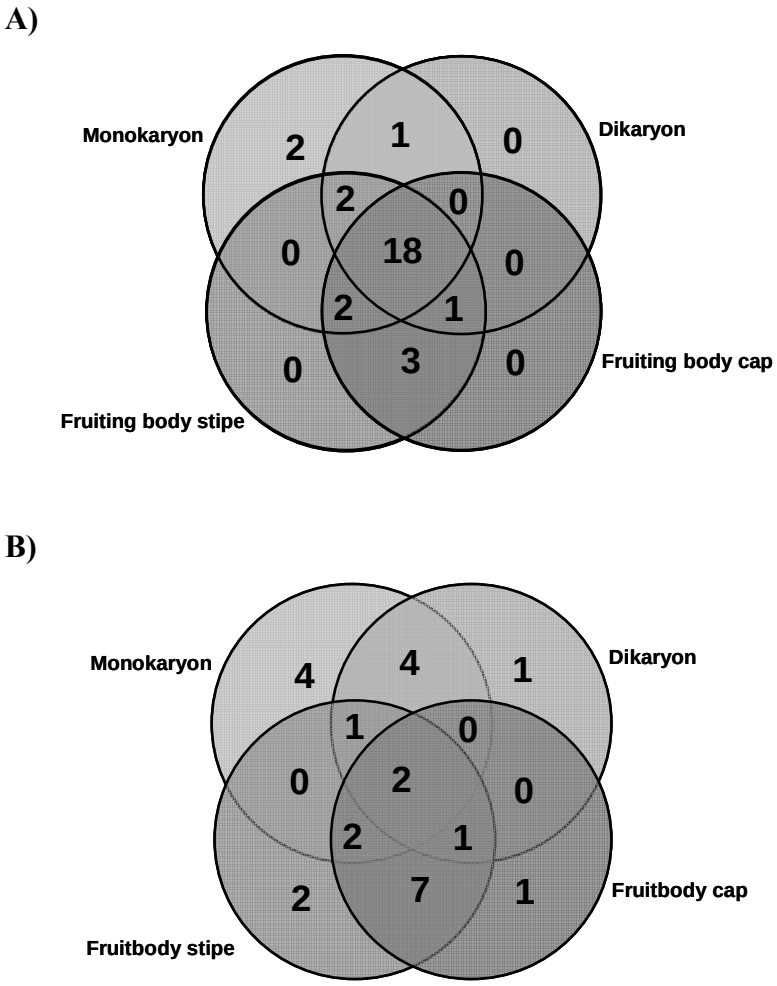


Figure 8. Comparison of hydrophobin expression patterns in *C. cinerea*. Genes showing any expression in different types of mycelium and/or in different types of tissues during fruiting body development (**A**) and the genes being highly expressed in different mycelial types and/or in stages of fruiting body development in cap tissues and/or stipe tissues (B).

gene (*coH*18) in the dikaryon and in caps and stipes of the fruiting body (Fig. 8b). Two genes (*coH*19 and *coH*33) were found expressed in all stages and samples.

The genes specifically highly expressed in mycelium as well as the genes specifically highly expressed in fruiting body stages come from different gene clusters (Fig. 2 in chapter 3) and their products are widely distributed in the phylogenetic tree of hydrophobins (Fig. 7 in chapter 3). This distribution of *C. cinerea* hydrophobins is not in accordance to the postulation of Wessels (1997) that hydrophobins evolve depending on functions in aerial mycelium and fruiting. All the deduced hydrophobins from *C. cinerea* belong to class I, according to their biophysical and hydropathy patterns (see chapter 5). The genome analysis revealed some interesting feature relating to their similarity and gene duplication events (see chapter 3). Genes that are clustered together showed high level of identity in sequence of their proteins (47-90% protein identity, 75-90% protein similarity

deduced from the sequences shown in chapter 3), suggesting that these genes were generated by simple recent duplications. In contrast, protein products coming from different gene clusters are more distantly related (25-40% protein identity, 40-70% protein similarity). At the gene level, the overall sequences from all the 34 deduced hydrophobin genes showed 84%-21% identity, whereas coding sequences in between different hydrophobin genes have an identity of 89%-26%. Protein sequences from all the 34 deduced hydrophobins showed an identity within the amino acid sequences between 15%-89% and the similarity of amino acid sequences ranges from 26%-99%. The current analysis of gene expression contradicts therefore also a possible hypothesis that the clustering reveals their developmental stage of expression as the proteins from different expression stages are spread all over the tree (Fig. 7 in chapter 3) and as within clusters of closer related genes, expression of individual genes do not follow all the same pattern (Figs. 2-4; for further reading, see chapter 5).

Previous studies on *C. cinerea* revealed two hydrophobin genes *coH*1 and *coH*2 localised within the near proximity in the genome, one of which has been shown to be well expressed in monokaryotic mycelium (Ásgeirsdóttir et al. 1997). In *S. commune*, three hydrophobin genes were isolated, whose expression was shown to be stage-specific during the fruiting body development. SC3, a well studied hydrophobin in *S. commune* was found to be expressed in the vegetative mycelium of mono- and dikaryons, whereas SC4 was expressed only in fruiting bodies (Wessels et al. 1995, van Wetter et al. 2000, Lugones et al. 1999). In *A. bisporus,* ABH1 is localized in the inner fruiting tissues at edges of the extracellular matrix and lines air channels (de Groot et al. 1996, 1999, Lugones et al. 1996, 1998). Depending on their presence in different tissues, different functions were postulated for hydrophobins, for example the presence of these proteins in air channels might prevent the inflow of water from outside and thereby they protect the fruiting body against water logging and allow gas exchange (Lugones et al. 1996). In *C. cinerea,* transcripts for 29 different hydrophobin genes were observed but for the majority of genes there was little specificity with regards to strain and stage of expression (Fig. 8). This might create much uncertainty regarding the specificity and the functional features of these multi-gene products, since from sequence analysis it is difficult to postulate any particular function to a specific gene.

Table 3. Comparison of expressed hydrophobin genes in different strains and stages in *C. cinerea* fruiting body development*.

Hydrophobin genes	Monokaryon	Dikaryon	Fruiting body stipe	Fruiting body cap
coH1	+++	++	++	+
coH2	+	+	++	+
coH3	+++	++	+	+
coH4	+++	+++	+	+
coH5	+++	+++	+	+
coH6	+++	+	+	-
coH7	+	+	+	+
coH8	+++	+	+	+
coH9	-	-	-	-
coH10	+	-	-	-
coH11	+	-	+	++
coH12	+	-	++	+
coH13	++	-	-	-
coH14	-	-	+++	+++
coH15	+	+	+++	+++
coH16	+++	+	+++	+++
coH17	+	++	+	+
coH18	+	++	+++	+++
coH19	+++	++	+++	+++
coH20	+	+	+	-
coH21	++	+	+++	+++
coH22	+	+	++	++
coH23	+	+	++	++
coH24	-	-	+++	+++
coH25	-	-	+++	+++
coH26	++	+	+	+
coH27	+++	++	+	+
coH28	-	-	-	-
coH29	-	-	-	-
coH30	-	-	-	-
coH31	-	-	-	-
coH32	+	+	-	-
coH33	+++	++	+++	+++
coH34	-	+	++	++

* - : no expression, +. ++. +++: low, medium and high expression.

In *S. commune*, *SC*1 and *SC*4 are regulated by mating type pathway in a very different way to that of *SC*3. The *A* and *B* mating type proteins together have positive effects on *SC*1 and *SC*4 expression, while the products of the *B* mating type genes act negatively on SC3 production (Wessels 1991, 1995, Schuurs et al. 1998, Ásgeirsdóttir et al. 1995). In *C. cinerea*, compatible *A*, respectively compatible *B* mating type proteins were claimed both to down-regulate expression of gene *coH*1 as observed in the monokaryon and possibly also that of gene *coH*2 (Ásgeirsdóttir et al. 1997). This postulate was deduced from Northern blot analysis of an untransformed monokaryon, an *A* transformed monokaryon of another genetic background and a *B* transformant of a third background. The analysis presented here shows that different monokaryons are not totally identical by gene expression (Fig. 4). Therefore, definite conclusions on mating gene regulation on expression of hydrophobins in *C. cinerea* can only be made when transformants of the same genetic background are compared. In this study, transformants of monokaryon AT8 were compared but influence of mating type gene regulation was not found for any hydrophobin gene.

This study gave a comprehensive knowledge regarding the transcript levels of multiple hydrophobin genes. To understand the differences in functional properties of each hydrophobin, more detailed studies have to be carried out in relation to protein expression and structural determination. Application of modern molecular biology tools like gene silencing and wide range gene knockout studies may reveal the physiological importance of these genes in growth and development of *C. cinerea* in the future. However, with the high number of different genes, one should not overlook that this is a highly demanding task, especially since knockouts in *C. cinerea* are difficult to achieve (Kües et al. 2004). With the recent development of RNAi in *C. cinerea* (Namekawa et al. 2005), this task might become easier.

4.6 Acknowledgements.

Patrik Hoegger kindly helped to complete the PCR analysis of expressed cDNAs of hydrophobin genes and to perform the final data analysis of expressed genes. We would like to thank Prayook Srivilai for the *C. cinerea* co-isogenic strains, and Monica Navarro-Gonzalez for sharing the knowledge and some pictures of fruiting body developmental stages in *C. cinerea*. The Deutsche Bundesstiftung Umwelt (DBU) financially supports our work.

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CHAPTER 5

General discussion: How many hydrophobins does a mushroom need?

5.1 Abstract

Hydrophobins are small fungal specific proteins, which play a significant role in fungal development and possibly in fungal ecology. With the rapid release of genomic sequences from different fungal species, comparative studies between sets of hydrophobin genes were made possible. In the following chapter, an analysis is made for the white-rot fungus *Phanerochaete chrysosporium*, the maize pathogen *Ustilago maydis,* the ectomycorrhizal fungus *Laccaria bicolor* and the human pathogen *Cryptococcus neoformans*, and the results are compared with those described earlier for *C. cinerea*. In total, we found 34 different hydrophobin genes in *Coprinopsis cinerea,* 20 in *P. chrysosporium*, and 14 in *L. bicolor*, two genes in *U. maydis*, and no gene in *C. neoformans*. By phylogenetic analysis, there is no strong evidence for clustering of proteins according to their stage of expression or according to the biotopes fungi occupy. The surprising high number of hydrophobin genes in some of the fungi has given a challenging question why so many different hydrophobin genes evolved and how many hydrophobins does a mushroom need for its survival as an individual and as a species. Furthermore, the question whether these different proteins distinguish in their properties and perform different functions within the life cycle of the fungi need to be thoroughly investigated. These questions are addressed in this chapter by phylogenetic analysis of proteins and expression data available from the work for this thesis and from literature. At the end as an extra point, the potential industrial applications of these interesting proteins are discussed that form amphiphatic films with hydrophobic and hydrophilic surface.

Keywords: Multi-gene family-Hydrophobins-Fruiting body development

5.2 Introduction

Hydrophobins are small secreted proteins of about 100 amino acids in length found expressed in fungi during hyphal growth and development (Wösten 2001, Walser et al. 2003). These proteins are secreted at hyphal tips as monomers and self-assemble to form amphipathic films at hydrophilic-hydrophobic interfaces (Wessels 1997, Wösten 2001). The typical primary structure of hydrophobins contains eight cysteine residues at well conserved positions (Fig. 1A). Apart from these conserved cysteine residues, the proteins have a very low sequence homology (Wessels 1994, 1997). The eight cysteine residues within the hydrophobins form four intramolecular disulfide bridges (Fig. 1B, de Vries et al. 1993, Hakanpää et al. 2004) preventing protein aggregation within solutions before they come in contact with air-water interfaces (Wösten et al. 1994). The cysteines are also needed in protein translocation out of the fungal cells. Cysteine mutants of hydrophobins showed a lethal effect on the host organism by retaining the protein in the endoplasmic reticulum (Kershaw et al. 2005).

Hydrophobins are classified into two groups, class I and class II, based on their biochemical properties and hydropathy patterns (Wessels 1997). Class I proteins are more stable and difficult to dissociate and it requires harsh treatment with solvents like trifluroacetic acid (TFA) or formic acid (FA) to bring the proteins into a soluble state (Wessels et al. 1991, de Vries et al. 1993). In contrast, class II proteins are easily dissolved in 96% ethanol or water (Wessels et al. 1991, Wösten and Willey 2000). Structural analysis of a class II hydrophobin (HFBII) from *Trichoderma reesei* revealed that, depending on number of hydrophobic amino acids present in between the 3rd and 4th cysteines and in between the 7th and 8th cysteines, the solubility of hydrophobins differs, and that this distinguishes between the two hydrophobin classes (Hakanpää et al. 2004, Linder et al. 2005). Class I hydrophobins are found in both asco- and basidiomycetes, class II are reported till now only in ascomycetes (Wösten 2001, Linder et al. 2005). Class I hydrophobins form films of characteristic mosaic bundles of 5-12 nm wide parallel rodlets, resembling amyloid fibrils (Wessels 1999, Wösten et al. 1993). In contrast, the films formed by class II proteins resemble crystalline fibrils (Hakanpää et al. 2004). Hydrophobin films help the hyphae to emerge from the moist substrates into the air to form aerial structures under adverse environmental conditions (Wösten 2001).

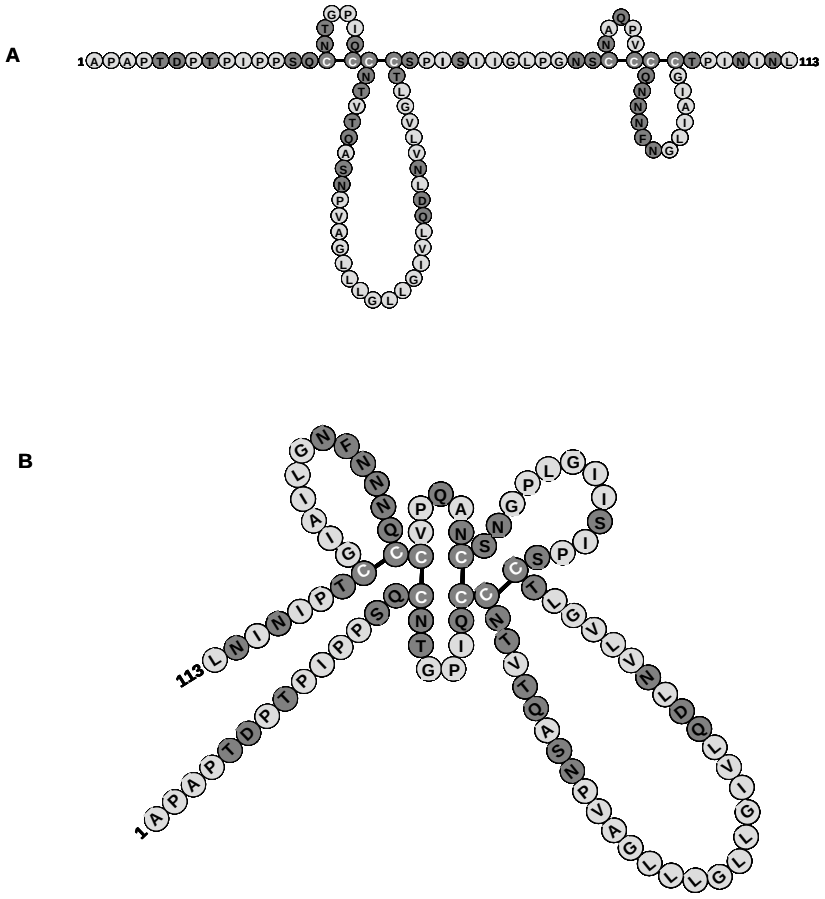

Figure 1. Models for folding of processed *Coprinopsis cinerea* CoH1. A Folding according to the model published first by Wessels for the *Schizophyllum commune* SC3 hydrophobin (Wessels 1997) and **B** according to biochemical data from HFBII a class II hydrophobin from *Trichoderma reesei* (Hakanpää et al. 2004). Disulphide bridges are indicated by bars. Cysteine are showed as circles with C, hydrophobic amino acids in dark shade and hydrophilic amino acids in light shade with a one letter code for corresponding amino acids. To define the specific positions of cysteines within the hydrophobin sequences (C1 to C8), count the number of cysteines starting from the N-terminal end.

Hydrophobins are the most surface-active proteins known so far (Vegt et al. 1996). They lower the surface tension of water from 72 mJm^{-2} into a range from 45 mJm^{-2} to 24 mJm^{-2}, thereby enabling fungal hyphae to emerge from the moist substrate into the air. Furtheron, by coating the hyphal surface, they protect the aerial hyphae from dehydration. Hydrophobins are further involved in fungal attachment to hydrophobic surfaces. In this way, they contribute for example to the interactions between fungi and plants, or to interaction between fungal cells in fruiting body tissues (Whiteford and Spanu 2002, see below). In plant-pathogen interaction, hydrophobins function in the attachment of the appressoria from the germinating spores to leaf surfaces (Talbot

et al. 1996). Coating of air channels in the fruiting bodies allows gas exchange within its tissues (Lugones et al. 1996). Hydrophobin coats on fungal spores make them hydrophobic and this eases their distribution by wind (Stringer and Timberlake 1995). Of the plant pathogen *Ophiostoma ulmi*, hydrophobin cerato ulmin (CU) showed toxic effects on the host trees attached by the fungus (Temple and Horgen 2000).

Multiple hydrophobin coding genes were identified in a number of basidiomycetes. For example, six different genes are known in *Pleurotus ostreatus* (Ásgeirsdóttir et al. 1998, Penas et al. 1998), four in *Agaricus bisporus* (de Groot et al. 1996, 1999, Lugones et al. 1996, 1998, 1999) and in *Schizophyllum commune* (Wessels et al. 1995), and each three in *Pisolithus tinctorius* (Tagu et al. 1996, 2001, Duplessis et al. 2001) and in the basidiomycete lichen *Dictyonema glabratum* (Trembley et al. 2002a, b). In ascomycetes, both class I and II hydrophobins were found expressed by a same organism. In the potato pathogen *Cladosporium fulvum*, at least four class I and two class II hydrophobins were shown to be expressed (Segers et al. 1999, Nielsen et al. 2001, Whiteford et al. 2004). In the corn pathogen *Fusarium verticillioides*, three class I and two class II hydrophobin coding genes were found (Fuchs et al. 2004). It is largely unknown how many hydrophobin genes are present in a single fungal species. The sequence diversity of hydrophobins made the isolation of hydrophobin genes impossible on the basis of homology (Wessels 1994, 1997). By applying biochemistry and molecular biology techniques, most abundantly expressed hydrophobins and respective genes were identified. With the availability of the complete genomic sequence from different fungal species, mycologists may now be interested in knowing the complete number of hydrophobin genes in a fungal genome.

In this thesis, one focus is given to the analysis of different basidiomycete genomes. The genomes of the saprophytic dung fungus *Coprinopsis cinerea* (http://www. broad. mit.edu/annotation/fungi/coprinus_cinereus/), of the white-rot fungus *Phanerochaete chrysosporium* (http://genome.jgi-psf.org/whiterot1/ whiterot1.home.html), of the maize pathogen *Ustilago maydis* (http://www.broad. mit.edu /annotation/ fungi/ustilago_ maydis/) and of the human pathogen *Cryptococcus neoformans* (http://www.broad. mit.edu/annotation/fungi/ cryptococcus_neoformans/) were publicly available for analysis. In addition, our laboratory was given access to the genome sequence of the ectomycorrhizal fungus *Laccaria bicolor* prior to publication by the *Laccaria* genome consortium at a password protected site (http://mycor.nancy.inra.fr/IMGC/LaccariaGenome/

Annotation/index.html, coordinated by Francis Martin, Nancy). All these genomes were Blast screened for hydrophobin genes by the same approach as described in chapter 3 for analysis of the *C. cinerea* genome.

5.3 Multiple hydrophobin genes in mushrooms

As mentioned above, hydrophobins are present in multiple numbers in many of the fungal species. In the genome of the model basidiomycete dung fungus *C. cinerea,* we found a total of 34 different hydrophobin genes, which is the highest number of hydrophobin genes known so far in a single species. Many of these genes are complied in the genome in clusters with one cluster of 7 genes (including the formerly known genes *coH*1 and *coH*2, Ásgeirsdóttir et al. 1997), one cluster of 6 genes, 3 clusters of 3 genes and 3 clusters of 2 genes. Moreover, seven genes were found in the genome at individual positions (see chapter 3). Those genes clustering closely together are in most cases also more closely related in protein sequence (see Fig. 3 in chapter 3), suggesting these genes were generated from each other by duplication events. The *C. cinerea* hydrophobin genes contain between 1-5 introns, two of which are found in most genes. Other intron positions are shared between few of the genes, usually between genes that cluster together in the genome or whose proteins are more similar to each other (Fig. 2).

In the published genome of the white-rot fungus *P. chrysosporium* (Martinez et al. 2004), hydrophobin genes are distributed on 10 different scaffolds with one cluster of 5 genes, two clusters of each 3 genes, 2 clusters of each 2 genes and five genes on individual scaffolds (Fig. 3). The genes have one or two introns at 4 different positions (Fig. 2), one of which shared with the nearly all hydrophobin genes of *C. cinerea* and one that is shared with gene *coH*14 of *C. cinerea*. However, 7 of the 20 potential hydrophobins deduced from the detected genes in *P. chrysosporium* have only 6 of the conserved cysteines. PcH1, PcH2, PcH3, PcH4, PcH8, PcH9 and PcH20 are lacking the cysteine at position 1 and the cysteine at position 6 (Fig. 4). With these two cysteines lacking, the proteins will not be able to adopt the normal folding involving a disulphide bridge between positions 1 and 6 (Hakanpää et al. 2004, Fig. 1B). Expression of these proteins within the fungus might therefore be lethal (Kershew at el. 2005). Thus, it is possible that these 7 genes (*PcH*1, *PcH*2, *PcH*3,

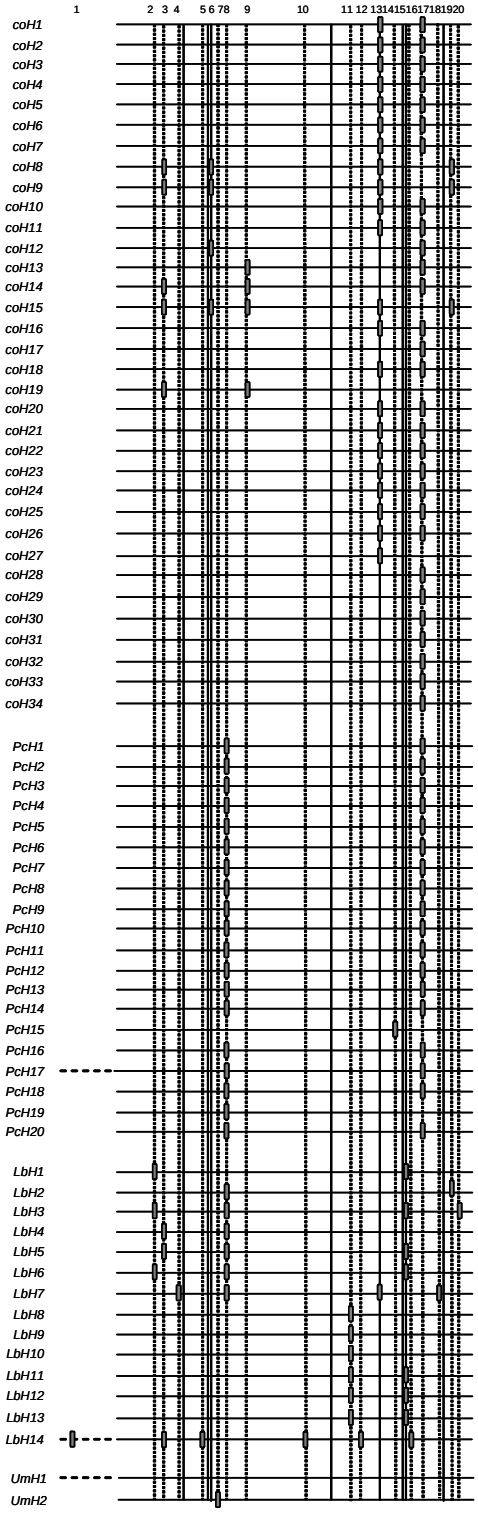

Figure 2. **Intron positions within deduced basidiomycete hydrophobin genes**. Intron positions within C*oprinopsis cinerea* (*coH*1-*coH*34), *Phanerochaete chrysoporium* (*PcH*1-*PcH*20), *Laccaria bicolor* (*LbH*1-*LbH*13) and *Ustilago maydis* (*UmH*1 and *UmH*2) hydrophobin genes. *Horizontal lines* indicate the hydrophobin genes whereby dashed parts symbolize prolonged 5´-ends of genes. Vertical closed lines mark positions of cysteine codons in which in some cases introns are inserted (indicated by grey rectangles). Dotted lines with grey rectangles mark other intron positions and ease recognition of those positions where introns interrupt the coding sequence of the different genes at exactly the same codon position.

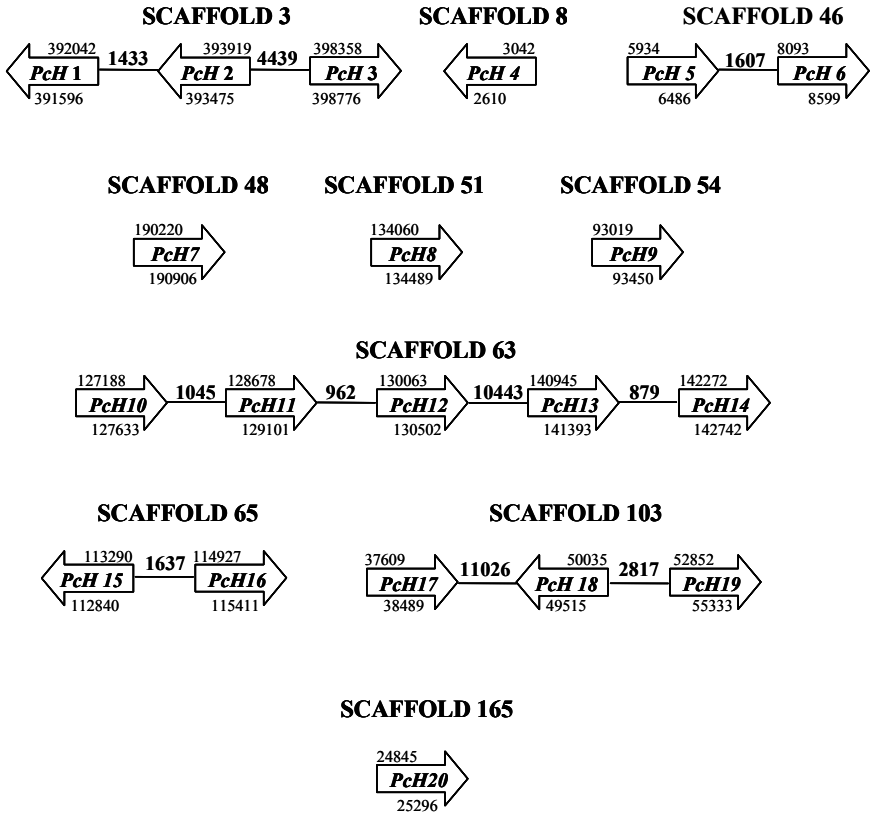

Figure 3. **Localization of hydrophobin genes in the genome of *Phanerochaete chrysosporium***.The distance between the neighboring genes were given in between the genes and the start and stop positions of the genes on the top and below of the broad arrows representing the genes, respectively.

*PcH*4, *PcH*8, *PcH*9 and *PcH*20) are non-functional. Of the 12 potentially functional hydrophobins of *P. chrysosporium* that possess all 8 cysteines,two others are special, PcH7 and PcH17, because of a short peptide (consensus GILPT/S) repeated 6 times full and two times partially at the N-terminal of PcH7 and 19 times perfect and two times imperfect at the N-terminal of PcH17 (Fig. 4). To our knowledge, such repeats have not yet been observed in any other hydrophobin. Related repeats occur in hypothetical proteins of other fungi e.g., in proteins deduced from the genome of *Neurospora crassa* (whose functions are still to be established, Galagan et al. 2003). In plants, within Brassicaceae species, a related motif is found in pollen coat oleosin helping in attachment of the pollen to the stigma of the plant and in subsequent swelling of the pollen (Fiebig et al. 2004). The protein suggests that the motif has an extracellular function probably in attachment to surfaces. Like all other deduced hydrophobins, the two hydrophobins with the repeated motif in *P. chyrsosporium*

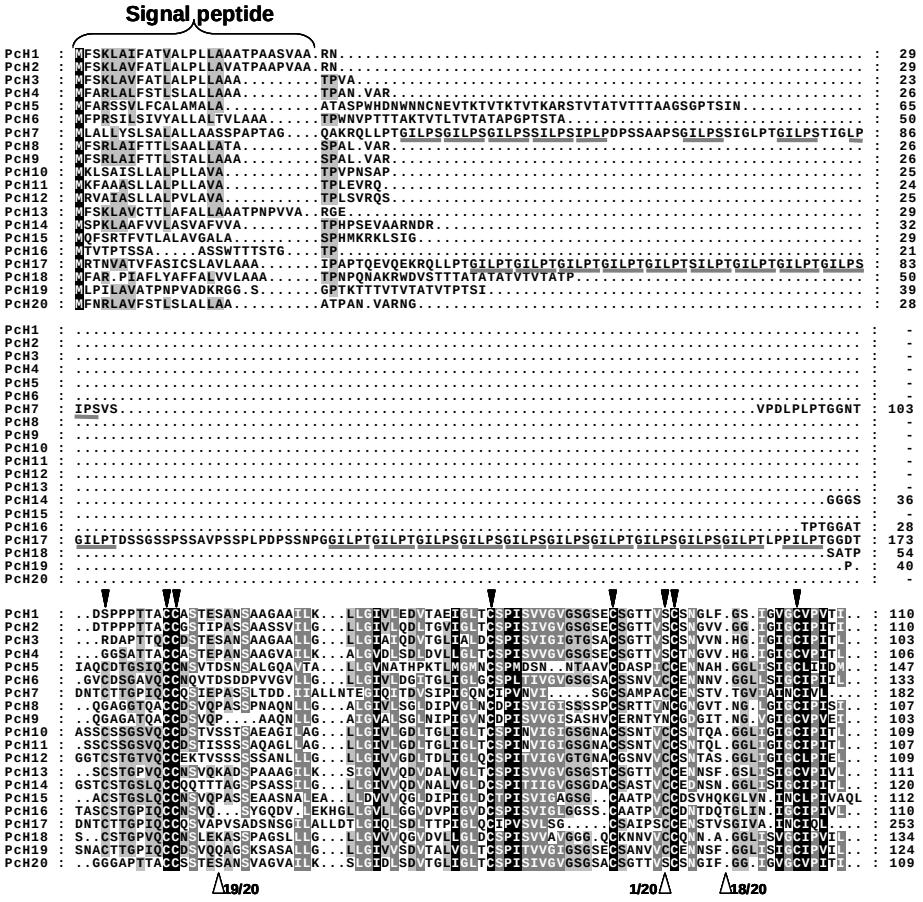

Figure 4. **Alignment of the deduced hydrophobin sequences from the *Phanerochaete chrysosporium* genome**. The alignment was done by using Clustal X (http//:ftp.embl-heidelberg.de) and the manual adjustments of the alignment were done using Gene doc software (http//:psc.edu/biomed/genedoc). Filled triangles indicate the eight cysteine residues at conserved positions, and the empty triangles showing the intron positions and the number of genes that have the conserved intron positions. The short signal peptides for hydrophobins were deduced showing that they are functional proteins (as indicated in figure). The shaded line below the sequence marks the repeated motifs (GILPT/S).

have an N-terminal secretary signal (with probability of 0.730 as determined with the program http://www.cbs.dtu.dk/services/SignalP/), suggesting that all proteins are secreted out of the cell. In the recently completed and yet not publicly available genome from the mycorrhizal fungus *Laccaria bicolor* (http://mycor.nancy.inra.fr/IMGC/Laccaria Genome/Annotation /index.html), we found 14 potential hydrophobin genes. Most of the genes are found in clusters, with one cluster of five genes, one cluster of 3 genes, and one cluster of two genes. The other four genes are spread individually on different contigs (Fig. 5). Introns within these genes are found at 5 different positions, all of which are shared with *C. cinerea* hydrophobin genes (Fig. 2). One of these 14 hydrophobin sequences (*LbH*14) is incomplete at the 3' end due to a short piece of DNA that is missing yet from the

genomic sequencing (Fig. 6). The sequences of genes *LbH*7 and *LbH*14 are complete but within the sequence of gene *LbH7* there is a codon for the conserved cysteine at position 1 missing and the codons for the conserved cysteines at positions 6 and 7, and *LbH14* appears to have no codons for the conserved cysteine at position 5 (Fig. 6). All other deduced hydrophobins of *L. bicolor* have the 8 conserved cysteines and an N-terminal signal peptide (probability 0.935) as standard class I hydrophobins. Interesting is further that with LbH14 there is also in the group of *L. bicolor* hydrophobins one protein with a much longer N-terminal sequence (Fig. 6). This N-terminal sequence is characterized by a 14 times more or less conserved repeated sequence motif (consensus sequence: PI/L/VTTTI/L/V/T).

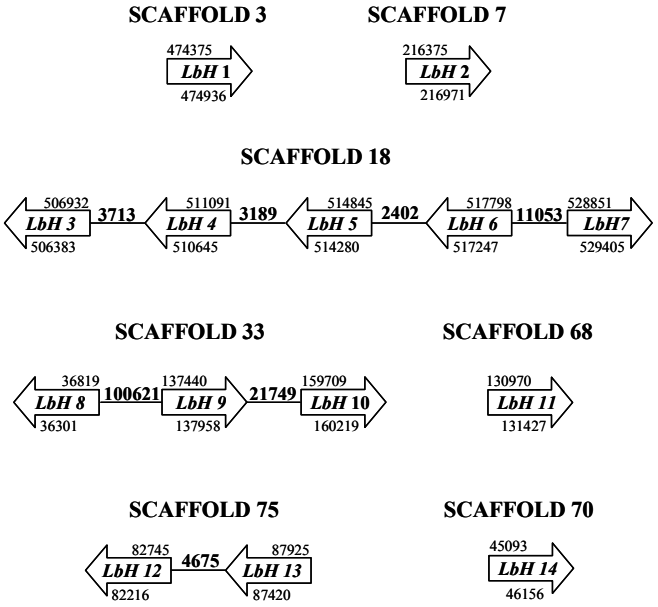

Figure 5. **Localization of hydrophobin genes in the genome of *Laccaria bicolor***. The distance between the neighboring genes were given in between the genes and the start and stop positions of the genes on the top and below of the broad arrows representing the genes, respectively.

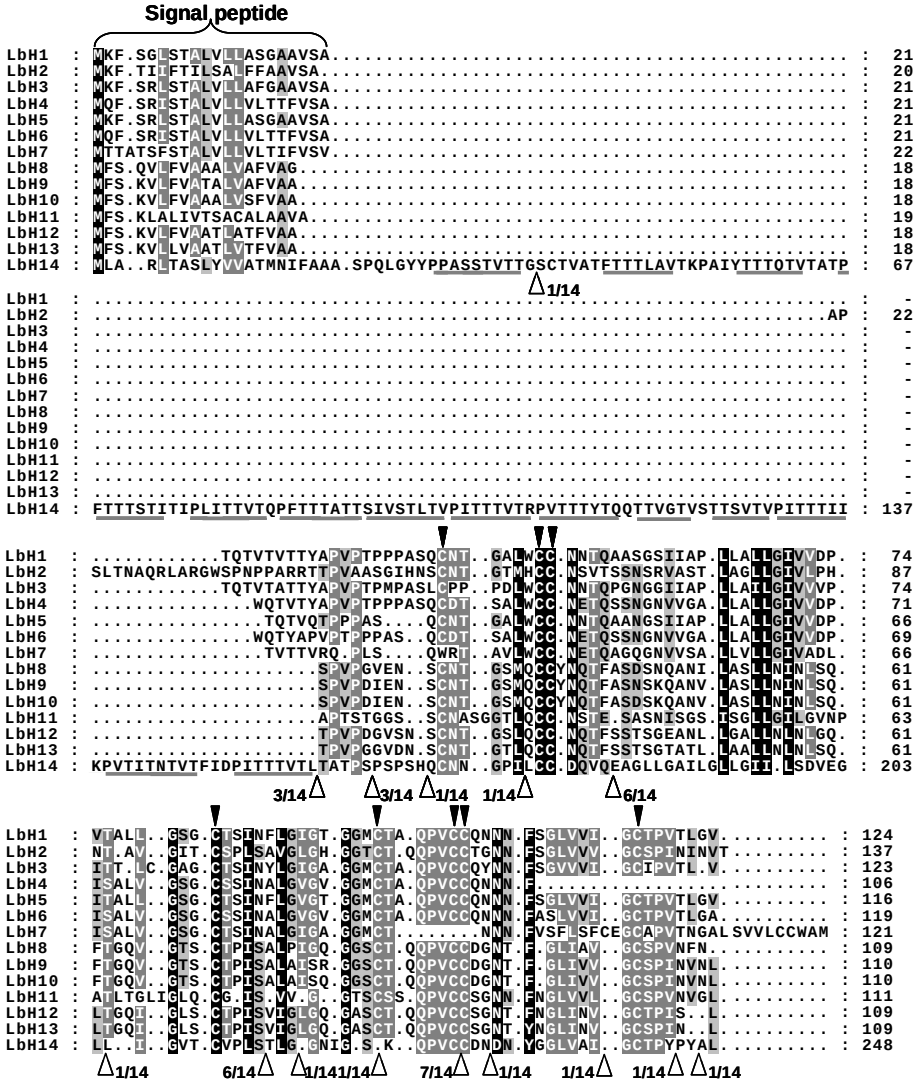

Figure 6. **Alignment of the deduced hydrophobin sequences from the *Laccaria bicolor* genome**. The eight conserved cysteines were shown in black color and are marked in positions with blank triangles above the sequences. The short signal peptides for hydrophobins were deduced showing that they are functional proteins (as indicated in figure). The open triangles below the sequences mark the intron positions in the corresponding gene sequences and number of genes that have the conserved intron positions. Note that LbH4 is an incomplete protein due to the missing DNA sequence in the genome of *L. bicolor*.

In a Blast search for hydrophobin genes in the *Ustilago maydis* genome (http://www.broad.mit.edu/annotation/genome/ustilago_maydis/Home.html), we found two potential hydrophobin genes at different positions within the genome, one of which encodes a standard class I hydrophobin already published before (*Hum2*, Bohlmann 1996; later ,the name was changed to *UmH*2, personal communication by Heine Deelstra) (Fig. 7). This gene has one intron at a unique position whereas the new gene *UmH*1 has no intron (Fig. 2). The product of *UmH*2 gene has a long N-terminal extension with repeated copies of 35 amino acids each (Fig. 8). Both *U. maydis* proteins have N-terminal signal peptides for secretion out of the cell.

Figure 7. **Localization of hydrophobin genes in genome of *U. maydis***. Two different hydrophobin genes were deduced from the genome of *U. maydis*. The start and stop positions of the genes on the top and below of the broad arrows representing the genes.

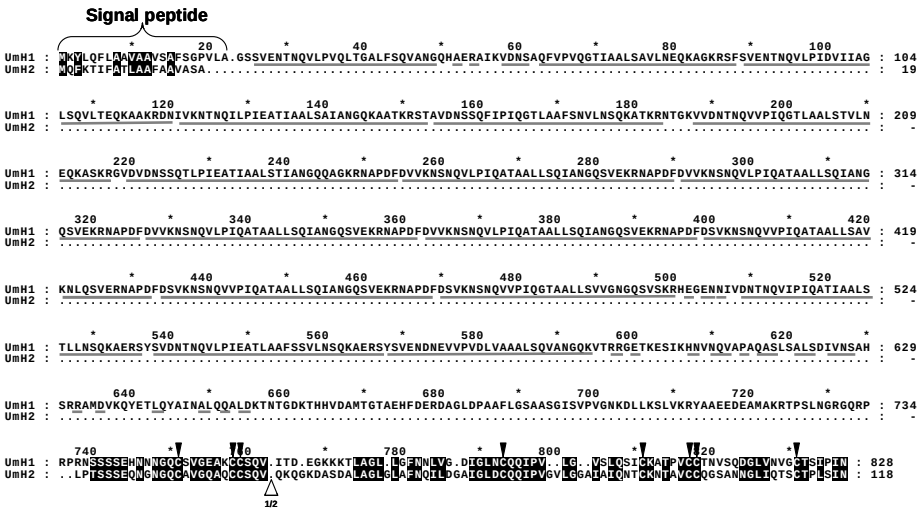

Figure 8. **Alignment of the deduced *Ustilago maydis* hydrophobins**. UmH1 is an untypical hydrophobin with a long N-terminal stretch of long peptide repeats of each 35 amino acids (underlined). The eight cysteines residues typical for hydrophobins are shown with filled triangles above the sequences and the signal peptides are indicated. UmH2 has only one intron which is indicated with an empty triangle, and UmH1 has no introns.

Interestingly, the last basidiomycetes of which we have a complete genome, *Cryptococcus neoformans* (http://www.broad.mit.edu/annotation/genome/ Cryptococcus_neoformans_b/Home.html), has no gene for a hydrophobin.

5.4 Phylogenetic grouping of hydrophobins from basidiomycetes

Using ClustalX alignment (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/) for aligning all hydrophobins deduced in this thesis from basidiomycete genomes, all available hydrophobin sequences from the NCBI database and a subset of ascomycete hydrophobins from the NCBI database, and these sequences were aligned, and a phylogenetic tree was created in order to get insight into the evolutionary relationships between basidiomycete hydrophobins (Fig. 9). Within the phylogenetic tree, all hydrophobins deduced in this study from the available basidiomycete genomes cluster among known class I hydrophobins from basidiomycetes, but CoH8 and CoH9 from *C. cinerea* that were found between class I hydrophobins of ascomycetes (Fig. 9). CoH8 and CoH9 differ in the hydropathy plots from other basidiomycete class I hydrophobins. They are generally less hydrophobic, particularly in the C-terminal half of the amino acid stretch between the cysteines at positions 3 and 4 and in the amino acid stretch between 4 and 5 cysteine positions (Fig. 10).

Ascomycete hydrophobins clustering with CoH8 and CoH9 in the phylogenetic trees (Fig. 9, 11) are all somewhat less hydrophobic than typical basidiomycetes hydrophobins such as SC3 of *S. commune* and CoH1 of *C. cinerea* (Fig. 10, Wessels 1997). Most other hydrophobins of *C. cinerea* have the typical hydropathy pattern as SC3 and CoH1 (see chapter 6). Only CoH33 and CoH34 diverge also from that pattern, but not as dramatically as CoH8 and CoH9 (Fig. 10). Within the typical class Ia hydrophobins of basidiomycetes, most of the *C. cinerea* hydrophobins are found in three subclusters (Ia3, Ia8 and Ia12) in the phylogenetic tree as shown in Fig. 9 and 11. In addition, CoH25 of *C. cinerea* clusters together with LbH11 from *L. bicolor* and hydrophobins from *Pholioka nameko* in subcluster Ia11´´. CoH2 of *C. cinerea* loosely clusters with subcluster Ia1 (as well as Le.Hyd1 from *Lentinula edodes*) together with various *P. chrysosporium* hydrophobins and CoH15 clusters only loosely with other hydrophobins in subcluster Ia8. *P. chrysoporium* hydrophobins are found only in three different subclusters (Ia1, Ia5 and Ia6)

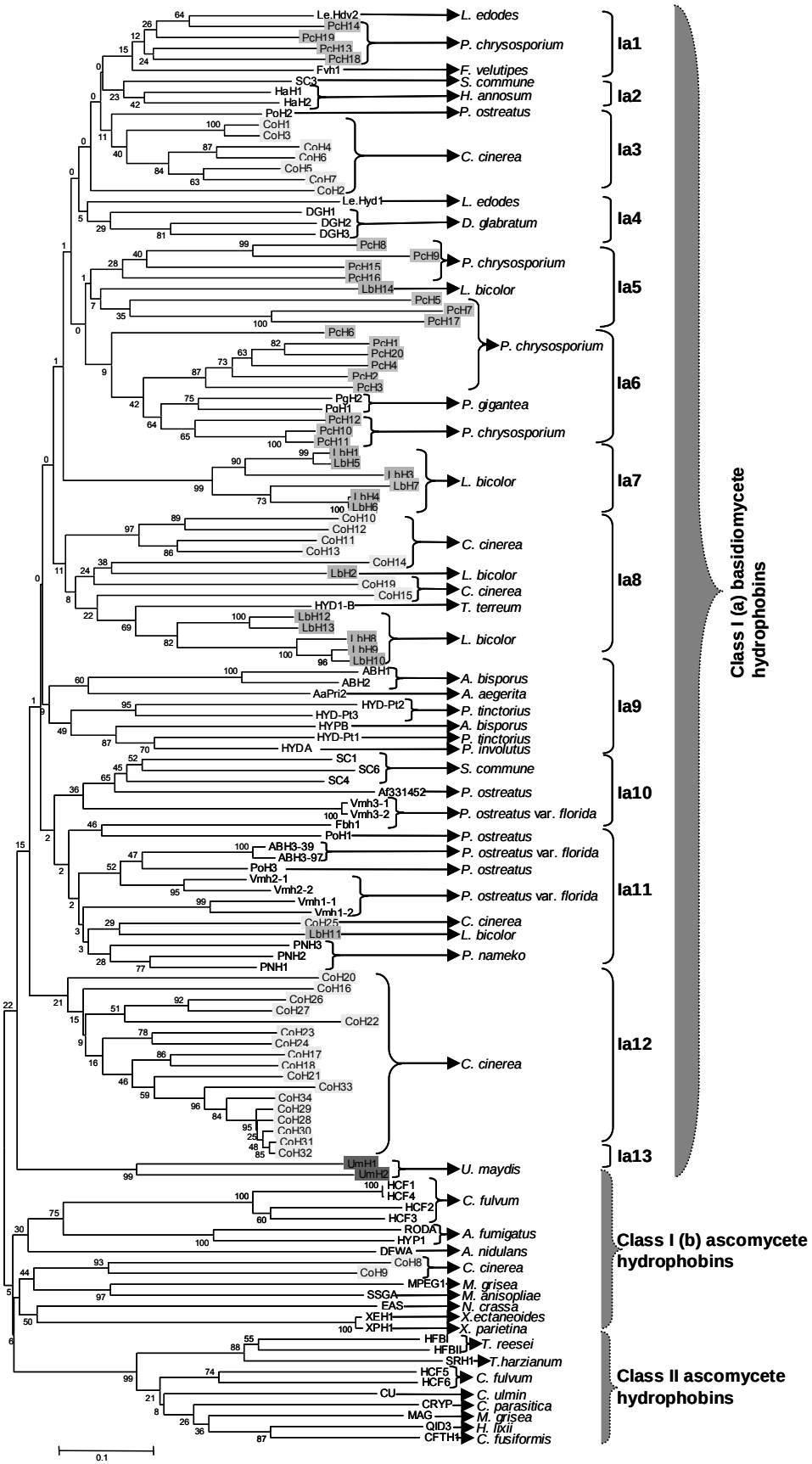

Figure 9. **Phylogenetic analysis of hydrophobins from basidio- and ascomycetes including all potential proteins deduced from basidiomycetes genomes**. The phylogenetic tree includes the deduced hydrophobins CoH1-CoH34 from *Coprinopsis cinerea,* PcH1-PcH20 from *Phanerochaete*

chrysosporium, LcH1-LcH14 from L*accaria bicolor* and UmH1 and UmH2 from *Ustilago maydis,* marked by different shades. The tree is calculated with *p*-distances using Mega ver. 2.1 (www.megasoftware.net), based on a ClustalX alignment (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/) using the hydrophobin core sequences, i.e. the sequences from the first conserved cysteine to the C-terminus (Wösten 2001). Amino acid residues in front of the first cysteine in all proteins were omitted for sequence comparison because this includes signal sequences for secretion and a more or less long N-terminal sequence left after secretion (Wösten 2001). Bootstrap values were calculated using 500 replications. Proteins marked by a grey color code refer to hydrophobins deduced in this study from the available basidiomycete genomes. GenBank accession number for ascomycete hydrophobins used in this study were: DEWA (U07935) and RODA (M61113) of *Aspergillus* (*Emericella*) *nidulans*, RODB (AY057385), HYPB (AB097448), and HYP1 (L25258, U06121) of *Aspergillus fumigatus*, ROLA (AB094496) of *Aspergillus oryzae*, HCF-1 (X98578), HCF-2 (AJ133700), HCF-3 (AJ566186), HCF-4 (AJ566187), HCF-5 (AJ133703), and HCF-6 (AJ251294) of *Cladosporium fulvum*, HCH-1 (AJ496190) of *C. herbarum*, CRP (L09559) of *Cryphonectria parasitica*, MPG1 (L20685) and MAG (AF126872) of *Magnaporthe grisea*, SSGA of *Metarhizium anisopliae* (M85281), EAS of *Neurospora crassa* (AAB24462, X67339), CU (U00963) of *Ophiostoma ulmi*, PbHYD1 (AF526275) and PbHYD2 (AY427793) of *Paracoccidioides brasiliensis*, SRH1 (Y11841) and QID3 (X71913) of *Trichoderma harzianum*, HFBI (Z68124) and HFBII (Y11894) of *Trichoderma reesei*, XEH1 (AJ250793) of *Xanthoria ectaneoides*, XPH1 (AJ250794) of *Xanthoria parietina*, For GenBank accession numbers of basidiomycete hydrophobins, see legend of Figure 1 in chapter 3, HaH1 (ABA46363) and HaH2 (ABA46362) of *Heterobasidion annosum* (Karlsson et al., unpublished), and PgH1 (AAY97884) and PgH2 (AAY97885) of *Phlebiopsis gigantea* (Adomas et al. 2006).

and *L. bicolor* hydrophobins mainly in two clusters (Ia7 and Ia8). The exceptions are LbH11 in subcluster Ia11 and LbH14 in subcluster Ia5. The two *U. maydis* hydrophobins formed an own subcluster, Ia13 (Fig. 9 and 11). However, many of the bootstrapping values are very low (Fig. 9 and 11), indicating that the data should not be over-interpreted. Indeed, when including further sequences from well known ascomycete hydrophobins from the NCBI GenBank (January 2006), grouping partially changed also within the clusters of class I hydrophobins from basidiomycetes (Fig. 11). CoH2 of *C. cinerea* moved closer into subclass Ia1 and Le.Hyd1 of *L. edodes* from subclass Ia4 into Ia1, and subclasses Ia9 and Ia11 split further into two separate groups, suggesting that at least some of the subclasses as seen in the Fig. 9 are very heterogeneous and might split even into further subclusters when more sequences are available. Most interestingly, SSGA and MPG1 from the two ascomycetes *Metarhizium anisopliae* and *Magnaporthe grisea* are now found with subclass Ia9 of the basidiomycete class I hydrophobins (compare Fig. 9 and 11). CoH8 and CoH9 of *C. cinerea* are however still grouping amongst ascomycete hydrophobins (Fig. 11). Possibly, these four proteins present the evolutionary transition between class I from asco- and basidiomycetes hydrophobins. The overall impression from the phylogenetic trees in Fig. 9 and Fig. 11 is that some divergence of hydrophobin genes in basidiomycetes occurred early in evolution, e.g. the separation between i. hydrophobins from *P. chrysosporium*, ii. hydrophobins from

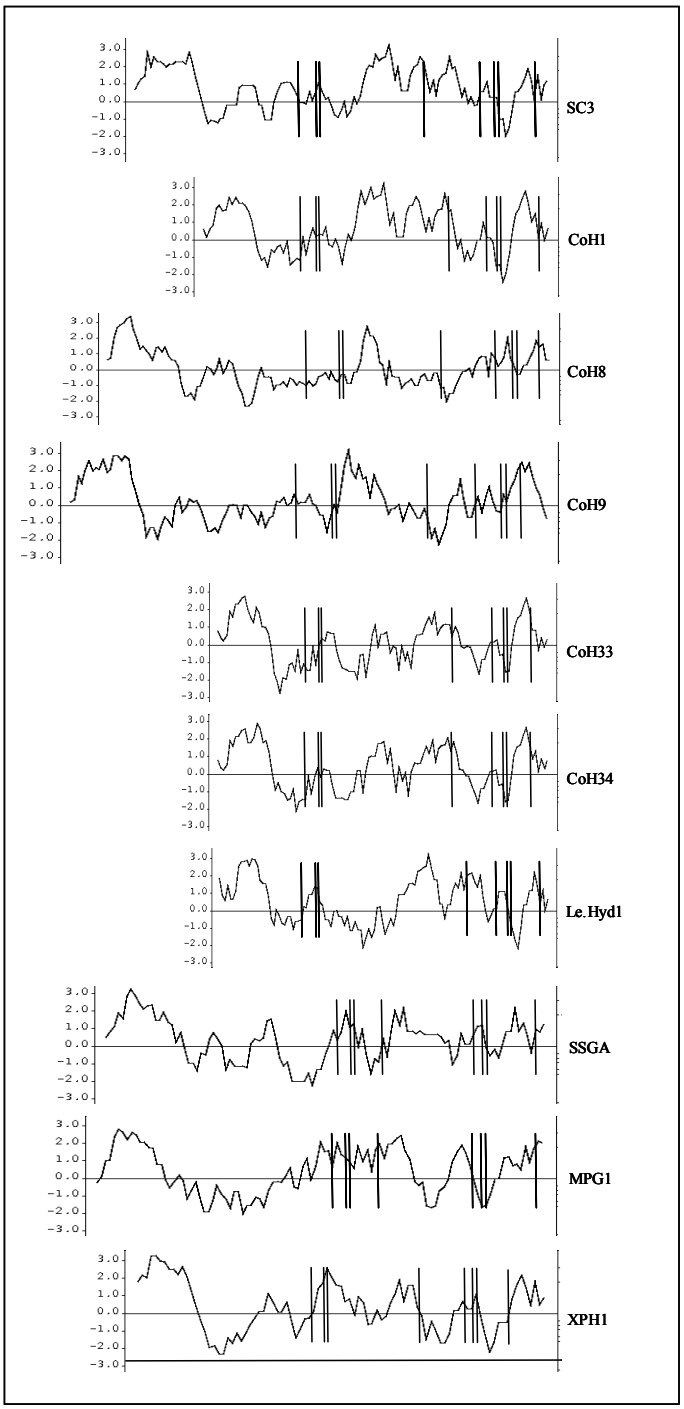

Figure 10. Comparison of hydropathy plots of selected hydrophobins. The plots were determined with the amino acid sequences of the proteins indicated by using the parameters of Kyte and Doolittle (1982) and the program (http://gcat.davidson.edu/rakarnik/kyte-doolittle.htm). A six amino acid window was used and the hydropathy values plotted against the deduced amino acid sequences. SC3 from *Schizophyllum commune*, CoH1, CoH8, CoH9, CoH33 and CoH34 from *Coprinopsis cinerea*, Le.Hyd1 from *Lentinula edodes* belong to class I hydrophobins. SSGA from Metarhizium *anisopliae*, MPG1 from *Magnaporthe grisea* and XPH1 from *Xanthoria parientina* from ascomycete class I hydrophobins are shown. The bars indicate the cysteine positions in the respective sequences.

U. maydis and iii. the hydrophobins from *C. cinerea* and *L. bicolor* (Fig. 9, 11). However, later in evolution, there appear to be several duplications leading to larger groups of closely related hydrophobins within a given species, e.g. 17 of the *C. cinerea* hydrophobins are present in subclass Ia12, and a group of each six in subclasses Ia3 and Ia8, respectively (Fig. 9). *P. chrysosporium* hydrophobins are clustered in three subclasses, subclass Ia1 with 4 *P. chrysosporium* hydrophobins grouping along with *Pleurotus ostreatus* and *L. edodes* hydrophobins, subclass Ia5 with 7 *P. chrysosporium* hydrophobins along with Lbh14 of *L. bicolor*, CoH15 from *C. cinerea* and subclass Ia6 with 9 *P. chysosporium* hydrophobins along with hydrophobins from *Plebiopsis gigantea*, respectively (Fig.9). Regarding *L. bicolor*, six hydrophobins cluster together in subclass Ia7 and six hydrophobins cluster together in subclass Ia8 (Fig. 9).

In conclusion, rather unique standing hydrophobins for *C. cinerea* are CoH2, CoH15 and CoH25, respectively, and for *L. bicolor* LbH11 and LbH14 (Fig. 9). In *C. cinerea*, the gene for CoH2 is found clustered with genes *coH*1 and *coH*3 to *coH*7, and gene *coH*25 is found in a cluster with *coH*24 (see Fig. 2 in chapter 3), suggesting that these genes diverged in higher extend after gene duplications from the closely neighboring genes of same origin. The genes for the two *L. bicolor* proteins on the other hand are found as single genes in the genome of the fungus (Fig. 5).

5.4.1 Biological roles of hydrophobins described in the literature for basidiomycete hydrophobins

Hydrophobins play distinguished roles in various processes during fungal development such as formation of aerial hyphae, fruiting structure development and dispersal of conidia (Talbot 1997). Biologically, these proteins help in lowering the water surface tension, thereby enabling the fungi to form the aerial structures (Wösten et al. 1999). In *S. commune*, SC3 is expressed in both the mono- and dikaryotic mycelium, whereas SC1 and SC6 are expressed in the dikaryotic mycelium only. SC4 is found in dikaryotic mycelium and in the fruiting tissues (Mulder and Wessels 1986). Differential expression of these hydrophobin genes is clearly under control of the mating-type genes (Wessels 1991, Wessels et al. 1995). Targeted deletion of *SC3* produced Δ*sc3* mutants that were deficient to produce aerial hyphae (van Wetter et al. 1996). SC3 also plays an important role in attachment of *S. commune* to hydrophobic surfaces. Δ*sc3* mutants are severely impaired in their ability to produce hyphae that

are capable of attaching to hydrophobic surfaces. Class I hydrophobins SC3 from *S. commune* (van Wetter et al. 1996), ABH3 from *Agaricus bisporus* (Lugones et al. 1998), CoH1 from *C. cinerea* (Ásgeirsdóttir et al. 1997), and PoH1 from *P. ostreatus* (Ásgeirsdóttir et al. 1998) have all been shown or proposed to be involved in formation of aerial hyphae. In tissues of fruiting bodies, hydrophobins line the air channels, thereby preventing tissues from soaking with water and guaranteeing the exchange of gas (Lugones et al. 1996). Judging from expression patterns within tissues, such function is likely performed by ABH1 (also called as HypA) of *A. bisporus* (Lugones et al. 1999), in *S. commune* by SC4 (van Wetter et al. 2000), in *L. edodes* by Le.hyd1 (Nishizawa et al. 2002) and in *P. ostreatus* by PoH1 (Ásgeirsdóttir et al. 1998). In *S. commune*, by knocking-out gene *SC*4, experimental evidence proved this function (van Wetter et al. 2000). In *A. bisporus*, in contrast to ABH1, expression of ABH2/HypB is spatially more defined to the extreme outer tissues of the developing cap in fruiting body primordia (de Groot et al. 1999). Thus, ABH2 may generally protect the whole structure from wetting during development. In the mature fruiting body, this role may be taken over by ABH1 that at this stage is highly expressed in the outer hyphal layer of the fruiting body (de Groot et al. 1999). In *S. commune,* a similar role is performed by SC3 and SC4 (Ásgeirsdóttir et al. 1995). This gives an impression that hydrophobins might play a role in cementing the hyphae together during the formation of complex fruiting structures. *ABH*1 and *ABH*2 genes from *A. bisporus* were found typically expressed in the fruiting bodies (Lugones et al. 1996; de Groot *et al*. 1996) whereas *ABH*3 was specially expressed in the substrate mycelium of *A. bisporus*. These expression patterns very much resemble the types of regulation of hydrophobin genes *SC*3, *SC*4, and *SC*6 in *S. commune* (Ásgeirsdóttir et al. 1995, van Wetter et al. 2000) and *PoH*1, *PoH*2, and *PoH*3 in *P. ostreatus* (Ásgeirsdóttir et al. 1998, Penas et al. 1998). The hydrophobins described so far showed differential expression during fungal development and more details of these and other basidiomycetes in the literature are given in Table 1. Differential expression might indicate adoption of different functions. Complementation of a *SC*3 knock-out in *S. commune* by the *S. commune SC*4 gene only partially restored the mycelial wild-type phenotype, most probably because SC4 is less efficient in lowering the water surface tension required to enable hyphae to make aerial structures and SC4 has a weaker interaction with the cell wall of aerial hyphae compared to that of SC3 (van Wetter et al. 2000).

5.5 Functions of hydrophobins in the saprophyte C. cinerea

5.5.1 Defining stage in fruiting body development for protein expression analysis

In *S. commune*, both SC3 and SC4 were shown to have lectin activity (van Wetter et al. 2000). In *C. cinerea*, interaction for fruiting body hydrophobin with galectins, a subgroup of lectins, was shown by Piers J. Walser (see chapter 1). The initial objective of this thesis in October 2002 was to analyze the expression of two types of extracellular matrix proteins, i.e. hydrophobins and fruiting body-specific galectins, and thereby to analyze their interaction in *in-vivo* conditions within fruiting bodies, in continuation to studies performed by Piers J. Walser (Walser et al. 2003). Galectins were shown to be expressed in the initial stages of fruiting, i.e. during primary and secondary hyphal knot formation (Boulianne et al. 2000). These initial stages are difficult to separate from the vegetative mycelium of the fungus and their cellular development is only partially understood (Matthews and Niederpruem 1972, 1973, Liu 2001 and Göbel 2003).

For better differentiation of expression of genes for extracellular matrix proteins within vegetative mycelium and youngest fruiting stages, a focus was therefore laid on understanding the early stages of fruiting body development in *C. cinerea* (see chapter 2). Little is known about the first cellular specifications, respectively differentiation of hyphae to initiate fruiting body development. Matthews and Niederpruem (1972, 1973) presented a few photos of hyphal aggregates (hyphal knots) as fruiting body initials. Lateron, Liu (2001) and Göbel (2003) tried to follow the development of such aggregates over the time. Their work suggested that at a single or at a few neighbored hyphae, hyphal knot formation starts by intense formation of short hyphal branches with restricted tip growth. These hyphae furtheron tend to swell and aggregate with each other.

Technically, this process turned out to be difficult to follow. The first steps of hyphal knot formation occur in complete darkness. When white light hits cultures, the

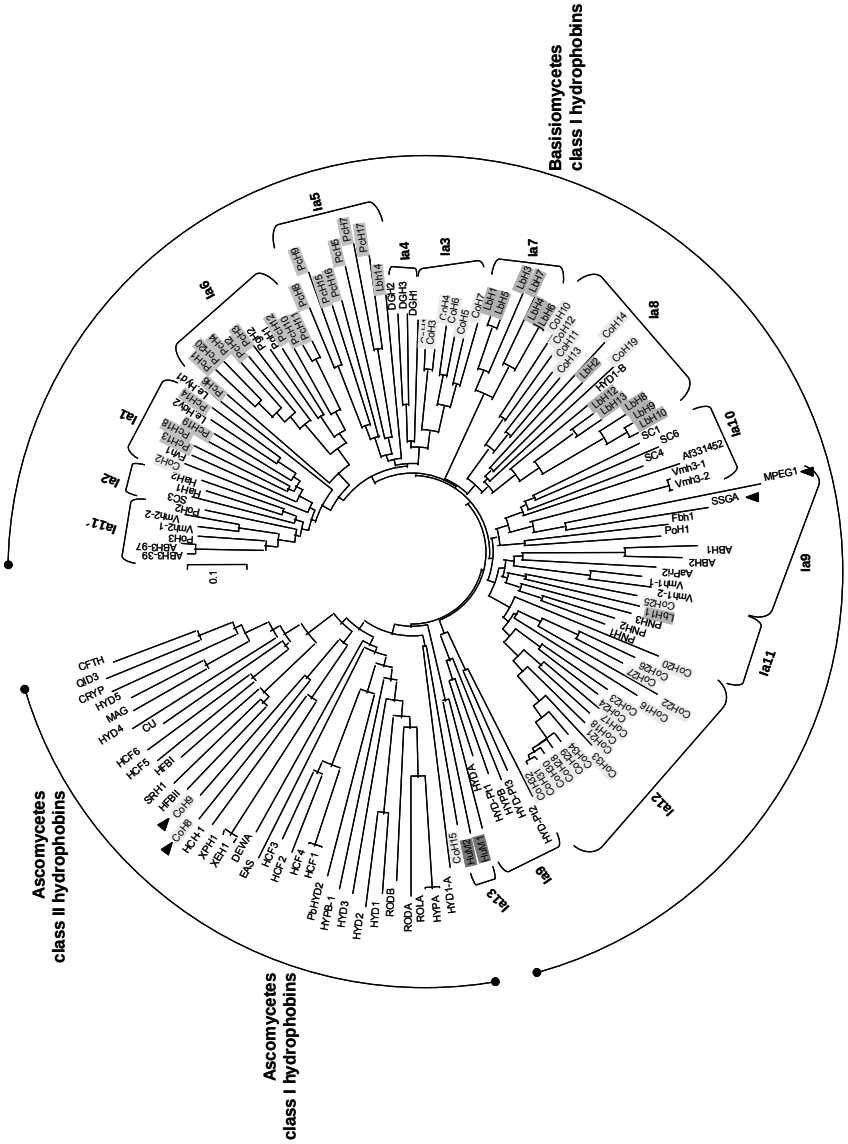

Figure 11. Phylogenetic tree of hydrophobins from all the asco- and basidiomycetes. Analysis is performed using some of the known asco- and basidiomycetes hydrophobins along with deduced hydrophobins from basidiomycete genomes. GenBank accession numbers for all the known proteins were given in Fig. 9. The different levels of shades mark the hydrophobins deduced from different basidiomycetes. The filled triangles indicate the proteins from basidiomycetes, respectively ascomycetes shuffling between hydrophobins from the other fungal group.

Table 1. Hydrophobins described from basidiomycetes in the literature

Organism	Protein	Expression stage	Physiological role	References
Agaricus bisporus	ABH1 (HYPA)	Fruiting specific, highly expressed in veil/peel and pileus	Lining air channels protecting fruiting body from water loading, protects fungi from bacterial parasites	de Groot et al.1996, 19999 Lugones et al. 1996
	ABH2	Poorly expressed in fruiting body tissues	?	de Groot et al.1996 Lugones et al. 1996
	ABH3-39	Outer mycelial cords	Might play a role in lignin degredation by helping hyphae to attach to the substrate	Lugones et al. 1998
	ABH3-97	Vegetative mycelium specific, no transcripts were detected in fruiting bodies	Might play a role in lignin degredation by helping hyphae to attach the substrate	Lugones et al. 1998
	HYPB	Fruiting specific, high in primordia, expressed in pins, inner regions of cap and at cap and stipe transition point	Coating air channels to exchange gases in fruiting bodies, protective barrier between cap and stipe	de Groot et al. 1999
Agrocybe aegerita	Aa-Pri1	Expressed during primordia and immature fruiting body stage, not in vegetative mycelium or later stages of fruiting body maturation	Allows hyphal aggregation	Fernandez and Labarere 1997
	Aa-pri2	Highly and specifically expressed during fruiting initiation	Allows hyphal aggregation and primordia formation	Santos et al. 1999
	Aa-pri3	Primordia stage specific	?	Pascal et al. 2002

Coprinopsis cinerea	CoH1	In vegetative monokaryotic mycelium	?	Ásgeirsdóttir et al. 1997
	CoH2	?	?	Ásgeirsdóttir et al. 1997
Dictyonema glabratum	DGH1	In mycobiont hyphae surrounding younger and older regions of fruiting bodies	?	Trembley et al. 2002a, b
	DGH2	Expressed by mycobiont and not by photobiont	?	Trembley et al. 2002a, b
	DGH3	Expressed by mycobiont and not by photobiont	?	Trembley et al. 2002a, b
Flammulina velutipes	FVH1	Abundantly expressed in mycelia, after the induction of fruiting and during fruiting body initiation	Plays a role in the recovery of mycelial damage after mycelial mat-scrapping	Ando et al. 2001
Lentinula edodes	Le.Hyd1	Highly during primordia development, active during development and enlargement of fruiting bodies	Involved in the formation of extracellular matrix lining the air channels	Ng et al. 2000 Nishizawa et al. 2002 Kaneko et al. 2001
	Le.Hdy2	High in dikaryotic mycelial tissues	?	Ng et al. 2000
Pleurotus ostreatus	Fbh1	Fruiting specific, transcripts in all tissues of mature fruiting body, absent in both mono- and dikaryotic vegetative mycelial samples	Protecting hyphae against desiccation and attack by fungal and bacterial parasites	Peñas et al. 1998
	PoH1	Fruiting body specific, not in vegetative mycelium	Expressed in fruiting body	Ásgeirsdóttir et al. 1998

	PoH2	Absent in fruiting body, but abundantly found in vegetative mycelium	Expressed in vegetative hyphae	Ásgeirsdóttir et al. 1998 Peñas et al. 2002
	PoH3	Absent in fruiting body, but abundantly found in mono- and dikaryotic vegetative mycelium	Expressed in vegetative hyphae	Ásgeirsdóttir et al. 1998 Peñas et al. 2002
	Vmh1-1	Only in vegetative mycelium	?	Peñas et al. 2002
	Vmh2-1	Only in vegetative mycelium	?	Peñas et al. 2002
	Vmh3-1	Transcripts in mono- and dikaryotic vegetative mycelium	?	Peñas et al. 2002
Pholiota nameko	PNH1	Induced in response to phosphate deficiency	?	Tasaki et al. 2004
	PNH2	?	?	Tasaki et al. 2004
	PNH3	?	?	Tasaki et al. 2004
Pisolithus tinctorius	HYD1	Vegetative mycelium, poorly expressed in submerged hyphae and abundant accumulation in aerial hyphae. Cell walls of hyphae forming mantle	Plays an important role in ectomycorrhiza development during adhesion and aggregation of fungal hyphae	Tagu et al. 1996, 2001
	HYD2	Vegetative mycelium, poorly expressed in submerged hyphae and abundant accumulation in aerial hyphae. Cell walls of hyphae forming mantle	Plays an important role in ectomycorrhiza development during adhesion and aggregation of fungal hyphae	Tagu et al. 1996, 2001

	HYD3	Early stages of ectomycorrhizal formation	Plays an important role in ectomycorrhiza development during adhesion and aggregation of fungal hyphae	Duplessis et al. 2001
Schizophyllum commune	SC1	During early fruiting stages	?	Mulder et al. 1986, Ruiters et al. 1989, Wessels et al. 1995
	SC3	In mono- and dikaryons and early fruiting stages	Involved in aerial hyphae formation	Wessels et al. 1991, van Wetter et al. 2000
	SC4	Early stages of fruiting body development, exclusively at the extra-cellular matrix boundaries	Lines air channels within fruiting tissue like ABH1	Lugones et al. 1999
	SC6	During fruiting body expansion	?	Wessels et al. 1995
Tricholoma terreum	Hyd1	Aerial mycelium of hyphal mantle and in Harting net hyphae	Host recognition and mycorrhizal formation	Mankel et al. 2002
Ustilago maydis	Hum2	?	?	Bohlmann 1996

development stops. Therefore, all observations under the microscope need to be done in a dark room and with a yellow filter in the light stream of the microscope. Further, cultures easily suffer desiccation during the handling under the microscope and also this leads to arrest in development. Therefore, inbetween observation periods, cultures have to be transferred into the moist chambers. This causes a further problem in finding back a minute place of observation under the microscope. In conclusion, it needs a certain degree of experience to be able to recognize and follow early stages of development. In this study, the techniques for observation were further improved so that cultures reliably form primary hyphal knots in the dark and upon transfer into light more compact secondary hyphal knots (see chapter 2). Secondary hyphal knots particularly form at the edges of the colonies. In this study, windows were cut into the agar of fungal cultures. Secondary hyphal knot formation and primorida formation were regularly observed at the outer edges of the window with respect to whole culture (see chapter 2). In the outer regions of a culture, at the stage of primary hyphal knot formation fruiting body-specific expression of galectins occurs, as shown before by Western blot analysis (Boulianne et al. 2000). Further evidence for localized expression of the fruiting specific galectins in regions of hyphal knot formation is given in this study by a reporter system, that expresses a laccase gene under control of the promoters of the galectin genes (see chapter 2). If there are hydrophobins expressed early in fruiting, also in these regions at the time point of primary and/or secondary hyphal knot formation transcripts would be expected.

Developmental stages following formation of secondary hyphal knots are as badly documented by photographic pictures in the literature as the very early stages of fruiting. Although the fungus has served for a century as a model species for fruiting body development (Buller 1931, Moore et al. 1979, Reijnders 1979, Kües 2000), the best available collection of photos in publications are in the review by Walser et al. (2003, see chapter 1.2), and in a similar set of photos in a book chapter by Kües et al. (2004). To better understand the hydrophobin expression patterns during fruiting, another more precise time course of the events was established in chapter 4.

With this cytological knowledge, studies were carried out to identify and characterize hydrophobins from fruiting bodies. Initial experiments with hydrophobin isolation lead to several constraints in protein purification and solubilization due to interference of contaminating melanin expressed in the later stages of fruiting body development (Peddireddi et al. 2005, see Appendix to this thesis). Further successful

experiments to isolate hydrophobins were therefore conducted with melanin-free primordia samples. Four different hydrophobins (CoH14, CoH23, CoH24 and CoH25) were identified from these samples by ESI-LC-MS analysis (see chapter 3) at a same developmental stage where galectins were shown to be expressed (Charlton et al. 1992, Boulianne et al. 2000). With the release of *C. cinerea* genome (by the Broad Institute on July 16th 2003, see chapter 3), we screened the genome for hydrophobin genes on homology bases with the known hydrophobin sequences from the GenBank database (see chapter 3). By the unexpected high number of genes, this was a longer task than originally anticipated. Unfortunately, the original idea to study potential functional interactions between two types of extracellular matrix proteins, i.e. galectins and hydrophobins, could therefore not be further followed up. Nevertheless, the work presented here shows that several hydrophobins are expressed in the caps and the stipes of fruiting bodies at places where we also find the galectins in *C. cinerea* (Boulianne et al. 2000), which supports the possibility that these two types of extracellular matrix proteins will interact with each other *in-vivo*.

5.5.2 Transcript analysis of hydrophobin genes

Transcripts were found for 29 of the 34 hydrophobin genes during different stages of development in *C. cinerea.* Unlike hydrophobin genes known from other basidiomycetes, most hydrophobin genes from *C. cinerea* showed little specificity towards stages of expression (see chapter 4).

From all obtained results, we can summarize for specific hydrophobin genes: Specific to mycelium are only *coH*10, *coH*13 and *coH*32 whereas *coH*14, *coH*24 and *coH*25 are found to be specific to fruiting. *coH*2 is highly expressed in stipes, throughout stipe tissue formation and elongation and found less expressed in cap tissues. *coH*2 is one of the seven genes found together in one gene cluster. The 6 other genes including *coH*7 (unfunctional by a frame shift at least in the Okayama 7 genome) are well expressed in vegetative mycelium of mono- and dikaryons (see chapter 4). In contrast, in fruiting stages, all these are not or only poorly transcribed but *coH*1 that expresses as *coH*2 well in stipe tissues. In the phylogenetic tree, protein CoH2 clusters to another subclass than the proteins of the six genes neighboured to *coH2* (Fig. 9). If originating from duplication of the same ancestor genes as suggested by the close localization of the genes in the genome, *coH*2 is clearly evolving away from the other genes (see above).

*coH*14, *coH*24 and *coH*25 are highly expressed in cap and stipe tissues of the fruiting bodies (see chapter 4). Hydrophobins from fungi have usually been detected on the transcript level and/or by a high protein levels. With genomic approaches we now see that there might be also numerous poorly expressed hydrophobins in the fungi that so far could escape from detection. It is possible that hydrophobin genes from other species are highly expressed in certain developmental stages and might be expressed in low levels in others that have them been left undetected by molecular approaches such as Northern blots, *in-situ* hybridization and protein detection by immuno blots, etc.

In this study with *C. cinerea*, we used RT-PCR for transcript detection which is much more sensitive than the other methods mentioned above. To perfectly judge expression levels, in the future a more precise detection technique might be applied, such as real-time PCR, where transcripts can be quantitatively detected (Bertossa et al. 2004). In such way, transcripts of galectins genes at the initiation of fruiting have been followed (Bertossa et al. 2004).

The high number of different hydrophobin genes within the higher basidiomycete genomes and different levels of transcripts raises several questions on the functional properties and the physiological role of these proteins within the fungus. It is of highest interest to know whether their products differ functionally depending on the differences in their primary structures. Generation of mutants is perhaps the most severe constraint on current progress on hydrophobin studies, as knock-out mutations in higher basidiomycetes are difficult to produce (Kües et al. 2004). Advanced molecular biology tools like RNAi silencing were recently established for *C. cinerea* (Namekawa et al. 2005, Wälti et al. 2006) and protein interaction assays such as pull-down assays (Graves and Haystead 2002) may help in the in-depth analysis of the physiological functions of the different hydrophobins.

5.5.3 Do positions of hydrophobins in the phylogenetic tree reflect potential functions?

Wösten (2001) and Wessels deduced before from phylogenetic analysis of a small group of hydrophobins with known expression patterns that the proteins evolved to adopt specific functions in fungal growth and development (Wessels 2000). Our somewhat larger analysis made in the year 2003 with more hydrophobins (in total 36

from different basidiomycetes) shed doubt onto this hypothesis, since proteins expressed in vegetative mycelium and mushroom partially intermingled with each other (Walser et al. 2003). The current phylogenetic analysis with 104 different basidiomycete proteins (taken from the NCBI database and 68 deduced hydrophobins from published basidiomycetes genomes) also failed to reveal a clear-cut division between proteins expressed in vegetative mycelium and proteins expressed in fruiting body-specific tissues (Fig. 9). With the expression patterns established for 34 different *C. cinerea* hydrophobins, we find proteins closely grouped in the subclass Ia8 that are only highly expressed and/or specific in vegetative mycelium (*coH*10, *coH*13) and others that are only highly expressed or specific to the fruiting stages (*coH*14). Thus, proteins of closer related amino acid sequence might have quite different developmental functions.

Concluding from the *C. cinerea* data with many genes expressed at all or nearly all developmental stages tested (see chapter 4), the phylogenetic tree (Fig. 9) cannot indicate at what developmental stage a protein might be expressed and what kind of different functions it might have. In contrast, smaller clusters of hydrophobins from other species may support the hypothesis of Wösten and Wessels. In subcluster Ia11, respectively Ia11´ (Fig. 9, 11), where no *C. cinerea* protein was found, the mycelium specific proteins ABH3-39 and ABH3-97 from *Agaricus bisporus*, POH3 from *Pleurotus ostreatus*, Vmh2-1 and Vmh2-1 from *P. ostreatus* var. *florida* group together, all of which are expressed in mono- and dikaryons (Walser et al. 2003). In subclass Ia10 (Fig. 9, 11), there are the fruiting-specific proteins SC1, SC4 and SC6 from *S. commune* and three proteins from *P. ostreatus,* two of which are known to be both mycelium and fruiting body expressed and one which came from a patent and is unclassified in its biological function (Walser et al. 2003). However, lack of proteins from other species in this subclass does not allow any deeper conclusions. Whether the three proteins SC1, SC4 and SC6 from *S. commune* grouped together because of expression in fruiting bodies or specific protein adaptations to the needs of the fruiting body or because of evolutionary duplications as seen in the genomes of other basidiomycete species needs to be addressed in the future when more complete genomes are available including that of *S. commune*. The mycelium-specific hydrophobin SC3 of *S. commune* on the other hand groups with mycelial hydrophobins from *Heterobasidion annosum* and closely with the mycelium-specific

proteins Le.hyd2 from *L. edodes*, and the fruiting-specific protein Fvh1 from *F. velutipes* (Fig. 9, 11).

5.5.4 Functions of hydrophobins in ecology of fungi

Next to expression in different developmental stages one other interesting question would be whether hydrophobins evolve in relation to the ecology of the habitat that a fungus occupies. With a complete genome of a saprophyte, a white-rot fungus, a mycorrhizal fungus and two pathogenic fungi, we can start to address this question. Between the deduced hydrophobins from *P. chrysosporium*, with one exception (LbH14 of *L. bicolor*) we do find only hydrophobins from other white-rot fungi (*L. edodes*, *F. velutipes*, *P. gigantea*). The hydrophobins from these species are known to be expressed in the monokaryons (Ng et al. 2000, Ando et al. 2001, Adomas et al. 2006) whilst nothing is known about expression of these hydrophobins within wood. In *S. commune*, a role for SC3 as a hydrophobic compound during invasive growth in lignified wood has been postulated (Wösten et al. 1994). However, a monokaryotic strain when grown on birch sawdust did not produce SC3 (Lugones et al. 2004) and wild type strains and SC3 knockout strains equally grow in wood (S. Peddireddi, personal communication). SC3 has been shown to affect the cell wall composition, in detail the percentage of glucans (van Wetter et al. 2000). SC3-free cultures produce extensive mucilage (water soluble glucan). Lugones et al. (2004) speculated that such mucilage can help to overcome desiccation in wood and/or to provide proper conditions for hydrolytic enzymes. In *P. chrysosporium*, formation of a mucilage layer encapsulating hyphae during growth on wood has been shown (Ruel and Joseleau 1991). By the two unusual hydrophobins PcH7 and PcH17 in *P. chrysosporium* with the N-terminal repeats (GLIPS/T), it is an interesting question to address whether these motifs mediate at some time attachment to cell walls of wood and swelling of hyphae to prevent desiccation, in a similar manner as has been observed for the pollen coat protein (Fiebig et al. 2004).

Hydrophobins expressed in mycelium root association as well as in dikaryon mycelium were reported for *Pisolithus tinctorius* (HYD-Pt1, HYD-Pt2 and HYD-Pt3) and *Tricholoma terreum* (Hyd1) (Duplessis et al. 2001, Mankel et al. 2002). In the phylogenetic tree of hydrophobins, only HYD1 of *Trichoderma terreum* is found in subcluster Ia8 amongst some of the *L. bicolor* hydrophobins and also hydrophobins from saprophytes that are variably expressed in vegetative mycelium or in fruiting

bodies (Fig. 9). The hydrophobins known to expressed by *P. tinctorius* in mycorrhizal association intermingle in subcluster Ia9 in the phylogenetic tree (Fig 9, 11) with a fruiting body-specific protein from *Agrocybe aegerita* (Santos et al. 1999) and fruiting body-specific and mycelium-specific proteins from *A. bisporus* (Lugones et al. 1996). As already mentioned above, many of the hydrophobins from the mycorrhizal species *L. bicolor* cluster with proteins from the saprophyte *C. cinerea*, suggesting that these might not be proteins that particularly evolved for reactions in establishing of and functioning in mycorrhiza. Mycorrhizal species as saprophytes form aerial hyphae and mushrooms. Thus, mycorrhizal species as saprophytes will need hydrophobins for such purposes and may use the same in mycorrhiza. There is however subcluster Ia7 whose proteins appear to be quite distinct from all hydrophobins of the other subclusters and the mebers of subcluster Ia7 are all from *L. bicolor*. It is thus tempting to speculate that these might present proteins specific to mycorrhizae.

The plant pathogenic fungus *U. maydis* might be seen as an out-group in the phylogenetic tree (Fig. 9), since the fungus is a heterobasidiomycete unlike the other basidiomycete species. Why there are only two hydrophobins in *U. maydis* and none in the human-pathogenic heterobasidiomycete *C. neoformans* may relate to the ways the fungi enter their hosts and survive in the hosts. Outside the hosts, both fungi grow yeast-like in moist environments, e.g. in soil. Entering of *C. neoformans* in mammalian tissues goes via inhalation into the lungs into a hydrophilic environment (Idnurm et al. 2005). *U. maydis* can enter plants only when two yeast-like cells of opposite mating types fuse. The resulting filaments enter the plants either through stomata or over appressoria. Within young plant tissues upon invasion, the fungus grows first as a biotroph intracellularly without destruction of the plant protoplasts and in older inner tissues intercellularly (Kahmann et al. 2000). These inner plant tissues with non-lignified cell walls are likely hydrophilic and hydrophobins in plants might not urgently be needed. Whether the hydrophobins of *U. maydis* contribute to appressoria formation to enter the plants is not yet known. Like as discussed above for specific *P. chrysosporium* hydrophobins, as an alternative the N-terminal repeats of UmH1 might give the protein particular properties adopt to specific conditions in planta. In this respect, it would also be of great interest to know whether LcH14 of the ectomycorrhizal fungus *L. bicolor* is expressed specifically in planta for reactions with the plant cell walls.

5.6 Conclusions and future perspectives

In this study, 34 hydrophobin genes from the saprophytic basidiomycetes *C. cinerea* were detected and analyzed in expression patterns. This is an amazing high number of proteins compared to the number of hydrophobins known so far from other fungi in the literature (Linder et al. 2005). Of the 34 genes found in the genome, 29 were shown to be active. The expression studies showed that for most genes there is no preference in expression at specific stages. Nothing is known yet whether they are all or at least some of them essential or whether some of them act as back-ups for others. With our current knowledge it is impossible to answer what all these different hydrophobins are for and whether they are all needed. As indicated above, knock-out or gene silencing studies and much more protein analyses are required.

In addition to *C. cinerea*, studies have to be done also for other species to clarify whether these multiple genes exhibit differential expression during development and growth in different biotopes. Other studies have to be focused on the functional roles of hydrophobins and the potential differences depending on the structural characters of the different hydrophobins. When more than one protein is expressed in a same tissue, it is likely that hydrophobins of the same kind as well as the different hydrophobins form with each other amphiphatic films. The films of the latter interaction will possibly be of different nature compared to the films from one type of hydrophobins. If such different proteins interact to perform in common a principle function, there is moreover the possibility that films differ in properties, depending on the relative amounts of the different hydrophobins in them. Thus, there is not only a large variety of different proteins with potential different properties but even a larger variety of possible protein combinations that offer even more potential in differentiation and adaptation to specific environmental and developmental conditions.

Whilst this thesis addressed solely the biological side of hydrophobins, there is also an application side to this kind of proteins. Hydrophobins are interesting candidates for industrial and medical application because of their biophysical properties. Hydrophobins may be used in tissue engineering for coating the hydrophobic surfaces to increase the biocompatibility of medical implants like artificial blood vessels and surgical instruments (Scholtmeijer et al. 2004). Genetic engineering may further improve coating by cell-binding domains from human proteins e.g. fibronectins. Growth of fibroblasts on Teflon could be improved by coating the solid with the

engineered hydrophobins (Janssen et al. 2002). Oil vesicles coated with hydrophobin film might be useful for delivering lipophilic drugs orally into target cells (Wessels 1997). Hydrophobins are safe for human consumption by the fact that large amounts of mushrooms are being consumed by people around the world every day. Hydrophobins can thus be used in foods and drinks. E.g., studies related to gushing in beer showed the presence of high amount of hydrophobins and stabilization of foam is of much interest for industrial applications (Talbot 2001, Hippeli and Elstner 2002). Other studies indicate that hydrophobins can be used in personal care to prolong the residence time of the additives in hair care and shampoo products (Vic G patent application), and in stabilizing the emulsions in creams and ointments (Wösten et al. 1994). In biosensor design, hydrophobins may be used as intermediates to attach cells, proteins, antibiotics and other small ligands to hydrophobic surfaces of electrodes (Bilewicz et al. 2001). Simple and reliable immobilization techniques preserve the activity of enzymes that are of interest in many technologies based catalysis (Palmo et al. 2003). Corvis *et al*. (2005) recently showed that these proteins can be used to immobilize small, electro-active molecules. Two redox enzymes, glucose oxidase from *Aspergillus niger* and horseradish peroxidase, were immobilized by physisorption on glassy carbon electrodes coated with *S. commune* hydrophobin SC3. The results obtained in this work show a way to easily manufacture stable, enzyme-based catalytic surfaces for applications in biosensing (Corvis *et al*. 2005). In another study, it has been demonstrated that enzymes can be immobilized on the hydrophobic side of the hydrophobin layer facilitating studies of lipases activity (Palmo et al. 2003). Using fungal hydrophobins as efficient tags for purification of recombinant fusion proteins by aqueous two-phase separation has many advantages such as high selectivity and good yield and it is technically very simple to perform (Linder et al. 2004). Hydrophobins can be used as anti-fouling applicants to prevent the growth of unwanted microorganisms on the surfaces (Hektor and Scholtmeijer 2005). In nanotechnology, alteration between two sides of amphiphatic hydrophobin films can give micro-chips required surface characters (Scholtmeijer et al. 2004).

The only limiting factor to a broad application of hydrophobins is the level of expression. In *S. commune*, 60 mg of purified hydrophobin SC3 can be obtained from 1 l of culture medium (Wessels 1994). In *C. cinerea*, hydrophobin yields from culture of monokaryons are around 100 mg per liter (S. Peddireddi, personal communication and own observations). Thus, the natural strains express hydrophobins at too low

level. With respect to class I hydrophobins, these proteins are very difficult to extract in order to get pure protein for application. Preventing the aggregate formation during extraction and purification is of major importance for commercial input (Scholtmeijer *et al.* 2001). With respect to recombinant overexpression of hydrophobins, under induced conditions the *Trichoderma reesei* strain HFBI gave a yield of 120 mg HFBI per 1 l of culture medium (Askolin *et al.* 2001). Recombinant yields in ascomycete *T. reesei* are currently only little better than natural yields in *C. cinerea*. So far, only SC3 from *S. commune* and HFBI of *T. reesei* have been tested for various applications (see above). By overcoming problems of aggregations of class I hydrophobin during purification, the fungus *C. cinerea* may become another good source for hydrophobins for applications. With a potential set of 34 different hydrophobins, the fungus offers a huge bank of hydrophobins with possibly different natural properties for testing in different applications.

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5.8 References

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Appendix

CHAPTER 6

Multiple hydrophobin genes in mushrooms

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The author of this thesis contributed Fig. 4 (hydropathy plots of hydrophobins); and the text and experimental data of the section "Hydrophobins and hydrophobin genes in *Coprinopsis cinerea*"to this publication

6.1 Abstract

Hydrophobins are small secreted fungal proteins that form amphipathic films on hyphal surfaces. In the wood-rotting fungus *Schizophyllum commune*, four different hydrophobins are known with well established functions during vegetative growth and fruiting body development. Our study aims at elucidating the role of these proteins in wood penetration and lignocellulose degradation. Blast searches of the genome of the dung fungus *Coprinopsis cinerea* revealed a surprising number of 34 different hydrophobin genes in this species. Functional analysis of these genes is in progress.

Key words: *Schizophyllum*-*Coprinopsis*-FTIR microscopy-Wood decay-Development

6.2 Introduction

Schizophyllum commune, the Split-Gill Mushroom, is one of the most widely distributed wood inhabiting basidiomycetes found throughout the tropical and temperate regions of the world (Raper et al. 1958, James et al. 1999, James and Vilgalys 2001). The species is regarded as mild rot in temperate regions and severe wood destroyer in the tropical regions (Schmidt and Liese 1980). Most commonly, *S. commune* is found in nature on fallen trunks and branches of deciduous trees (Fig. 1, left), less often on wood of conifers (Cooke 1961, Breitenbach and Kränzlin 1991). Moreover, the fungus can act as a pathogen on standing trees (Latham 1970, Adaskaveg et al. 1993; Fig. 1, middle and right).

Figure 1. *Schizophyllum commune* fruiting bodies found on a fallen beech tree in the Billingshäuser Schlucht, Göttingen (left; May 2004) and on a living *Juglans ailantifolia* tree growing next to the GZMB (Göttinger Zentrum für Molekularbiologie) on the ground of the Georg-August-University Göttingen (middle; November 2004). Note that in spring 2005 the tree produced leaves on all healthy branches but not or only as an exception on branches infected with *S. commune* (right; May 2005).

S. commune is usually considered to be a white rot fungus. However, wood decay tests in the laboratory in most instances did not result in considerable weight losses (Hegarty et al. 1987, Nsolomo et al. 2000, Humar et al. 2001, 2002, Schirp et al. 2003). Schmidt and Liese (1980), Nilsson and Daniel (1983) and Leithoff and Peek (2001) measured between 1 to 6% weight loss for different strains and concluded the fungus is a rather weak wood-destroyer. In rare cases, mass losses of nearly 50% were observed mainly due to lignin degradation and to a low degree due to cellulose degradation (Hong 1982). Although there might be no weight loss in wood decay tests, toughness of wood can be negatively affected. 32% strength loss has been measured (Schirp et al. 2003). In

flake board tests with *S. commune*, weight loss of 9.6% occurred together with strength loss (67.5% modulus of rupture loss; Hadi et al. 1995). A monokaryon of *S. commune* was shown to some extent to demethylate lignin. This was not due to production of laccases, peroxidases or ligninases (Trojanowski et al. 1986). Poor lignin degradation in another study was thought to result from the inability of the fungus to solubilize lignin (Boyle et al. 1992). On wheat straw, some tropical isolates were shown to degrade lignin (up to 15%) and to cause simultaneous mass loss of up to 23.5%. Another strain showed even higher mass loss (26.7 %) but no lignin decay (Capelari and Zadrazil 1997). Lignin in olive pomace was efficiently degraded by a *S. commune* isolate (up to 52.7 % of lignin breakdown). During pomace degradation, high laccase activities were recorded (Haddadin et al. 2002). Many *S. commune* strains have xylanase and cellulase activities (Schmidt and Liese 1980, Clarke and Yaguchi 1986, Hegarty et al. 1987, Bray and Clarke 1995, Haltrich et al. 1995, Thygesen et al. 2003). Most of them have also laccase and peroxidase activities (Schmidt and Liese 1980). de Vries et al. (1986) described laccase activity specific to the dikaryotic state of *S. commune* whilst in the parental monokaryons, they could not detect this activity. Phenoloxidase activities were not found in other studies, also not upon treatment with phenolic inducers (Boyle et al. 1992, Nsolomo et al. 2000). By the well established xylanase and cellulase activities it has been suggested that the main role in nature is to recycle carbon by breaking down cellulose and xylans in fallen wood (Raper and Fowler 2004). In conclusion, the ability of *S. commune* to degrade wood remains to be a puzzle.

S. commune is used as a model fungus to study mating types and fungal development in basidiomycetes (Raper and Fowler 2004). Well known is the discovery of hydrophobins in this fungus, small-secreted fungal proteins of about 100 to 140 amino acids with 8 conserved cysteine residues. Upon secretion, hydrophobins self-assemble into amphipathic stable films that cover fungal cells and make their surfaces hydrophobic. Therefore, these films enable fungal structures to grow into the air and protect them from adverse environmental conditions. Due to the hydrophobin coating, aerial hyphae are repellent towards water (Fig. 2). Within mushrooms, hydrophobin films coat the air channels and prevent them from filling with water. In total, four different hydrophobins have been described in *S. commune* (SC1, SC3, SC4 and SC6). SC3, the best characterized hydrophobin, is expressed in the vegetative mycelium of

monokaryons and dikaryons. The other three, SC1, SC4 and SC6 are dikaryon specific. SC4 is low expressed in the mycelium and well in mushroom tissues. SC1 and SC6 are specific to fruiting stages (Wösten 2001, Walser et al. 2003).

It is not known whether hydrophobins participate also in wood colonization and pathogenicity of *S. commune* and in degradation of lignin as a hydrophobic component (see also discussion by Wösten et al. 1994). Is it necessary that the hyphae have a

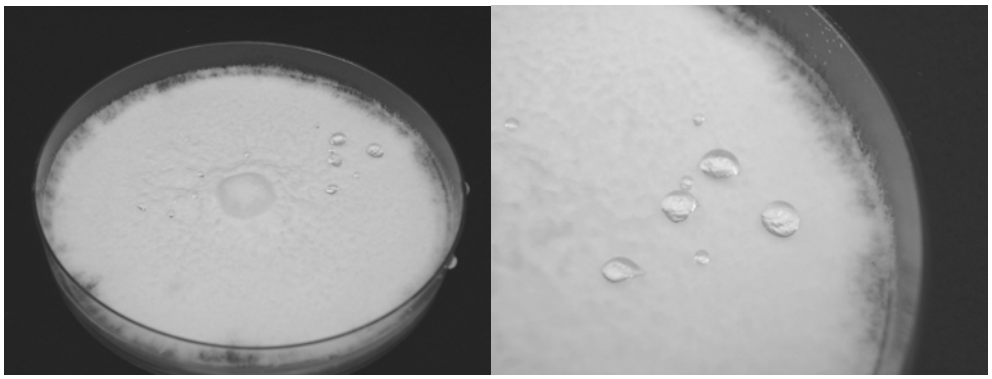

Figure 2. Aerial mycelium of *S. commune* monokaryon 4-39 is water-repellent due to the amphipathic SC3 film covering the surfaces of the hyphae.

hydrophobic surface when growing in wood? Hyphae of the species have been shown to grow within the lumen of tracheids and vessels and attack the wood by loosening the S_3 layer from the rest of the wood cell walls before localized dissolution of cell wall substance results in narrow slits within the S_2 layer. Pronounced lamellation of the S_2 layer occurs in later stages of degradation and only then hyphae were found in the slits of the S_2 layer (Nilsson and Daniel 1983).

Another model fungus for studying development in basidiomycetes is the dung fungus *Coprinopsis cinerea* (Kües 2000, Kües et al. 2002, 2004). So far, only one hydrophobin (CoH1; Fig. 3) has been described in this species. CoH1 is expressed in the vegetative mycelium of monokaryons and, less efficient, of dikaryons. Although shorter in length than the *S. commune* hydrophobin SC3, the hydropathy plots of the two proteins are very similar (Fig. 4). A gene for another potential hydrophobin (CoH2) had been detected by sequencing on a 10 kb DNA fragment carrying gene *coH1*

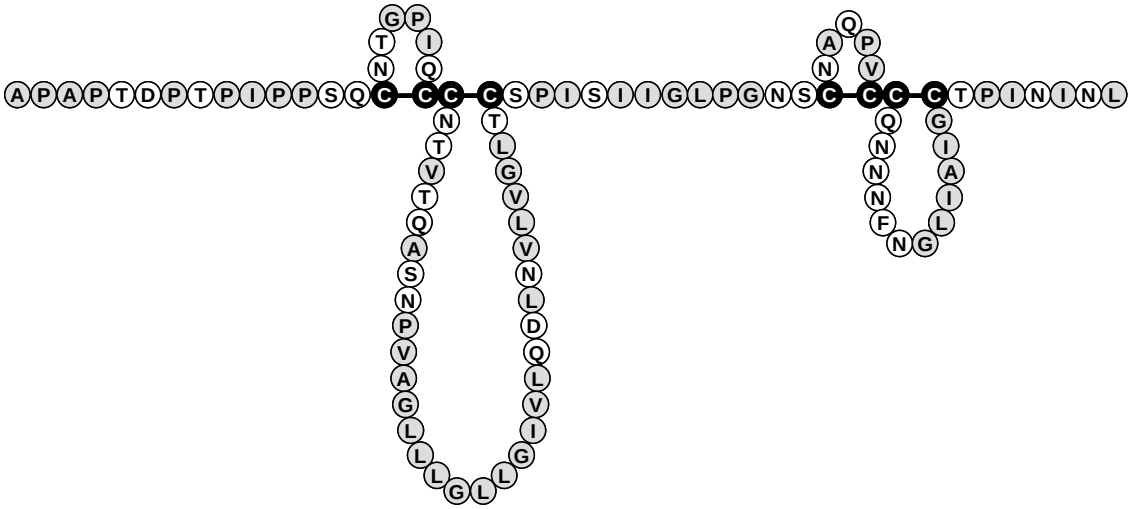

Figure 3. Model of *C. cinerea* hydrophobin CoH1 analogous to a *S. commune* SC3 model presented by Wessels (2000). The eight conserved cysteines (shown as white letters in filled black circles) are thought to interact to give the typical hydrophobin folding. Open circles with black letters indicate hydrophilic and filled grey circles with black letters hydrophobic residues.

(Ásgeirsdóttir et al. 1997). Studies in our lab aim at further understanding roles of *S. commune* and *C. cinerea* hydrophobins in growth and development.

6.3 Material and methods

6.3.1 Strains, culture conditions and light and FTIR microscopy**.**

S. commune monokaryon 4-39 (*A41*, *B41*) was kindly supplied by Han A.B. Wösten. To infest wood, the strain was grown at 25°C in light on minimal medium (Dons et al. 1979). Sterilized beech wood blocks (3 x 1 x 0.5 cm) were placed onto 1 mm thick stainless steal grids layed on established mycelium to avoid direct contact between the wood and the agar. Wood blocks were incubated with the fungus for 20 weeks before sections of 25 µm thickness were prepared with a microtome. Sections were transferred onto gelatin-coated glass slides, stained for 10 min with lactophenol blue (Esser 2001), dried at 60°C and cleaned with water. Coverslips were fixed on the samples with Depex (Serva Electrophoresis, GmbH, Heidelberg) and dried overnight with a weight placed on top of the cover slip. Samples were analyzed with a Zeiss Axiophot photomicroscope equipped with a Soft Imaging Color View II digital camera. For FTIR microscopy, mycelium grown on the surface of the wood blocks was air-dried. An FTIR spectrometer Equinox 55 in combination with an IR microscope Hyperion 3000 (Bruker

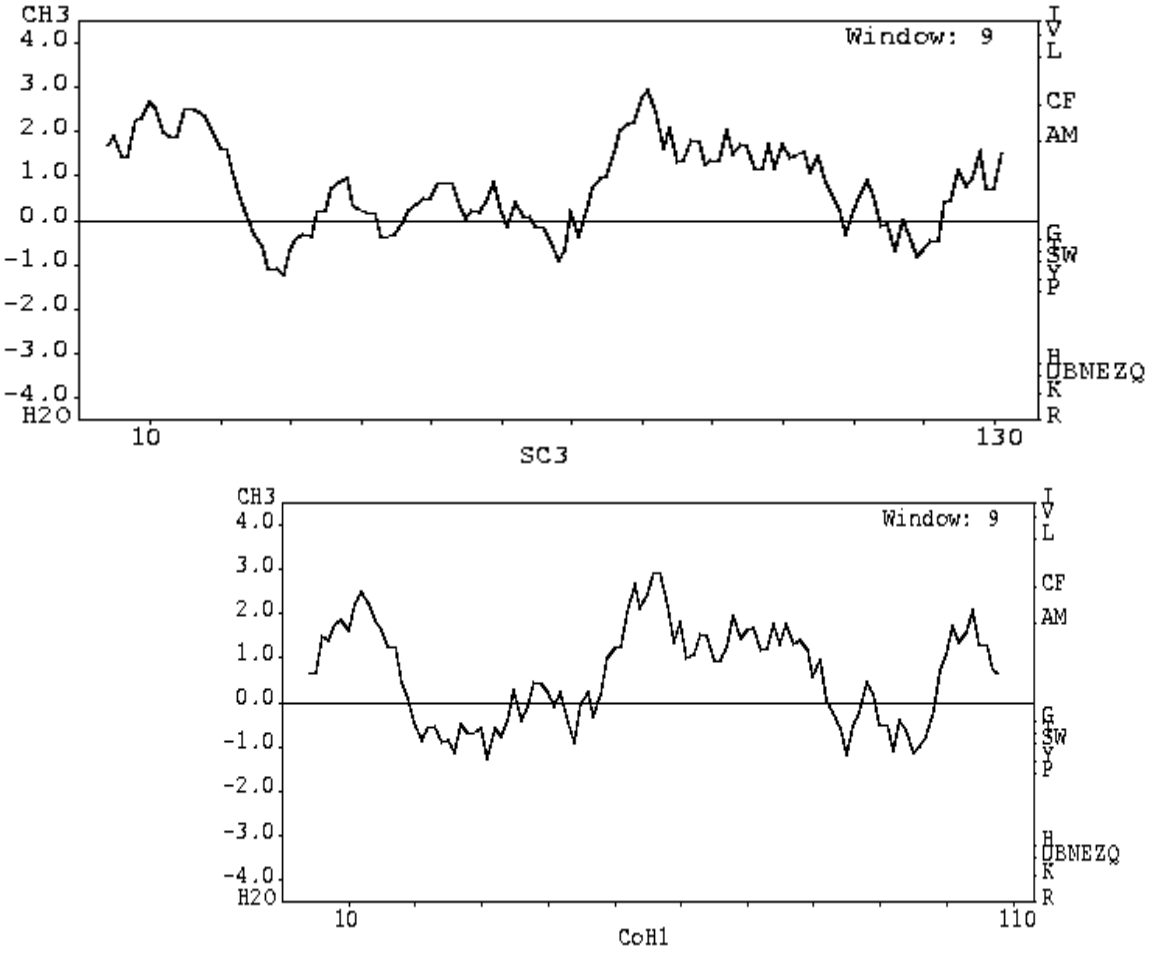

Figure 4. Hydropathy plots for *S. commune* hydrophobin SC3 (top) and *C. cinerea* hydrophobin CoH1 (bottom) calculated according to Kyte and Doolittle (1982).

Optics, Ettlingen, Germany) with a single channel MCT detector was used to record FTIR spectra of the mycelium on KBr windows (2 mm) in transmission mode with a 15 x Cassegrain-objective, knife edge aperture of 20 x 45 µm, resolution of 4 cm^{-1}, 16 scans (Naumann et al. 2005).

C. cinerea strain AmutBmut is a self-compatible homokaryon that forms fruiting bodies without prior mating to another strain (Boulianne et al. 2000) under standard fruiting conditions (Granado et al. 1997). Primordia and fruiting bodies were harvested for hydrophobin isolation following the protocol of Ásgeirsdóttir et al (1998). Isolated proteins were separated by SDS-PAGE (Garffin 1990).

6.4 Results and discussion

6.4.1 Growth of *Schizophyllum commune* strain 4-39 in beech wood.

In sections of beech wood incubated for 20 weeks with *S. commune* strain 4-39, lactophenol-stained fungal hyphae were visible within vessels (Fig. 5). Signs for attack of the wood by the fungus were not obvious from optical inspection of the samples. Spectra recorded by FTIR microscopy distinguish wood and mycelium from each other (Naumann et al. 2005). Spectra from mycelium grown within vessels are similar to each other (results not shown). Likewise, spectra from mycelium grown on the wood surface are similar to each other (for an example, see Fig. 6).

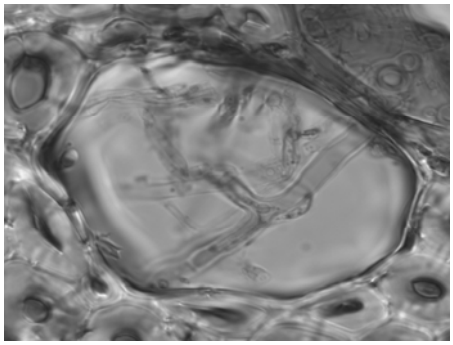

Figure 5. Lactophenol blue-stained hyphae of *Schizophyllum commune* strain 4-39 in a beech wood vessel.

Wood and mycelium grown in and on the beech wood in unstained sections was analyzed by Fourier transform infrared microscopy (FTIR) microscopy (Naumann et al. 2005). FTIR microscopy allows local resolution of the chemical composition of a sample. The absorption of infrared light by dipolar molecular bonds of polysaccharides, proteins, lipids, aromatic and many other compounds results in a typical absorption spectrum for the sample (Fig. 6).

The two groups of spectra relate to each other. However, compared to mycelium within vessels, spectra of surface mycelium showed a more pronounced peak at 1640 cm^{-1} (Naumann et al. 2005). This wave number corresponds to the amide I band of peptide bonds (de Vocht et al. 1998). Purified SC3 hydrophobin, the most abundant protein on the aerial hyphae of *S. commune* 4-39 strain (Wessels et al. 1991, de Vries et al. 1993), has a characteristic peak at this wave number (de Vocht et al. 1998).

Next to wildtype strains, there exist *SC3* knockout strains of *S. commune* (van Wetter et al. 1996) that will be very useful in analyzing whether the fungus needs

hydrophobins for growth in wood. By comparison, these mutants will be useful to establish in the FTIR spectra of *S. commune* wildtype strains specific peaks corresponding to hydrophobins. Knowing this, it might be possible to deduce whether detected differences in spectra of mycelium grown in and on wood result from differences in hydrophobin expression.

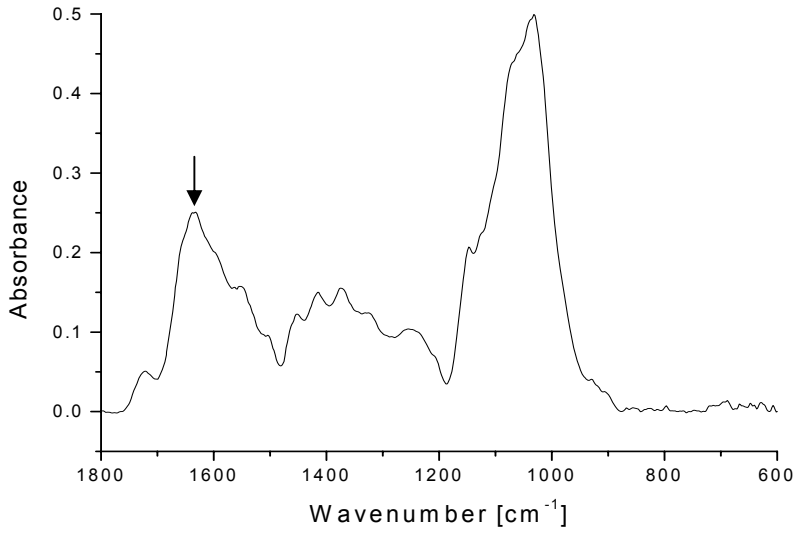

Figure 6. FTIR spectrum of mycelium of *Schizophyllum commune* grown on the surface of wood blocks, recorded with an FTIR microscope with MCT single channel detector. The spectrum has been baseline corrected. The arrow points to 1640 cm^{-1} where hydrophobin SC3 has a characteristic peak.

6.4.2 Hydrophobins and hydrophobin genes in Coprinopsis cinerea.

Hydrophobins in *S. commune* and other basidiomycetes have important structural functions in mushroom development (Wösten 2001, Walser et al. 2003). Since in *C. cinerea* nothing was known on hydrophobins in mushroom development, by standard protocols (Ásgeirsdóttir et al. 1998) we tried to isolate such proteins from young fruiting bodies from homokaryon AmutBmut (stages of rapid stipe elongation and basidiospore pigmentation). However, we were unable to solubilise hydrophobins isolated from such late stages in fruiting body development. Such probes were contaminated with melanin that probably was produced for spore blackening in the mushroom maturation process. Since not properly been dissolved, on SDS gels such hydrophobin samples caused smear (Fig. 7). In contrast, we could solubilize hydrophobins isolated from earlier

stages of primordia development prior to spore formation and separate them on SDS gels (Fig. 8). Multiple hydrophobin bands were visible ranging in sizes from 10 to 15 kDa, which is the typical size for this class of proteins (Wösten 2001, Walser et al. 2003) with the only exception of *S. commune* SC3 that is a 24 kDa protein (Wösten et al. 1993).

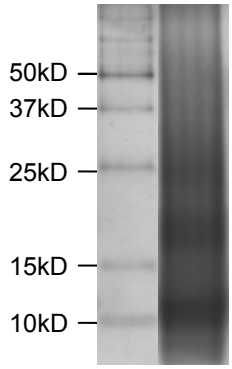

Figure 7. 15% SDS-PAGE showing poorly dissolved hydrophobins from young fruiting bodies of *Coprinopsis cinerea* homokaryon AmutBmut in a dark smear (right lane). At the left, molecular size marker.

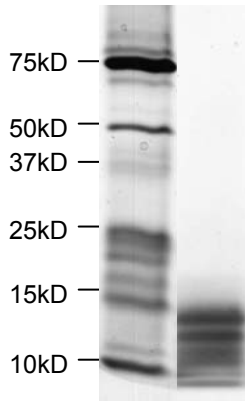

Figure 8. 15% SDS-PAGE showing well dissolved and several well separated hydrophobins from primordia of *Coprinopsis cinerea* homokaryon AmutBmut (right lane). At the left, molecular size marker.

With the release of the genomic sequence of *C. cinerea* strain Okayama 7 by the Broad Institute (http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/), we were able to Blast search the genome for hydrophobin genes and found the amazing total number of 34 different genes. All potential gene products contain the eight conserved cysteines involved in protein folding (Fig. 3) and their hydropathy plots resemble those of *S. commune* SC3 and *C. cinerea* CoH1 (Fig. 4). Expression studies of the different *C. cinerea* genes will have to verify whether they are all functional or whether there are pseudogenes amongst them. The protein gel shown in Fig. 8 supports that many of them will be functional.

The situation of multiple genes as in *C. cinerea* appears not be uncommon in higher basidiomycetes since in the genome of the wood-rotting fungus *Phanerochaete*

chrysosporium (Martinez et al. 2004), we found 20 different hydrophobin genes by Blast searches. Whilst four different hydrophobin genes are currently known in *S. commune* (Wessels et al. 1995), also this fungus may have many more than is so far evident from experimental work.

6.5 Conclusions

Basidiomycetes can have many different hydrophobins. Work in *S. commune* showed before that hydrophobins are differently expressed and have been evolved for different development functions (Wösten 2001, Walser et al. 2003). So far, it is not known whether hydrophobins are needed for growth in and degradation of lignocellulosic substrates such like wood and straw. In *C. cinerea*, we found the amazing number of 34 different hydrophobin genes and it will be a demanding task to determine all their functions in growth and development.

6.6 Acknowledgements.

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Oral presentations

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2005 Extracellular matrix proteins in basidiomycete fruiting body development.
University of Zurich, Division of Psychiatry Research, on 14th November, Zurich, Switzerland

2005 Multiple hydrophobin genes in mushrooms - what for?
Basidio2005, Warwick-HRI, on 15th April Wellesbourne (University of Warwick) -UK

2004 Extracellular matrix proteins in mushroom development.
Göttingen Centre for Molecular Biosciences (GZMB), on 16th June, University of Göttingen, Germany

Conference contributions (Posters)

2005 **Velagapudi R**, Peddireddi S, Hoegger PJ, Majcherczyk A, Naumann A, Olbrich A, Polle A, Kües U. The mushroom *Coprinopsis cinerea* has multiple class I hydrophobins but no class II hydrophobins. 7th VAAM-Symposium Molecular Biology of Fungi, 4-6 September 2005, Bochum, Germany

Abstract: 7th VAAM Symposium 2005, Bochum, Program and Abstract Book: Molecular Biology of Fungi (Kück, U., Pöggeler, S., Nouwrousian, M., Hoff, B., Eds), Bochumer Universitätsverlag, Europäischer Universitätsverlag GmbH (ISBN 3-89966-018-8)

Peddireddi S, **Velagapudi R**, Hoegger PJ, Majcherczyk A, Naumann A, Olbrich A, Polle A, Kües U. Multiple hydrophobin genes in mushrooms. Genetics and cellular Biology of Basidiomycetes VI. Pamplona, 3-6 June, 2005, Spain

Abstract: congress VI Genetics and Cellular Biology of Basidiomycetes, Pamplona 3 - 6 June, Part II Posters (AG Pisabarro, ed.)

Velagapudi R, Kilaru S, Pemmasani JK, Kaur K, Hoegger PJ, Kües U. Spatial and temporal expression of laccase in *Coprinopsis cinerea* using galectin promoters. Genetics and cellular Biology of Basidiomycetes VI. Pamplona, 3-6 June, 2005, Spain

Abstract: congress VI Genetics and Cellular Biology of Basidiomycetes, Pamplona 3 - 6 June, Part II Posters (AG Pisabarro, ed.)

Velagapudi R, Hoegger PJ, Majcherczyk A, Kües U. How many hydrophobins does a mushroom need? Basidio 2005, Warwick-HRI, Wellesbourne (University of Warwick) on Friday 15th April 2005, UK

Abstract: in conference book: Basidio 2005 Conference on the biology of Basidiomycete fungi. A forum for the UK Basidiomycetes research community, 15 April, Warwick HRI, University of Warwick

Velagapudi R, Kilaru S, Pemmasani JK, Kaur K, Hoegger P, Kües U. Spatial and temporal expression of laccase in *Coprinopsis cinerea* using galectin promoters. Basidio 2005, Warwick-HRI, Wellesbourne (University of Warwick) on Friday 15th April 2005, UK

Abstract: in conference book: Basidio 2005 Conference on the biology of Basidiomycete fungi. A forum for the UK Basidiomycetes research community, 15 April, Warwick HRI, University of Warwick

Velagapudi R, Peddireddi S, Hoegger PJ, Majcherczyk A, Polle A, Kües U. How many hydrophobins does a mushroom need? Twenty Third Fungal Genetics Conference, 15-20 March, 2005, Asilomar Conference Center, Pacific Grove, California, USA

Abstract: Fungal Genetics Newsletter 52

Pemmasani JK, **Velagapudi R**, Kilaru S, Kaur K, Hoegger P, Kües U. Spatial and temporal expression of laccase in *Coprinopsis cinerea* using galectin promoters. Twenty Third Fungal Genetics Conference 15-20 March, 2005, Asilomar Conference Center, Pacific Grove, California, USA

Abstract: Fungal Genetics Newsletter 52

2004 **Velagapudi R**, Peddireddi S, Pemmasani JK, Majcherczyk A, Hoegger P, Naumann A, Polle A, Kües U. Hydrophobins in white rot fungi. Zukunftsweisende Umweltforschung Stiftungslehrstühle der DBU stellen sich vor, 6-7 December 2004, Zentrum für Umweltkommunikation der DBU- Osnabrück, Germany

Abstract: Abstractsammlung: Zukunftsweisende Umweltforschung - Stiftungslehrstühle der DBU stellen sich vor

Kilaru S, Pemmasani JK, Kaur K, Saathoff A, Schwedhelm N, Lange K, **Velagapudi R**, Krothapalli RSS, Hoegger PJ, Kües U. Promoter screening for high laccase expression in the basidiomycete *Coprinopsis cinerea* Zukunftsweisende Umweltforschung Stiftungslehrstühle der DBU stellen sich vor, 6-7 December 2004, Zentrum für Umweltkommunikation der DBU- Osnabrück, Germany

Abstract: Abstractsammlung: Zukunftsweisende Umweltforschung - Stiftungslehrstühle der DBU stellen sich vor

Kilaru S, Pemmasani JK, Kaur K, Schwedhelm N, **Velagapudi R**, Krothapalli RSS, Hoegger PJ, Kües U. Promoter screening for high laccase expression in the basidiomycete *Coprinopsis cinerea*. VAAM Workshop, Stoffwechsel der Pilze, Fachhochschule Lausitz 16-18 September 2004, Senftenberg, Germany

Abstract: Tagungsband VAAM-Workshop Stoffwechsel der Pilze, Fachhochschule Lausitz 16 - 18 September 2004, Organizer: Fachbereich Bio-, Chemie und Verfahrenstechnik, Lausitzer Technologie Transferstelle, Lausitzer Biotech-Initiative zur Förderung der regionalen Biotechnologie

Velagapudi R, Peddireddi S, Kilaru S, Hoegger P, Majcherczyk A, Polle A, Kües U. Hydrophobin genes in higher basidiomycetes. VAAM Workshop, Stoffwechsel der Pilze, Fachhochschule Lausitz 16-18 September 2004, Senftenberg, Germany

Abstract: Tagungsband VAAM-Workshop Stoffwechsel der Pilze, Fachhochschule Lausitz 16 - 18 September 2004, Organizer: Fachbereich Bio-, Chemie und Verfahrenstechnik, Lausitzer Technologie Transferstelle, Lausitzer Biotech-Initiative zur Förderung der regionalen Biotechnologie

Velagapudi R, Peddireddi S, Kilaru S, Hoegger P, Majcherczyk A, Polle A, Kües U. Hydrophobin genes in higher basidiomycetes. 2nd International *Ustilago maydis* Meeting, 25-29 August 2004, Rauischholzhausen, Germany

Abstarct: in conference book - Poster Session II Abstracts

Velagapudi R, Kilaru S, Hoegger PJ, Dwivedi RC, Srivilai P, Peddireddi S, Navarro-González M, Pemmasani JK, Zomorrodi M, Lange K, Majcherczyk A, Kües U. Multi-gene families in the basidiomycete *Coprinopsis cinerea*. Gordon Research Conferences, 20-25 June 2004, Plymouth, USA

Abstract: Fungal Genetics Newsletter 50

Peddireddi S, Pemmasani JK, **Velagapudi R**, Majcherczyk A, Polle A, Kües U. Hydrophobins in white rot fungi. VAAM-2004, Sonderausgabe, 28-31 March, 2004, Braunschweig, Germany

Abstract: in Biospektrum Sonderausgabe 2004

2003 **Velagapudi R**, Walser PJ, Majcherczyk A, Aebi M, Kües U. Galectins and hydrophobins, extracellular matrix proteins in fruiting body development of the basidiomycete *C. cinereus*. Molecular biology of fungi 6th VAAM-Conference ''Molekularbiologie der Pilze'' 3-5 September 2003, Göttingen, Germany

Abstract: Conference book: Molecular biology of fungi. 6th VAAM-Conference "Molekularbiologie der Pilze", 3-5 September 2003, Göttingen, Germany, Wissenschaftlicher Fachverlag Dr. Peter Fleck, Langgöns (Niederkleen), Germany

Göbel S, Clergeot PH, Ruprich-Robert G, Liu Y, Loos S, Srivilai P, **Velagapudi R**, Künzler M, Aebi M, Kües U. Mutants in initiation of fruiting body development of the basidiomycete *Coprinus cinereus*. Molecular biology of fungi 6th VAAM-Conference ''Molekularbiologie der Pilze'' 3-5 September 2003 Göttingen, Germany

Abstract: Conference book: Molecular biology of fungi. 6th VAAM-Conference "Molekularbiologie der Pilze", 3 - 5 September 2003, Göttingen, Germany, Wissenschaftlicher Fachverlag Dr. Peter Fleck, Langgöns (Niederkleen), Germany

Clergeot PH, Ruprich-Robert G, Liu Y, Loos S, Srivilai P, **Velagapudi R**, Göbel S, Künzler M, Aebi M, Kües U. Mutants in initiation of fruiting body development of the basidiomycete *Coprinus cinereus*. 22nd Fungal Genetics Conference. 18-23 March, 2003, Pacific Grove, California, USA.

Abstract: Fungal Genetics Newsletter 50.

Conference contributions (abstracts to oral presentations of others)

2005 Kües U*, Pukkila P, Stajich J, Dietrich F, Gathman A, Kilaru S, Hoegger PJ, James TY, Lilly W, **Velagapudi R**, Chaisaena W, Dwivedi RC, Holzwarth F, Majcherczyk A, Navarro-Gonzalez M, Peddireddi S, Pemmasani KJ, Rühl M, Saathoff A, Srivilai P (2005) A mushroom in the genomic era. VAAM-Tagung: Molekularbiologie der Pilze, 4 -7 September, Bochum, Germany (* speaker)

Abstract: 7th VAAM Symposium 2005, Bochum, Program and Abstract Book: Molecular Biology of Fungi (Kück U, Pöggeler S, Nouwrousian M, Hoff B, eds.), Bochumer Universitätsverlag, Europäischer Universitätsverlag GmbH (ISBN 3-89966-018-8)