Regulation of mushroom formation in *Schizophyllum commune*

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Front cover:
Fruiting bodies (mushrooms) of *Schizophyllum commune*, seen from below

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Regulation of mushroom formation in *Schizophyllum commune*

Regulatie van paddenstoelvorming in
Schizophyllum commune
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 22 december 2010 des middags te 4.15 uur

door

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Chapter 1

General introduction

MUSHROOMS

The kingdom of the fungi is a morphologically and physiologically diverse group of eukaryotes. Morphologically, the fungal kingdom can be divided into (generally unicellular) yeasts and (generally multi-cellular) filamentous fungi. Filamentous fungi form mycelia that consist of a network of branching hyphae. These hyphae grow by apical extension. After a period of vegetative growth, filamentous fungi produce asexual and/or sexual spores, which are often produced by specialized structures. The mushroom fruiting body is the most conspicuous form of these structures and is found primarily in the phylum Basidiomycota. Approximately 14,000 mushroom-forming species have been described. Many of these fungi are ectomycorrhizae (Chang, 2004). They form a mutually beneficial symbiosis with roots of plants. For instance, the fly agaric (*Amanita muscaria*) establishes a symbiosis with various deciduous and coniferous trees including pine, birch, beach, and oak. Mushroom-forming fungi can also be pathogens of plants and animals. For instance, the honey mushroom (*Armillaria bulbosa*), which is among the largest and oldest living organisms (Smith *et al.*, 1992), is a pathogen of hardwood and conifer trees. Apart from forming a pathogenic or mutual beneficial symbiosis, mushroom-forming fungi can live as saprotrophs by degrading dead organic material. The inky cap mushroom (*Coprinopsis cinerea*, formerly *Coprinus cinereus*), the turkey tail (*Trametes versicolor*), the split gill (*Schizophyllum commune*) and many other mushrooms (see below) are examples of such fungi.

The main economic value of mushrooms is their use as food (Kües & Liu, 2000; Kothe, 2001). Notable examples of edible mushrooms are the white button mushroom (*Agaricus bisporus*), the oyster mushroom (*Pleurotus ostreatus*), shiitake (*Lentinula edodes*), enokitake (*Flammulina velutipes*) and chanterelle (*Cantharellus cibarius*). Mushroom-growing has a tradition of many centuries, especially in South-East Asia and Europe, yet few species can be cultured commercially. The white button mushroom, the oyster mushroom, shiitake and enokitake are examples of such mushrooms. The world-wide production of edible mushrooms amounts approximately 2.5 million tons annually. Apart from being a food source, fruiting bodies produce anti-tumor and immuno-stimulatory molecules (Kües & Liu, 2000; Kothe, 2001) and enzymes that can be used for bioconversions (Lomascolo *et al.*, 1999). Recently, mushrooms have been identified as promising cell factories for the production of pharmaceutical proteins (Berends *et al.*, 2009).

Despite their economical interest, relatively little is known about how mushrooms are formed. Many mushroom-forming fungi cannot be cultured in

the lab nor genetically modified, which makes studying these organisms on the molecular level challenging. Notable exceptions are *S. commune* and *C. cinerea*. These basidiomycetes are used as models systems to study the mechanisms behind mushroom development (Kües, 2000; Wösten & Wessels, 2006). Yet, the molecular amenability of these fungi is still limiting these studies. For instance, genes cannot be inactivated by homologous recombination in *C. cinerea*, whereas the efficiency in *S. commune* is low.

SCHIZOPHYLLUM COMMUNE

The mushrooms of *S. commune* are used as a traditional food source in Africa and Asia. *S. commune* is characterized by its split gills, hence the genus name *Schizo-phyllum*. It can be found on all continents except Antarctica, which explains the species name *commune*. *S. commune* has occasionally been reported as an opportunistic pathogen of humans (Kern & Uecker, 1986) and living trees (Oprea *et al.*, 1995). It is generally found as a saprobic fungus on fallen branches and logs of hardwood (*e.g.* birch, beech and oak). At least 150 genera of woody plants have been shown to be substrates for *S. commune*, but it also colonizes softwood and grass silage (O'Brien *et al.*, 2005; de Jong, 2006). *S. commune* is a white rot fungus (Schmidt & Liese, 1980) because it not only degrades (hemi)cellulose (like the brown rotters) but also lignin.

S. commune completes its life cycle in approximately 10 days. Its basidiospores germinate to form a sterile mycelium. The hyphae within this network are compartmentalized by porous septa (van Peer *et al.*, 2010). The compartments contain one nucleus (*i.e.* it is a monokaryotic mycelium) and all nuclei within the mycelium are identical (*i.e.* it is a homokaryotic mycelium). Initially, the mycelium grows submerged, but after a certain biomass has been reached, aerial hyphae are formed (Wösten & Wessels, 2006). These aerial hyphae have a role in the colonization of new substrates (de Jong, 2006). Two monokaryons can fuse to form a dikaryon. This dikaryon is fertile when the alleles of the *matA* and *matB* mating type loci of the fusing partners differ. This dikaryon can form fruiting bodies under appropriate environmental conditions (see below) providing that a feeding substrate mycelium has been formed. The first stage of mushroom development is the aggregation of aerial hyphae into aggregates (Figure 1A). These aggregates develop into primordia (Figure 1B) and subsequently into mature fruiting bodies (Figure 1C and 1D). Nuclear fusion (karyogamy) and meiosis take place in the basidia within the mature fruiting body. The resulting basidiospores can give rise to new monokaryotic mycelia (Leonard & Dick, 1968; Wösten & Wessels, 2006).

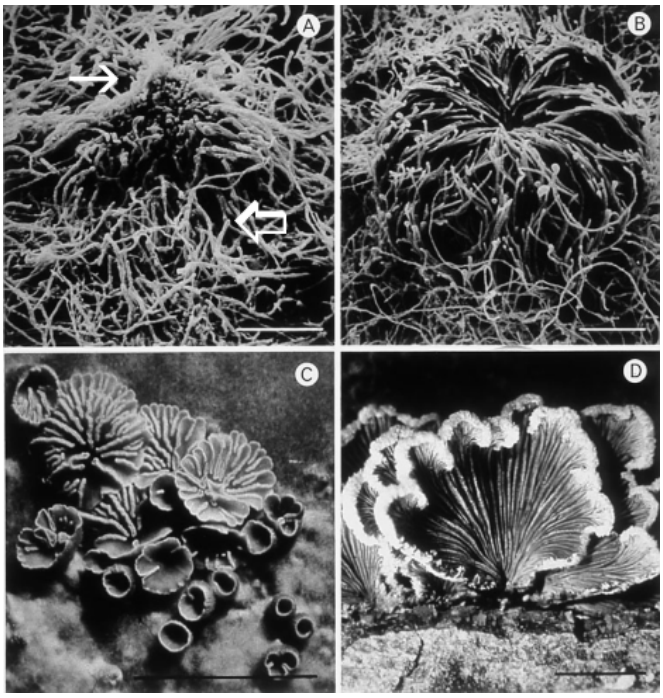

Figure 1. Fruiting body development in *S. commune*. Monokaryons and dikaryons form aerial hyphae (thick arrow) after a vegetative substrate mycelium has been formed (**A**). In the dikaryon, but not in the monokaryon, these aerial hyphae can aggregate (thin arrow) (**A**) and further develop into a primordium (**B**). The primordium develops into a simple cup with the hymenium lining the entire inner surface (**C**). The split gills arise by marginal profileration of the cup (**D**) (Taken from Wessels, 1993).

The relatively fast life cycle of *S. commune* and the fact that it can be grown on synthetic media make this basidiomycete an ideal model system to study mushroom development. Moreover, several molecular tools have been developed for this organism. It can be transformed using auxotrophic markers (Munoz-Rivas *et al.*, 1986; Kothe *et al.*, 1993) or one of the three available antibiotic resistance markers (*i.e* phleomycin (Schuren & Wessels, 1994), hygromycin (Mooibroek *et al.*, 1990; Scholtmeijer *et al.*, 2001) and nourseothricin (van Peer *et al.*, 2009)). Genes can be inactivated by homologous recombination (Marion *et al.*, 1996; Robertson *et al.*, 1996; van Wetter *et al.*, 1996; Horton *et al.*, 1999; Lengeler & Kothe, 1999a; Lengeler & Kothe, 1999b; van Wetter *et al.*, 2000a; Lugones *et al.*, 2004; Schubert *et al.*, 2006), although the efficiency is generally low. RNAi has been shown to be an alternative for gene inactivation by homologous recombination (de Jong *et al.*, 2006), but this does not seem to be the case for every gene (Lugones, unpublished results).

REGULATION OF MUSHROOM FORMATION IN *S. COMMUNE*

Formation of a fertile dikaryon is regulated by the *matA* and *matB* mating type loci. Proteins encoded in these loci activate signaling cascades and regulate genes encoding transcription factors. These transcription factors

regulate target genes which encode proteins that fulfill enzymatic or structural functions. The activity of the mating type loci combined with the sensing of environmental signals results in the formation of fruiting bodies. Here, I focus on environmental signals, the mating type genes, transcription factors, other regulatory genes involved in mushroom development, as well as their downstream target genes.

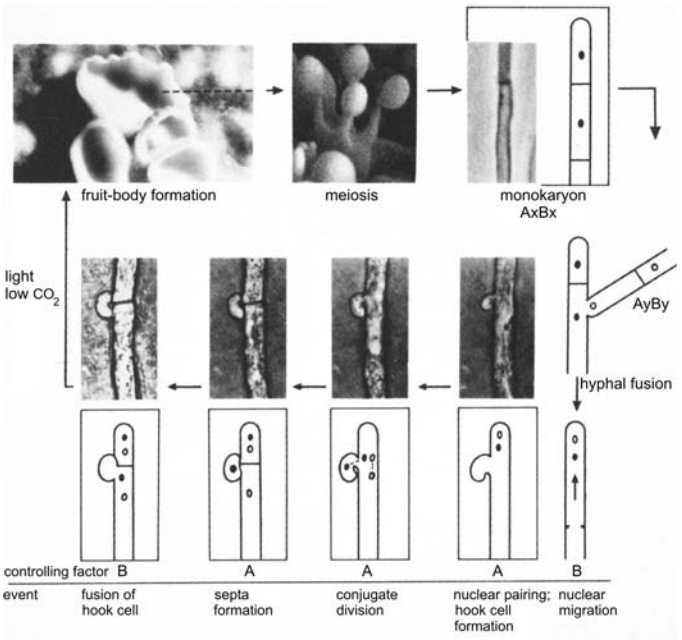

Figure 2. The life cycle of *S. commune*. Basidiospores germinate and form a monokaryotic mycelium (*top right*). A fertile dikaryon is established when monokaryons with compatible *mat*A and *mat*B mating types fuse. The mating type locus that controls each of the steps in this process is indicated. Fruiting bodies are formed under the appropriate conditions. In the hymenium of the fruiting body basidia are formed, in which meiosis takes place. This results in four basidiospores (adapted from Ásgeirsdóttir, 1994; de Jong, 2006).

Environmental signals

The environmental signals that promote mushroom development are generally species-specific. Light is an important factor for both *S. commune* and *C. cinerea*. In *C. cinerea* light/dark cycles are required for mushroom development and at least 5 light-sensitive phases can be distinguished (Kües, 2000; Lu, 2000). For example, one stage precedes the formation of initials, another the maturation of primordia. Blue light was shown to activate mushroom development in *C. cinerea* via a blue light receptor encoded by *dst1* (Terashima *et al.*, 2005) (see below). In *S. commune*, a short exposure to light is sufficient to induce the formation of primordia (Perkins, 1969) and thereby the formation of mature fruiting bodies. It was shown that light in the blue part of the spectrum is most effective in inducing mushroom development in *S. commune* (Perkins & Gordon, 1969). This suggests that a

blue light receptor is involved in mushroom development as is the case in *C. cinerea*.

High concentrations of carbon dioxide are inhibitory for mushroom initiation in *S. commune* (Niederpruem, 1963). For instance, mushroom formation is blocked by sealing culture plates or by growing in air with increased levels (5%) of carbon dioxide (de Jong, unpublished results). The optimum temperature for mushroom development in *S. commune* is around 25 °C, higher temperatures are inhibitory (Wessels, 1965).

Mating type genes

The mating type genes are the master regulators of sexual development in fungi. In *S. commune*, mushrooms generally do not develop in monokaryotic cultures. Monokaryons first have to fuse with a partner that harbors compatible mating type loci. The process of establishment of a fertile dikaryon is regulated by the genes on the mating type loci *matA* and *matB* and follows several discrete steps (Figure 2). First, a nucleus enters the acceptor mycelium at the site of hyphal fusion. This nucleus migrates to the apex of the hypha, which is enabled by dissolution of the septa that separate the compartments. At the hyphal apex the nuclei of both partners pair and divide synchronously. This process is facilitated by the formation of a hook cell (at the site of the future septum). One nucleus divides in the longitudinal axis of the hypha at the position where the new septum will be formed, whereas the other nucleus divides within the plane of the hypha and the hook cell. Formation of the septum (which is resistant to the lytic enzymes that dissolve the septa during nuclear migration) is followed by fusion of the hook cell with the underlying compartment. As a result, the apical and subapical compartments both have a set of compatible nuclei (for a review see Raudaskoski & Kothe, 2010). Nuclear migration and fusion of the hook cell are regulated by the *matB* mating type locus, whereas the *matA* locus regulates nuclear pairing, hook cell formation, conjugate nuclear division and septa formation.

The molecular structure of the mating type system of *S. commune* is tetrapolar. The *matA* and *matB* locus both consist of an α and a β sub-locus. Nine and thirty two alellic specificities are assumed to exist in nature for *Aα* and *Aß*, respectively. Nine allelic specificities have also been identified for both *Bα* and *Bβ* (Raper, 1966; Koltin *et al.*, 1967). A fertile dikaryon is formed when at least one of the *matA* and one of the *matB* subloci is different. Thus, more than 23000 different compatible interactions can occur in nature.

The *matA* locus has been well studied in *C. cinerea.* Its subloci contain gene pairs encoding homeodomain transcription factors of the HD1 and HD2

family. HD1 binds weakly to DNA and it has a nuclear localization signal and an activation domain. HD2 shows higher binding affinity to DNA but has no nuclear localization signal or activation domain. When monokaryons fuse that contain different allelic versions of at least one of the subloci, a heterodimer of HD1 and HD2 forms between allelic versions (*i.e.* from different nuclei) of a gene pair via dimerization domains that are located in the N-terminal region of the proteins. These heterodimers are not formed between pairs within the same nucleus, or between allelic versions of different pairs. The heterodimer is a transcription factor that activates the target genes of the *A* mating type pathway (Kües *et al.*, 1992; Kües *et al.*, 1994; Asante-Owusu *et al.*, 1996; Pardo *et al.*, 1996; Spit *et al.*, 1998; Raudaskoski & Kothe, 2010). The *matA* locus of *S. commune* has been less well studied. So far, three genes have been identified on the *matA* locus. The genes encoding Y and Z (HD2 and HD1, respectively) are located on the Aα sub-locus and the gene encoding V (HD2) is situated on the Aβ sub-locus. Y and Z form a pair, but the partner of V has not yet been identified (Specht *et al.*, 1992; Stankis *et al.*, 1992; Shen *et al.*, 1996).

The genes on the *MatBα* and *MatBβ* loci encode pheromones and their receptors. Pheromones can bind to receptors encoded by a different allelic version of the same sub-locus. As a result, a signaling cascade is activated that includes heterotrimeric G-proteins and a mitogen-activated protein kinase (MAPK) cascade (Reviewed in Raudaskoski & Kothe, 2010).

Transcription factors involved in mushroom formation

Little is known about the transcription factors that are involved in mushroom formation. Apart from the homeodomain genes in de *matA* loci, few transcription factors have been linked to mushroom development. The transcription factor gene *priB* was isolated as a gene that is up-regulated during the formation of primordia in *Lentinula edodes* (Endo *et al.*, 1994). No deletion strain has been made, so the function of this transcription factor remains unknown. The role of transcription factors in mushroom formation has been better studied in *C. cinerea*. Gene *pcc1* encodes a protein with a HMG (high-mobility group) DNA binding domain. The encoded protein functions in *A* regulated development (Murata *et al.*, 1998; Murata & Kamada, 2009). Mutations in *pcc1* result in a complete program of sexual differentiation in the homokaryon independent of the mating type genes. Its exact function is unknown, however. The *exp1* gene of *C. cinerea* encodes a transcription factor and is strongly induced in the cap tissue 3 h prior to its expansion. A recessive mutation in the *exp1* gene results in a phenotype with abnormal development of the cap and absence of autolysis (Muraguchi *et al.*,

2008). The *dst1* gene of *C. cinerea* encodes a homolog of the blue light receptor WC-1 of the ascomycete *Neurospora crassa* (Terashima *et al.*, 2005). In *N. crassa*, WC-1 and WC-2 form the white collar complex (WCC), which functions as a transcription factor upon induction by light (Ballario *et al.*, 1996; Linden & Macino, 1997; Linden *et al.*, 1997; Chen *et al.*, 2009). The *dst1-1* mutation in *C. cinerea* results in a truncated Dst1 protein, the consequence of which is a blind phenotype. In the dark, the wild type forms a 'dark stipe' (the cap and stipe tissue at the upper part of the primordium remain rudimentary, while the basal part elongates). The same is observed for the *dst1-1* mutant grown in either light or darkness. The genome of *C. cinerea* also has a homolog of WC-2 (Stajich *et al.*, 2010), but its function has not yet been assessed.

Other regulators involved in mushroom development

Several other regulatory genes have been identified that are involved in mushroom development, in particular in *S. commune*. A mutation in the *thn* gene occurs spontaneously in some strains of *S. commune* (Raper & Miles, 1958; Wessels *et al.*, 1991). This is due to a transposon (*Scooter*) that integrates into the locus of this gene (Fowler & Mitton, 2000). The *thn* mutant forms a strongly reduced number of aerial hyphae, does not form mushrooms and produces a pungent smell. The *thn* gene encodes a predicted regulator of G protein signaling. It was proposed that the THN protein exerts its function by interacting with the α-subunit of a heterotrimeric G protein. In this way, THN would regulate the expression of the hydrophobin gene *sc3* in the monokaryon and of the hydrophobin genes *sc3, sc1,* and *sc4* in the dikaryon (see below) (Wessels *et al.*, 1991). Constitutively active forms of two G-protein α–subunits (ScGP-A and ScGP-C) resulted in a similar phenotype as the *thn* mutant, suggesting that they are targets of THN (Yamagishi *et al.*, 2002). Another gene involved in signaling has also been implicated in mushroom development. Inactivation of *gap1* (GTPase-activating protein for Ras) of *S. commune* resulted in an accumulation of the active form of Ras. As a result, more primordia were formed but the number of mature fruiting bodies remained similar to the wild type. Yet, gill development was abnormal or even absent. Other effects of the *gap1* deletion included a reduced growth rate in both the monokaryon and dikaryon, and the inability of hook cells to fuse with the hypha (Schubert *et al.*, 2006).

FRT1 of *S. commune* encodes a protein with a predicted RNA exonuclease domain. Dikaryotic strains in which *frt1* was deleted by homologous recombination showed no apparent phenotype. In contrast, aggregates were formed in monokaryotic strains in which *frt1* had been

deleted. This was accompanied with accumulation of RNA of dikaryon-specific genes (such as *sc1*, *sc4* and *sc7*) (Horton & Raper, 1991; Horton & Raper, 1995; Horton *et al.*, 1999). Remarkably, *frt1* induced fruiting when it was transformed to certain monokaryons of *S. commune* (Horton & Raper, 1991). This was the case when the acceptor strain had a different allele of the gene (named *frt1-2* as opposed to *frt1-1*). In dikaryons fruiting was also accelerated. How fruiting is induced by the presence of two different alleles of FRT1 is unknown, but it has been hypothesized that the proteins dimerize, relieving the pressure on dikaryon-specific genes. Alternatively, this heterodimer could activate other genes, resulting in the development of mushrooms in a monokaryon (Horton *et al.*, 1999).

Structural genes involved in mushroom development

Several classes of proteins have been implicated in mushroom development. This is mainly based on the finding that they are up-regulated during this developmental process. So far, a function in mushroom development has only been shown for the hydrophobins.

Hydrophobins

Hydrophobins have been shown to fulfill a wide spectrum of functions in filamentous fungi in general and in formation of aerial hyphae and fruiting bodies in particular (Wösten & de Vocht, 2000; de Jong, 2006). It seems that hydrophobins are present in all studied mushroom-forming fungi (de Vries *et al.*, 1993). For instance, hydrophobins and their encoding genes have been isolated from *C. cinerea* (Ásgeirsdóttir *et al.*, 1997), *P. ostreatus* (Ásgeirsdóttir *et al.*, 1998) and *A. bisporus* (Lugones *et al.*, 1998). However, the best studied hydrophobins are SC3 and SC4 of *S. commune*.

The water soluble form of SC3 is secreted into the cell wall and into the medium. SC3 in this form affects cell wall composition (van Wetter *et al.*, 2000b). The amount of water-soluble β-(1-3)-glucan with single glucose residues attached by β-(1-6)-linkages is increased considerably in a Δ*sc3* monokaryon. On the other hand, the amount of alkali-resistant β-(1-3)-glucan (*i.e.* glucan linked to chitin) is reduced. SC3 can also assemble into an amphipathic membrane at a hydrophilic-hydrophobic interface such as that between the cell wall and the air or between the moist (or aqueous) substrate and the air (Wösten *et al.*, 1993; Wösten *et al.*, 1994; Wösten *et al.*, 1999). Assembly of SC3 is accompanied with conformational changes. In the stable end form, SC3 adopts an amyloid-like structure (de Vocht *et al.*, 1998; de Vocht *et al.*, 2000; Wösten & de Vocht, 2000; Butko *et al.*, 2001; de Vocht *et al.*, 2002; Scholtmeijer *et al.*, 2009). SC3 in this state forms a mosaic of

bundles of 10 nm wide rodlets, the so-called rodlet layer. The amphipathic amyloid film of SC3 is highly surface active (van der Vegt *et al.*, 1996; Wösten *et al.*, 1999). As a result, the surface tension at the interface between the aqueous substrate and the air is strongly decreased, enabling hyphae to escape the water to grow into the air (Figure 3). Not only substrate hyphae, but also aerial hyphae secrete the SC3 hydrophobin. In this case, the hydrophobins can not diffuse into the medium but rather assemble at the interface between the cell wall and the air (Wösten *et al.*, 1994; Wösten *et al.*, 1999). The hydrophilic side of the amphipathic SC3 membrane orients itself to the cell wall, whereas the hydrophobic side is exposed to the air. As a result, aerial hyphae become hydrophobic. This prevents hyphae to grow back into the aqueous substrate or to fall back when it rains or in the presence of dew.

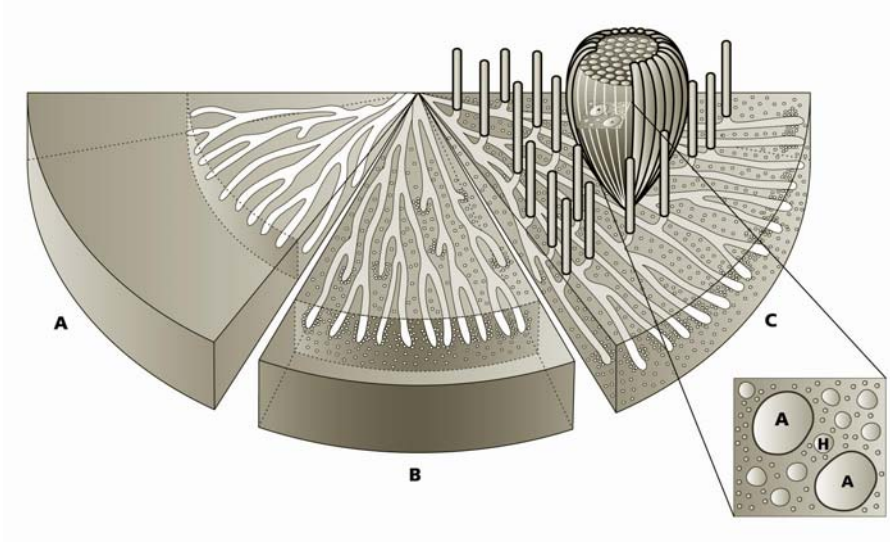

Figure 3. The role of hydrophobins in formation of aerial hyphae and fruiting bodies in a *S. commune* dikaryon (note that formation of aerial hyphae in a monokaryon follows the same mechanism). A. Hydrophobin genes are not active in young mycelia. Such mycelia grow only submerged. **B.** After a mycelium is formed that can feed aerial hyphae and fruiting bodies, the hydrophobin genes are switched on. The SC3 hydrophobin (and, to a lesser extent, the SC4 hydrophobin) is secreted into the medium in a water-soluble form (*stipples*). SC3 self-assembles at the interface between the moist substrate and the air. As a result, the water surface tension is reduced, thus enabling hyphae to breach the interface to grow into the air (**C**). Aerial hyphae continue to secrete SC3, which assembles at the interface between the cell wall and the air (hyphal walls drawn as *thick lines*). SC3 also coats the hyphae surrounding the fruiting bodies (here represented by a fruiting body whose cap has not yet expanded). In contrast, SC4 is secreted within the fruiting body tissue. Here, it coats the air channels. As a result the air channels are water repellent, thus ensuring gas exchange (*inset;* 'A' represents an air channel; 'H' represents a hypha) (Taken from Wösten & Wessels, 2006).

The Δ*sc3* monokaryon and the Δ*sc3*Δ*sc3* dikaryon form few aerial hyphae (van Wetter *et al.*, 1996; van Wetter *et al.*, 2000a). Formation of fruiting bodies is not affected in the Δ*sc3*Δ*sc3* dikaryon. However, the surfaces of the aerial hyphae and the fruiting bodies are wettable. This can be explained by the finding that SC3 is expressed by both the aerial hyphae and the hyphae at the surface of fruiting bodies.

Formation of aerial hyphae can be further decreased by inactivation of the *sc4* hydrophobin gene (van Wetter *et al.*, 2000a) or the hydrophobin-like *sc15* gene (Lugones *et al.*, 2004). Apparently, the proteins encoded by these genes can partly take over the function of the SC3 hydrophobin. Fruiting body formation was not affected in the Δ*sc4*Δ*sc4* dikaryon. The encoded hydrophobin was shown to self-assemble at the surface of air channels within the fruiting bodies (Lugones *et al.*, 1999). The resulting hydrophobicity prevents that these channels fill with water under humid conditions. As a consequence, gas exchange is affected in Δ*sc4*Δ*sc4* fruiting bodies. When both *sc3* and *sc4* are inactivated, this resulted in an absence of fruiting body formation in dikaryons (van Wetter *et al.*, 2000a).

SC7 and SC14

Genes *sc7* and *sc14* were identified as genes highly expressed in fruiting bodies of *S. commune* (Mulder & Wessels, 1986). The encoded proteins are rather hydrophilic and it was shown that SC7 is secreted into the medium and loosely binds to the extracellular matrix that embeds the hyphae in the mushroom (Schuren *et al.*, 1993). SC7 and SC14 contain an SCP domain. Such a domain is also found in the plant pathogenesis-related protein PR-1. This protein has antifungal activity and accumulates after an infection of tomato or tobacco plants with the fungal pathogen *Phytophthora infestans* (Niderman *et al.*, 1995). Homologs of SC7 and SC14 are present in the genomes of other mushroom-forming fungi and it is tempting to speculate that these proteins play a role in the defense of mushrooms against pathogens.

Lectins

Lectins are defined as non-immunoglobulin, carbohydrate-binding proteins without catalytic activity towards the recognized carbohydrate (Peumans & Van Damme, 1995). Several types of lectins have been identified in mushrooms (Wang *et al.*, 1998). Many mushroom lectins are up-regulated during fruiting body formation, which led to the hypothesis that they are involved in this process, for example by linking hyphae to each other (Boulianne *et al.*, 2000). Recently, however, it was shown that the β-

galactoside binding lectin CGL2 is toxic for the nematode *Caenorhabditis elegans* (Butschi *et al.*, 2010), implying a role for this class of proteins in the defense against pathogens.

Laccases and other oxidative enzymes

Laccases are copper-containing oxidases that are found in plants, fungi and bacteria. Increased laccase activity has been reported during fruiting in *S. commune* (Leonard, 1971; Leonard & Phillips, 1973; Phillips & Leonard, 1976) and *L. edodes* (Zhao & Kwan, 1999). This suggests a role for this class of proteins in mushroom development, for example by oxidative cross-linking of hyphae. Alternatively, laccase activity may stimulate aerial growth by making the hyphal cell walls more hydrophobic due to the deposition of phenolic compounds.

Members of another class of oxidative proteins, cytochrome P450, have been isolated from mushrooms of *C. cinerea* (Muraguchi & Kamada, 2000), *A. bisporus* (De Groot *et al.*, 1997) and *L. edodes* (Akiyama *et al.*, 2002). Deletion of the nucleotides encoding the 18 C-terminal amino acids of Eln2 in *C. cinerea* resulted in abnormally small primordia (Muraguchi & Kamada, 2000). To date, the underlying mechanism of cytochrome P450 in mushroom development is unknown.

SCOPE AND OUTLINE OF THIS THESIS

The regulatory genes that have been identified to fulfill a function in mushroom development clearly do not present a complete picture of how fruiting body formation is regulated at the molecular level. In this Thesis, the regulation of mushroom formation was studied with the aim to present a model of mushroom formation. To this end, a whole genome expression analysis was performed during several stages of development. Transcription factor genes were inactivated that showed a differential expression during development of *S. commune*. The phenotypes were studied and, in some cases, a whole genome expression analysis was performed. In addition, tools were developed that facilitate the functional analysis of genes.

Chapters 2 and **3** describe a more efficient procedure for gene inactivation by homologous recombination. In **Chapter 2** the plasmid pDelcas is described, which allows easy cloning of the flanking regions of a gene that is to be deleted around a nourseothricin resistance marker. Furthermore, pDelcas contains a phleomycin resistance cassette outside the flanking regions. Phleomycin and nourseothricin resistant colonies result from a single cross-over event of the pDelcas deletion vector into one of the chromosomes.

Such colonies will not have a gene deletion. In contrast, nourseothricin resistant, phleomycin sensitive strains are candidates to have a gene deletion. A fast colony PCR protocol was developed to confirm the gene deletion in such strains. A strain with a deletion in the *ku80* gene is described in **Chapter 3**. Single cross-over events rarely occur in this strain due to the fact that the non-homologous end joining pathway has been inactivated. As a result, the number of transformants is reduced up to 100-fold in this strain. However, the relative number of transformants with a gene inactivation is increased dramatically.

In **Chapter 4** the first inducible promoter for a mushroom-forming fungus is described. The promoter of the heat shock protein encoding gene *hsp3* is not active at 25 °C. However, it is active at a temperature above 37 °C. The coding sequence of the red fluorescent protein dTomato was expressed from the *hsp3* promoter. Red fluorescence was not observed during growth at 25 °C. In contrast, fluorescence of the reporter was observed after a local or systemic heat treatment (*e.g.* 1 h at 42 °C).

Chapter 5 describes the genome sequence of *S. commune*. The 38.5 MB assembled genome contains 13,210 predicted genes. Gene expression was analyzed in various developmental stages, allowing the identification of differentially expressed groups of genes during mushroom formation. A total of 472 transcription factors were identified. Two of these were inactivated, resulting in more but smaller mushrooms (in the case of deletion of *fst3*) or in absence of mushrooms (in the case of deletion of *fst4*). Furthermore, it was shown that antisense transcripts may play an important role in mushroom development.

Five additional transcription factor genes were inactivated in **Chapter 6**. Colony morphology of a dikaryon was similar to that of a monokaryon and no aggregates were formed when either *hom2* or *bri1* were deleted. Development stopped in the aggregate stage when *c2h2* was inactivated. In contrast, more (but smaller) mushrooms developed when either *hom1* or *gat1* was removed from the genome. Genome-wide expression analysis of dikaryons in which either the *hom2* or *fst4* genes were inactivated revealed differentially expressed groups of genes, such as hydrophobins. A regulatory model of mushroom formation is presented, which is based on the phenotypes and the expression profiles of the mutant strains.

Chapter 7 describes the effect of light on gene expression and the role of the blue light receptor WC-2 in mushroom development. Inactivation of the encoding gene resulted in a blind phenotype and therefore in the absence of mushroom development. The gene was reintroduced into the deletion strain under the control of the inducible *hsp3* promoter. Mushroom

formation could be induced in this strain by applying a daily heat shock. Gene expression analysis resulted in the identification of several downstream targets of WC-2, as well as a putative promoter binding element. The regulatory model of mushroom formation presented in **Chapter 6** is extended with the results described in **Chapter 7**.

In **Chapter 8** the effects of the deletion of the hydrophobins genes *sc3* and *sc4* on genome-wide expression is analyzed. The global expression profile of a dikaryon with *sc3* and *sc4* gene deletions (which forms few aerial hyphae and no mushrooms) was more similar to that of a monokaryon than to a dikaryon of the same age. However, expression of the transcription factors that were shown to be involved in mushroom formation (**Chapters 5**, **6** and **7**) was more similar to that of the wild type dikaryon. This suggests that the regulatory mechanism for mushroom formation is in a stand-by mode, waiting for a signal originating from aerial structures.

The results are summarized and discussed in **Chapter 9**.

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Chapter 2

An efficient gene deletion procedure for the mushroom-forming basidiomycete *Schizophyllum commune*

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ABSTRACT

Gene deletion in *Schizophyllum commune* is hampered by a low incidence of homologous integration. As a consequence, extensive screening is required to identify a transformant with the desired genotype. To alleviate this and to facilitate the assembly of deletion plasmids, vector pDelcas was constructed. This construct has a set of restriction sites, which allows for directional cloning of the flanking sequences at both sides of a nourseothricin resistance cassette. Moreover, it contains a phleomycin resistance cassette elsewhere in the plasmid, which is used to screen for transformants with an ectopic integration of the pDelcas derivative. The use of pDelcas derivatives in combination with an improved PCR screening protocol permitted the efficient identification of *S. commune* deletion strains. This procedure may also function in other basidiomycetes.

INTRODUCTION

Gene inactivation is an important tool in functional genetics and the most reliable way of implementing it is gene deletion through homologous recombination. The efficiency of this process depends on the pathway that is used by the organism to repair double stranded DNA breaks (Ninomiya *et al.*, 2004). Homologous integration occurs with a high frequency in organisms such as *Saccharomyces cerevisae* that primarily use the homologous recombination pathway. Plants, animals and filamentous fungi use mainly non homologous end joining for DNA repair. As a result, ectopic integration is the most frequent outcome of a DNA transformation. Ten gene deletions have been reported in the basidiomycete *Schizophyllum commune.* A gene of the *mat*Aα mating type locus and the *mtd1* gene were inactivated with a frequency of 33% (Marion *et al.*, 1996) and 50% (Lengeler & Kothe, 1999b), respectively. The other genes were deleted with an average frequency of only 3% (Robertson *et al.*, 1996; van Wetter *et al.*, 1996; Horton *et al.*, 1999; Lengeler & Kothe, 1999a; van Wetter *et al.*, 2000; Lugones *et al.*, 2004; Schubert *et al.*, 2006). The low incidence of homologous integration in most of the cases where a gene was deleted prompted us to set up a screening system for transformants with such an integration event. To this end, pDelcas (accession number GQ184463) was constructed. This vector allows easy directional cloning of flanking sequences at either side of a nourseothricin resistance cassette. The presence of a phleomycin resistance cassette elsewhere in the construct enables elimination of transformants with an integration originating from a single cross-over (either homologous or ectopic)

due to their resistance to the antibiotic phleomycin. In the second step, phleomycin sensitive colonies are screened using a fast colony PCR protocol to confirm gene inactivation by homologous integration.

MATERIALS AND METHODS

Genes in the genome of S. commune

Genes (indicated by ProteinID) of *S. commune* are available through the interactive JGI Genome Portal at http://jgi.doe.gov/Scommune (see Chapter 5; Ohm *et al.*, 2010). The predicted gene models and annotations are also deposited at DDBJ/EMBL/GenBank under the project accession ADMJ00000000.

Construction of pDelcas

Vector pDelcas consists of a backbone of pUC20 encompassing the *S. commune* phleomycin and nourseothricine resistance cassettes (Schuren & Wessels, 1994; van Peer *et al.*, 2009) (Figure 1). Flanking regions of 1-2 kb of the gene of interest can be cloned at either side of the nourseothricin resistance cassette by replacing a 2.4 kb Van91I fragment and a 0.64 kb SfiI fragment, respectively. Van91I and SfiI recognize the sequences CCANNNNNTGG and GGCCNNNNNGGCC, respectively, and both cut within the variable middle part. This allows the generation of tailored sticky ends. As a result, fragments cut with SfiI (which is chosen because it is an 8 cutter whose recognition sites rarely occur in genomic DNA) can be directionally cloned into a vector cut with either Van91I or SfiI. To this end, both genomic flanking sequences of the gene of interest are amplified with primers that contain a SfiI site. The forward and reverse primers should have SfiI sites that differ in their variable region, resulting in different sticky ends (Figure 1). In the first step, one of the flanks with the SfiI sticky ends is directionally cloned in between the Van91I sites of pDelcas. In the second step, the other flank is cloned in between the SfiI sites. In the case a restriction site for SfiI is present within one of the flanking sequences, primers can be used that contain sites for BstAPI (GCANNNNNTGC), DraIII (CACNNNTGC) or Van91I (CCANNNNNTGG) and that obey the rules for the variable region as depicted in Figure 1.

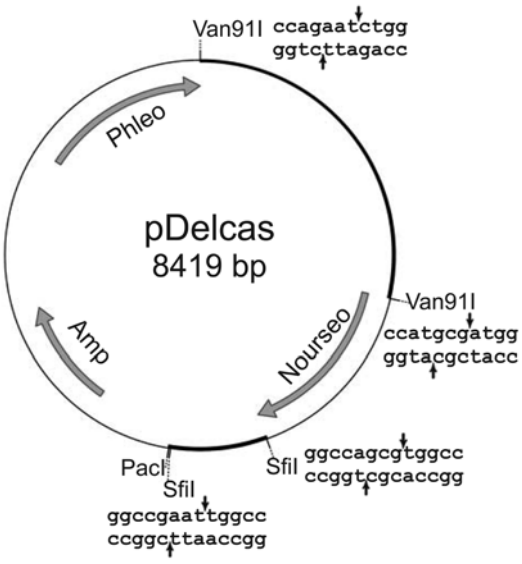
Transformation and first screening

Circular DNA of derivatives of pDelcas was introduced in *S. commune* strain 4-8 (*MAT*A43 *MAT*B41) (Fowler *et al.*, 1999), as described previously (van Peer *et al.*, 2009). Selection was done on minimal medium (Dons *et al.*, 1979) with

8 µg ml^{-1} nourseothricin (Jena Biosciences, Jena, Germany). Transformants were transferred to a second selection plate with nourseothricin and then screened on minimal medium plates with 25 µg ml^{-1} phleomycin (Cayla S.A.R.L., Toulouse, France). Transformants that failed to grow on phleomycin were candidates to have a gene deletion due to homologous integration resulting from a double cross-over.

PCR screening

From the transformants that failed to grow on phleomycin, a small piece of mycelium (2 – 25 mm^2) was taken that had grown on a nourseothricin selection plate. The fragment was placed in a 2 ml Eppendorf tube together with a new metal ball (Marabu, 3/16" or approximately 5 mm in diameter). The mycelium was frozen in liquid nitrogen and homogenized for 1 minute at 25 Hz using a Retsch Tissue Lyser II. One ml of CTAB-buffer (2% CTAB, 0.1 M Tris-HCl pH 8.0, 1.4 M NaCl) was added to the powered mycelium, after which the metal ball was removed with a magnet. The tubes were placed at 65 °C for 20 min. Cell debris was pelleted by centrifugation for 1 min at 20000 g and half volume chloroform was added to the supernatant. After mixing, the samples were centrifuged for 5 min at 20000 g. 800 µl of the water (upper) phase was transferred to a clean tube and DNA was precipitated by adding 640 µl isopropanol. After centrifugation for 5 min at 20000 g, the pellet was washed with 500 µl 70% ethanol and dissolved in 50 µl TE buffer. For PCR, 1 µl of the DNA was mixed with primers, Taq polymerase, Taq buffer (as supplied by the



Flank	Primer	Sequence
Upstream	Forward	GGCCNAATNGGCCN$_{18}$
	Reverse	GGCCNCGCNGGCCN$_{18}$
Downstream	Forward	GGCCNGCGNGGCCN$_{18}$
	Reverse	GGCCNATTNGGCCN$_{18}$

Figure 1. Vector pDelcas is based on a pUC20 backbone. It contains a phleomycin and a nourseothricin resistance cassette. Van91I and SfiI restriction sites flank fragments from bacteriophage λ of 2.4 and 0.64 kb, respectively (indicated in bold). Custom made sticky ends in these sites allow directional cloning when combining the primers depicted in the lower panel. The underlined sequence of the primers represent the recognition site of SfiI, while the N_{18} region represent the flank specific sequence.

manufacturer), 1.5% DMSO, 1.5 mM $MgCl_2$ and 200 µM of each nucleotide. For screening, primers were used that only render a product in case of homologous integration of the flanking sequences of the deletion construct (Figure 2). The forward primer pr1 anneals to the genomic DNA immediately outside the upstream flank. Its sequence depends on the gene that is inactivated. The reverse primer pr1' (Table 1) anneals in the nourseothricin resistance cassette and is used for screening of all gene deletions. In the same way primers pr2 (anneals in the nourseothricin resistance cassette) (Table 1) and pr2' (gene specific) are used to check the downstream flank. As a negative control, a PCR is performed with primers pr1 and pr3' (anneals in the deleted fragment of the gene). Generally, 35 PCR cycles were sufficient to amplify a specific band. Agar from the medium did not inhibit the PCR.

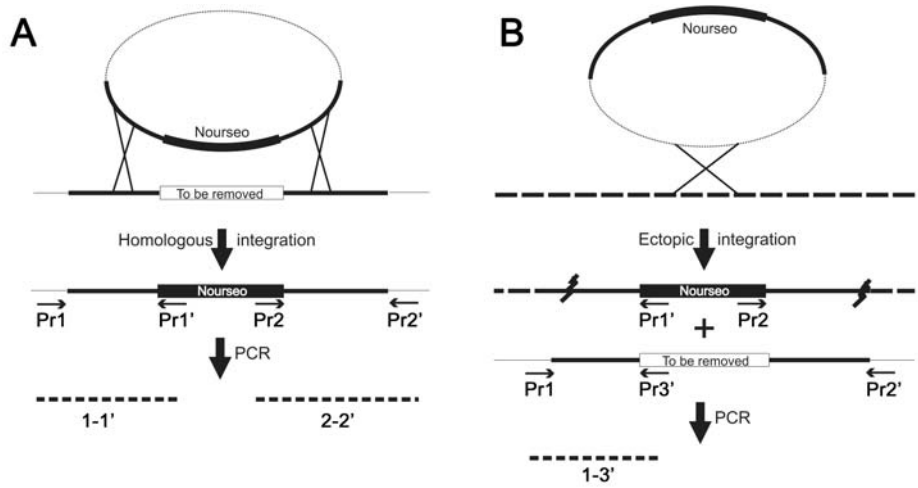


Figure 2. Screening of transformants in which a pDelcas derivative has been introduced. Integration will be either homologous **(A)** or ectopic **(B)**. PCR with three primer pairs reveals which integration event has occurred. A third possibility not depicted here is homologous integration which resulted from a single crossing over. In this case product 1-1' or 2-2' will be found together with product 1-3'.

Crossings

Derivatives of strain H4-8 with a gene inactivation were crossed with the compatible isogenic strain H4-8b (*MAT*A41 *MAT*B43). Segregation of nourseothricin resistance in the F1 was assessed to exclude multiple integrations (integration in a single locus should give a 1:1 ratio of resistant and sensitive colonies). Crossing was also used to clean heterokaryons that harbor transformed and untransformed nuclei. Such heterokaryons can result

from fusion of transformed and non-transformed (regenerating) protoplasts and give PCR products in both the positive and negative controls.

Southern analysis

Chromosomal DNA was isolated as previously described (van Peer *et al.*, 2009). Southern analysis was performed with PstI digested DNA as previously described (Schuren *et al.*, 1993). Hybridization was done using DNA fragments that were purified using the NucleoSpin Extract II kit (Macherey Nagel) and labelled with α-^{32}P-dCTP using standard protocols (Sambrook *et al.*, 1989).

Table 1. Primers used in this study.

Primer	Sequence (5' – 3')
MYN6_ufw	GGCCGAATCGGCCCGCCTTCTCCAG
MYN6_urv	GGCCACGCTGGCCCAAGACACCTC
MYN6_dfw	GGCCAGCGGGGCCTTCGCACGTC
MYN6_drv	GGCCGATTAGGCCGTCCTTTGCTTTC
MYN6_outfw (pr1)	CCGCCAGGGTACAACTACTC
Nourdelrev (pr1')	TTGGTGACCTCCAGCCAGAG
SC3TerminatorPr (pr2)	GGCTGAGTCGTGGACTAAAG
MYN6_outrev (pr2')	GGGCGGACGAGATGTACAAG
MYN6_control (pr3)	TATAGCTCTCGAGCGGCGTCAGAGATG

RESULTS

A pDelcas derivative was constructed with the flanking regions of a 524 bp coding region fragment of *myn6* (Protein ID: 114035), which is up-regulated in stage II aggregates (Chapter 5; Ohm *et al.*, 2010). Primers MYN6_ufw, MYN6_urv, MYN6_dfw and MYN6_drv (Table 1) were used to amplify up- and downstream flanks, respectively. These flanking regions were cloned into pDelcas resulting in vector pDelcas-MYN6updw. 309 nourseothricin resistant colonies were obtained after introduction of the construct in *S. commune*. Of these, 22 were sensitive to phleomycin. Phleomycin sensitivity may be due to a homologous integration of the flanking regions via a double cross-over. Alternatively, it may be caused by an ectopic integration which took place within the phleomycin resistance cassette. To discriminate between these events, PCR was performed using three primer pairs (Figure 2 and Table 1). Genomic DNA of one of the transformants gave a band of 1338 bp with primers SC3TerminatorPr and MYN6_outrev and a band of 1805 bp with primers MYN6_outfw and nourdelrev. These bands are explained by the homologous recombination between pDelcas-MYN6updw and the *myn6* locus. Primers MYN6_outfw and MYN6_control did not result in a 1150 bp fragment,

which is expected when the *myn6* fragment has been deleted. Southern analysis of chromosomal DNA confirmed the deletion of *myn6* (Figure 3). A fragment of 1913 bp hybridized with the upstream flank of *myn6* in the case of the wild-type DNA, whereas a 3428 bp fragment hybridized in the case of genomic DNA of the Δ*myn6* strain. Like gene *myn6*, three other genes were inactivated using pDelcas (Table 2). In general, 20-25% of the nourseothricin resistant colonies are phleomycin sensitive. Of these transformants, about 4% have a gene deletion. Up to 60% of the false positives can be eliminated by decreasing the concentration of phleomycin during the screening to 5 μg ml^{-1}. By using this concentration also transformants are eliminated that express the phleomycin resistance cassette to a low extent (data not shown).

DISCUSSION

Efficient gene deletion protocols are essential for functional gene analysis. However, due to the high percentage of ectopic integrations in *S. commune* extensive screening is required to identify a transformant with the desired genotype. Here, a dedicated vector and an efficient screening method were developed to inactivate genes *myn6*, *spc33*, *sc15* and *fst3*. The phenotypes of *spc33*, *sc15* and *fst3* have been reported (Lugones *et al.*, 2004; van Peer *et al.*, 2010; Ohm *et al.*, 2010; Chapter 5). Inactivation of *myn6* did not affect growth and development in monokaryons or in hetero- or homozygous dikaryons (data not shown). The function of this gene, which is up-regulated during formation of stage II aggregates, is therefore not clear yet.

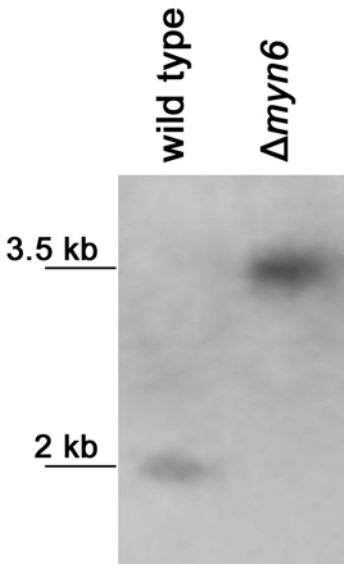


Figure 3. Southern analysis of a transformant with a deletion in *myn6*. A 1913 and a 3428 bp PstI fragment hybridized in the case of genomic DNA of the wild type and the Δ*myn6* strain, respectively.

Vector pDelcas consists of a backbone of pUC20 encompassing the *S. commune* phleomycin and nourseothricine resistance cassettes (Schuren & Wessels, 1994; van Peer *et al.*, 2009). Flanking regions of a gene of interest can be cloned at either side of the nourseothricin resistance cassette by replacing a 2.4 kb Van91I fragment and a 0.64 kb SfiI fragment, respectively. Strains that have been transformed with a pDelcas derivative via a single cross-over (either homologous or

ectopic) will be nourseothricin and phleomycin resistant. On the other hand, strains that are nourseothricin resistant but phleomycin sensitive are the result of a double homologous cross-over or a single cross-over within the phleomycin cassette. In the former case a gene inactivation is expected. This can be confirmed using a colony PCR method that was developed for *S. commune.* This method consists of DNA isolation from the mycelium of colonies grown on agar medium, followed by a PCR with primer pairs that amplify a product either from the wild type gene or from the correctly integrated nourseothricin resistance gene. The fact that mycelium can be used that have been grown on agar medium allows the screening of large number of transformants.

Table 2. Results of the screening of transformants from four different gene deletion experiments. Nourseo = Nourseothricin; Phleo = Phleomycin. A (+) indicates resistance and a (-) indicates sensitivity to the antibiotic.

Gene name	Protein ID	Nourseo (+) colonies	Nourseo (+) Phleo (-) colonies (% of Nourseo (+) colonies)	Transformants with a gene deletion (% of Nourseo (+) Phleo (-) colonies)
myn6	114035	309	22 (7.1%)	1 (4.5%)
spc33	74104	495	140 (28.3%)	2 (1.4%)
fst3	257422	68	16 (23.5%)	1 (6.3%)
sc15	82353	76	20 (26.3%)	1 (5.0%)
Average percentage			21.3%	4.3%

Recently, we described that the number of *S. commune* transformants can be increased 10-fold by adding a low non-selective concentration of phleomycin in the regeneration medium (van Peer *et al.*, 2009). This increase can be explained by the fact that this antibiotic introduces double strand breaks in the DNA. We have also observed that the low concentration of phleomycin promotes single integrations. However, addition of a low concentration of phleomycin in the regeneration medium did not improve efficiency of gene deletion using pDelcas. In fact, no gene deletions were observed in nearly a thousand transformants that had been transformed with pDelcas derivatives. These data and the fact that phleomycin increases expression of genes involved in non homologous end joining (van Peer *et al.*, 2009) indicates that this antibiotic promotes ectopic integrations.

The genomes of the mushroom forming fungi *Phanerochaete chrysosporium* (Martinez *et al.*, 2004), *Coprinopsis cinerea* (Stajich *et al.*, 2010), *Laccaria bicolor* (Martin *et al.*, 2008) and *S. commune* (Chapter 5; Ohm *et al.*, 2010) have been sequenced and those of *Pleurotus ostreatus* and

Agaricus bisporus will become available as well. Vector pDelcas and the described PCR procedure may allow rapid screening of transformants in these basidiomycetes. This is indicated by the fact that expression cassettes of *S. commune* are functional in other basidiomycetes like *Pycnoporus cinnabarinus* (Alves *et al.*, 2004), *Agaricus bisporus* and *Pleurotus ostreatus* (Lugones, unpublished results).

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Chapter 3

Inactivation of *ku80* in the mushroom-forming fungus *Schizophyllum commune* increases the relative incidence of homologous recombination

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ABSTRACT

***Schizophyllum commune* is the only mushroom-forming fungus in which targeted gene deletions by homologous recombination have been reported. However, these deletions occur with a low frequency. To overcome this, the *ku80* gene of *S. commune* was deleted. This gene is involved in the non-homologous end joining system for DNA repair. The Δ*ku80* strain was not affected in growth and development. However, the transformation efficiency was reduced up to 100-fold. This was accompanied by a strong increase in the relative number of transformants with a homologous integration of a knock-out construct. Genes *sc15*, *jmj3* and *pri2* were deleted in the Δ*ku80* strain. In total, 7 out of 10 transformants showed a gene deletion. This frequency will facilitate a systematic analysis of gene function in *S. commune*.**

INTRODUCTION

Schizophyllum commune is used as a model system to study mushroom development. This basidiomycete can complete its life cycle on defined media in approximately 10 days and molecular tools have been developed to study its growth and development. In fact, it is the only mushroom-forming fungus in which genes have been inactivated by homologous recombination. The importance of *S. commune* as a model system is also exemplified by the fact that its constructs will express in other mushroom-forming fungi (Alves *et al.*, 2004).

Targeted gene disruption in *S. commune* is hampered by a low incidence of homologous recombination (HR). So far, 12 gene deletions have been reported in *S. commune.* A gene in the *matAα* mating type locus and the *mtd1* gene were inactivated with an efficiency of 33% (Marion *et al.*, 1996) and 50% (Lengeler & Kothe, 1999b), respectively. The other genes were deleted with an average frequency of only 3% (Robertson *et al.*, 1996; van Wetter *et al.*, 1996; Horton *et al.*, 1999; Lengeler & Kothe, 1999a; van Wetter *et al.*, 2000; Lugones *et al.*, 2004; Schubert *et al.*, 2006; Ohm *et al.*, 2010a; van Peer *et al.*, 2010).

Recently, a dedicated deletion vector has been described, which reduces screening for transformants with a gene inactivation (Chapter 2; Ohm *et al.*, 2010a). This construct, called pDelcas, consists of two antibiotic resistance cassettes. The nourseothricin resistance cassette is positioned in between the flanks of the gene that is to be deleted. On the other hand, the phleomycin resistance cassette is positioned elsewhere in the construct.

Consequently, phleomycin resistance is indicative for an integration of the construct by a single cross-over; *i.e.* an event not leading to a gene inactivation. By replica plating on a medium containing phleomycin, about 70% of the transformants could be eliminated in the screening process for a strain with a gene deletion. However, 30% of the transformants still had to be screened by PCR and/or Southern hybridization. This is the reason why we decided to inactivate the *ku80* gene that is part of the non homologous end joining (NHEJ) pathway.

The frequency of targeted gene inactivation by HR is related to the default pathway used by the organism to repair double stranded DNA breaks (Ninomiya *et al.*, 2004). *Saccharomyces cerevisae*, for instance, uses mainly HR, which is mediated by the concerted action of Rad51 and Rad52 (New *et al.*, 1998). This explains the high incidence of homologous integration in this organism. In most filamentous fungi, ectopic integrations are much more frequent (Fincham, 1989). Such integrations are mediated by NHEJ. NHEJ can be initiated by PARP-1, which recruits the XRCC1-DNA ligase III complex (Audebert *et al.*, 2004). Alternatively, NHEJ results from the action of the Ku70/Ku80 heterodimer (for a review see Weterings & Chen, 2008). This heterodimer binds to free DNA ends, and recruits and activates the DNA-dependent protein kinase catalytic subunit (DNA-Pkcs). Consequently, DNA ligase IV binds to the formed complex together with XRCC4, which results in the ligation of the DNA ends. Inactivation of *ku70*, *ku80* or both, has greatly increased targeted gene inactivation in a number of filamentous fungi (Ninomiya *et al.*, 2004; Krappmann *et al.*, 2006; Nayak *et al.*, 2006; Poggeler & Kuck, 2006; Takahashi *et al.*, 2006; Choquer *et al.*, 2008; Haarmann *et al.*, 2008).

Here, we report for the first time the inactivation of *ku80* in a mushroom-forming fungus and the use of the resulting strain for the deletion of *sc15* (Lugones *et al.*, 2004) and the putative transcription factors *jmj3* (containing a Jumonji DNA binding domain) and *pri2* (containing a Zn(II)2Cys6 zinc cluster DNA-binding domain).

MATERIALS AND METHODS

Strains, growth conditions and transformation

Monokaryotic and dikaryotic strains of *S. commune* were grown at 25 °C in the light on minimal medium (MM, Dons *et al.*, 1979). The monokaryotic strain H4-8 (Fowler *et al.*, 1999) was transformed as described (van Peer *et al.*, 2009). Twenty µg of vector DNA was incubated with $5{\cdot}10^7$ protoplasts.

After regeneration in medium without antibiotic, transformants were selected on plates containing 20 µg ml^{-1} hygromycin or nourseothricin, as indicated.

Genes in the genome of S. commune

Genes (indicated by ProteinID) of *S. commune* are available through the interactive JGI Genome Portal at http://jgi.doe.gov/Scommune (see Chapter 5; Ohm *et al.*, 2010b). The predicted gene models and annotations are also deposited at DDBJ/EMBL/GenBank under the project accession ADMJ00000000.

Deletion construct for ku80

Up- and downstream flanking regions of the coding sequence of *ku80* (proteinID 61992) were amplified using primer pairs dku80upfw/dku80uprv and dku80dwfw/dku80dwrv, respectively (Figure 1). The 1570 bp upstream fragment was cut with EcoRI and HindIII. The resulting 1374 bp fragment was cloned in plasmid pHYM1.2 (Scholtmeijer *et al.*, 2001) that had been cut with HindIII and MunI. This resulted in plasmid pHymk80u. The 1300 bp downstream flank was cloned in the EcoR1 site of pESC (Alves *et al.*, 2004) in between the *sc3* terminator and the phleomycin resistance cassette. To this end, the EcoRI site downstream of the phleomycin resistance cassette was removed. In the next step an artificial intron was inserted in the BamHI site of the pESC derivative (*i.e.* at the beginning of the *sc3* terminator). This resulted in vector pEPK80D. The 2700 bp HindIII/BamHI fragment of pHymk80u, which consists of the upstream flank of *ku80*, the *gpd* promoter, and the hygromycin coding sequence, was cloned in the respective sites of pEPK80D. The final construct pKu80del contains a hygromycin resistance cassette that is surrounded by the flanking regions of *ku80* as well as a phleomycin resistance cassette that is located outside the flanking regions of *ku80*.

Deletion constructs for sc15, jmj3 *and* pri2

Deletion constructs for *sc15* (ProteinID 82353), *jmj3* (ProteinID 103341) and *pri2* (ProteinID 269936), were based on vector pDelcas. These deletion constructs, called pDelcas-sc15, pDelcas-jmjC and pDelcas-priB were made as described (Chapter 2; Ohm *et al.*, 2010a). Primers that were used to amplify the flanking regions are indicated in Table 1.

Screening of transformants

Colony PCR was conducted to screen transformants (Chapter 2; Ohm *et al.*, 2010a). A PCR product of 1400 bp is obtained with genomic DNA of a *ku80* deletion strain with primer pair 1-1' (Figure 1). These primers anneal to the

genomic DNA outside the upstream flank of *ku80* and to the *gpd* promoter of the hygromycin resistance cassette. Similarly, a 1300 bp fragment is obtained with the primer pair 2-2', which anneals to the downstream region of the *sc3* terminator of the hygromycin cassette and the region immediately downstream of the downstream flank of *ku80*. Primer pair 3-3', which anneals just outside the deleted region of *ku80,* should give a band of about 500 bp and 1700 bp in a wild type and a deletion strain, respectively. The size difference of the PCR products is due to the size of the hygromycin resistance cassette and the deleted region of the coding sequence of *ku80*.

Phenotypic analysis

Morphology of the monokaryotic Δ*ku80* strain was compared with the wild type after 6 days of growth on MM plates. To assess the phenotype of the Δ*ku80* or the Δ*ku80*Δ*ku80* dikaryon, the Δ*ku8*0 strain was crossed with the compatible co-isogenic strain H4-8b (*MAT*A41 *MAT*B43; Chapter 2; Ohm *et al.*, 2010a). After 6 days of growth, spores were collected from the lids of the plates (plates had been placed inverted in the growth chamber) and counted with a haemocytometer. 500 spores were plated out on complete medium (glucose 20 g/l; $MgSO_4$ 2mM; KH_2PO_4 3.4 mM; K_2HPO_4 5.7 mM; pepton 2 g/l; yeast extract 2 g/l, 1.5% agar) to assess whether antibiotic resistance and antibiotic sensitivity segregated 1:1. To this end, 100 1-day-old colonies were transferred to MM plates and grown for two days. The colonies were replicated on plates containing 20 µg/ml antibiotic (hygromycin or nourseothricin depending on the strain) and growth was monitored after two days. In the next step, antibiotic sensitive and antibiotic resistant siblings were selected that had mating types of strains H4-8 and H4-8b. To this end, siblings were crossed with these wild type strains and clamp formation and fruiting body formation was monitored. Growth and fruiting body formation of dikaryons that contained a single or a double deleted *ku80* gene was followed in time on MM plates and compared to that of a wild type dikaryon. Spore formation was assessed by growing the dikaryons on plates that had been placed inverted in the growth chamber and spore viability was checked by determining the colony forming units of 100 spores.

The phenotypes of the homozygous monokaryotic and dikaryotic *jmj3* and *pri2* strains were similarly assessed as the Δ*ku80* strains. However, in this case the *ku80* gene was reintroduced before phenotypic analysis. To this end, a wild type was crossed with monokaryons in which *jmj3* or *pri2* had been deleted (both types of deletion strains were nourseothricin and hygromycin resistant). Spores that were nourseothricin resistant but

hygromycin sensitive had a *jmj3* or *pri2* deletion but contained a wild type *ku80* gene.

Quantitative PCR

RNA isolation and qPCR were performed as described (van Peer *et al.*, 2009). After DNAse treatment, cDNA was synthesized using random hexamer primers and M-MuLV reverse transcriptase according to the manufacturer's instructions (Fermentas; St. Leon-Rot, Germany). Real-time PCR was performed using the ABI Prism 7900HT SDS and SYBR Green chemistry (Applied Biosystems, Foster City, CA, USA). Expression levels were related to that of the actin gene *act1*. Levels of *act1* (proteinID 83632) and *rad52* (proteinID 38411) cDNA were determined using the primer pairs act1_fw/act1_rev and rad52_fw/rad52_rev, respectively.

RESULTS

To inactivate the *ku80* gene, *S. commune* monokaryon H4-8 was transformed with deletion construct pKu80del. This vector consists of the hygromycin resistance cassette that is flanked by the up and downstream regions of the coding sequence of *ku80* and by a phleomycin resistance cassette that is positioned elsewhere in the vector. 600 hygromycin resistant transformants were replicated on plates containing 5 μg ml^{-1} phleomycin. 30 transformants were phleomycin-sensitive, thus representing strains potentially having a *ku80* deletion. To confirm the gene deletion, transformants were screened by colony PCR. One of the transformants gave a product of about 1400 bp with primer pair 1-1', a product of about 1300 bp with primer pair 2-2' and a product of about 1700 bp with primer pair 3-3'. PCR products of these sizes are expected in the case of a gene deletion of *ku80* (see Materials and methods and Figure 1).

The Δ*ku80* monokaryon was phenotypically indistinguishable from the wild type. The Δ*ku80*Δ*ku80* dikaryon formed normal fruiting bodies that produced similar numbers of spores with a similar viability when compared with the wild type. Moreover, like in the wild type, 10^9 protoplasts could be obtained from 5 g wet weight mycelium (data not shown).

To assess whether the homologous recombination pathway was up-regulated in the Δ*ku80* mutant qPCR was performed. The expression of *rad52* (which is a gene involved in HR) was similar in the Δ*ku80* strain (Ct = 29.24 ± 0.33) when compared with the wild type strain (Ct = 28.90 ± 0.16). Apparently, inactivation of the NHEJ pathway does not induce an up-regulation of the HR pathway.

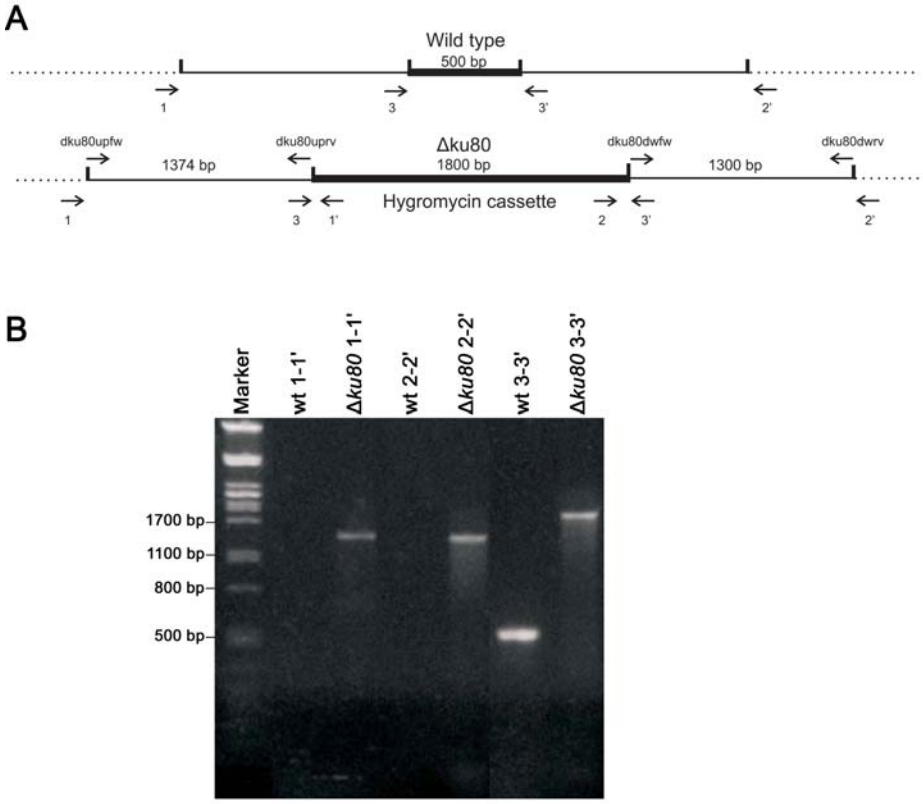

Figure 1. PCR screening of transformants of *S. commune* to confirm the deletion of *ku80*. A. The screening is performed essentially as described (Chapter 2; Ohm *et al.*, 2010a). The upper panel represents the wild type situation, the lower panel represents the situation in which the *ku80* gene is replaced by a hygromycin cassette. The thin lines represent the flanking sequences of the gene that is to be deleted. The thick lines represent the hygromycin resistance cassette and the coding sequence that is to be replaced by this selection cassette. Primer positions and names that are used in a PCR to confirm the gene deletion are indicated. Names and sequences of primers as well as the short names are given in Table 1. **B.** Primer pairs 1-1' and 2-2' yield no product in the wild type, but do so in the Δ*ku80* strain. The product of primer pair 3-3' is larger in the Δ*ku80* strain.

The Δ*ku80* strain was transformed with the knock out constructs pDelcas-sc15, pDelcas-jmjC and pDelcas-priB. The deletion constructs had been linearised with the restriction enzyme SspI (pDelcas-JmjC and pDelcas-priB) or PacI (pDelcas-SC15) before they were introduced in the Δ*ku80* H4-8 strain. Four out of 7 transformants had a deletion of *sc15,* while 1 out of 1 and 2 out of 2 transformants had a deletion of *jmj3* and *pri2*, respectively (Table 2). Typically, 100 transformants are obtained with protoplasts of the wild type

strain and these transformants would contain one, if any, knock out strain (illustrated by the efficiency of the inactivation of *ku80*). The number of transformants obtained with the Δ*ku80* strain is a 100-fold lower (Table 2). However, most of these transformants have a gene deletion. Transformation of a Δ*ku80* strain in which a wild type *ku80* gene had been re-introduced by crossing had a similar transformation frequency as observed for the wild type. This confirms that the low transformation frequency was due to the deletion of the *ku80* gene.

Table 1. Primers used in this study.

Primer Name	Sequence	Short name (see Figure 1)
dku80upfw	CGACGAACCATACCATAGAG	
dku80uprv	CAATTGGAGATCGAGGACTTCTTCAG	
dku80dwfw	GAATTCCCCGACGTGGATTGTTTGC	
dku80dwrv	GAATTCTGCTCTGCCGATTTATGCC	
ku80outf	CCGGAAGACAGACAAATG	1
gpdprr5	GCGACGCTCTATTCTATC	1’
sc3tersqf	GCCTCAGGTCCCGAAGTAAG	2
ku80doutrv	GGCAGCTTCGTTGTCTAGATG	2’
dku80f1	CGACTCCGACGCTGAAGAAG	3
dku80rv	GCAGCTCAGTTAAGCCCTAC	3’
dsc15_ufw	GGCCAAATTGGCCGGACTCCGCGAACGAAG	
dsc15_urv	GGCCTCGCGGGCCCATCGTGGGAGTCCTTG	
dsc15_dfw	GGCCAGCGTGGCCAGCTGCGCGAATGAATG	
dsc15_drv	GGCCAATTAGGCCAAGGCTGAGGAGAAGG	
dJmjc_ufw	GGCCTAAAGGGCCATGAGGGTGGTATC	
dJmjc_urv	GGCCGCGCAGGCCGGACTATACTAAC	
dJmjc_dfw	GGCCAGCGTGGCCGCCGGAGAACGTGGAGC	
dJmjc_drv	GGCCAATTAGGCCGCCGCCTCAAGTCCCTG	
dPrib_ufw	GGCCTAATTGGCCCTTGGTGTGCTGTAACC	
dPrib_urv	GGCCTCGCAGGCCACCGGCAAATAACGAGG	
dPrib_dfw	GGCCAGCGAGGCCGCCCGAGAAGTACAAGGAG	
dPrib_drv	GGCCTATTAGGCCAGCATGCCGATAATGTTTG	
act1_fw	TGGTATCCTCACGTTGAAGTA	
act1_rev	GTGTGGTGCCAGATCTT	
rad52_fw	GAAGAGTGGGCGGTTTA	
rad52_rev	CCTGCCCGTACCCAATA	

The phenotype of the Δ*sc15* strain has been described (Lugones *et al.*, 2004). Before determining the phenotypes of the Δ*jmj3* and Δ*pri2* strains, the wild type *ku80* gene was re-introduced by crossing. Monokaryotic and homozygous dikaryotic Δ*pri2* strains showed no phenotypic differences when compared to

the wild type. The monokaryotic Δ*jmj3* strain was also indistinguishable from the wild type strain. However, the dikaryotic Δ*jmj3*Δ*jmj3* strain grew somewhat slower than the wild type and formed a less dense mycelium. Yet, the mutant did form sporulating fruiting bodies (data not shown).

DISCUSSION

Efficient gene deletion protocols are essential for functional gene analysis. However, due to the high percentage of ectopic integrations in *S. commune* extensive screening is required to identify a transformant with the desired genotype. Recently, a dedicated deletion vector and colony PCR screening method have been described, which make screening for transformants with a gene inactivation more efficient (Chapter 2; Ohm *et al.*, 2010a). Here, the gene *ku80* was inactivated and it is shown that this decreases transformation frequency, but increases the relative incidence of homologous recombination. Deletion of *ku70* in *Aspergillus oryzae* (Takahashi *et al.*, 2006) and *Sordaria macrospora* (Poggeler & Kuck, 2006) also led to a reduction in the transformation frequency and in an increase of the relative incidence of homologous recombination. In these cases, a 7- and 40-fold reduction in the number of transformants was obtained, which was accompanied by an incidence of more than 60 and 90% of gene inactivation, respectively. Inactivation of *ku80* in *S. commune* did not result in an increase in the expression of *rad52,* which is a gene encoding a protein of the HR machinery. This and the reduced transformation frequency suggests that the activity of HR is not increased in the Δ*ku80* strain of *S. commune*.

Table 2. Transformation of the Δ*ku80* strain with linear plasmid DNA results in a high relative incidence of targeted gene inactivation.

Gene (protein ID)	Transformants	Knock-outs
sc15 (82353)	7	4
jmj3 (103341)	1	1
pri2 (269936)	2	2

The Δ*ku80* strain of *S. commune* was used to inactivate genes *sc15*, *jmj3* and *pri2*. Four out of 7 transformants had a deletion of *sc15,* while 1 out of 1 and 2 out of 2 transformants had a deletion of *jmj3* and *pri2*, respectively. The phenotype of the Δ*sc15* strain has been described (Lugones *et al.*, 2004). Gene *pri2* was identified as a gene whose activity was up-regulated during formation of primordia and fruiting bodies. In stage I aggregates, expression was 29 tags per million (tpm), this increased to 309 tpm in stage II primordia and 273 tpm in mature mushrooms (Chapter 5; Ohm *et al.*, 2010b). Inactivation of *pri2* did not result in a phenotype. Previously, the *pri2* homologue in *Lentinula edodes,* called *priB,* was shown to be up-regulated in

primordia and early fruiting bodies (Endo *et al.*, 1994). We can not exclude that in contrast to *pri2* in *S. commune, priB* does play a role in mushroom formation in *L. edodes.* Gene *jmj3* was shown to be up-regulated during formation of primordia. In stage I aggregates, expression was 18 tpm and this increased to 46 tpm in stage II primordia (Chapter 5; Ohm *et al.*, 2010b). The function of *jmj3* does not become clear from these experiments, but it does not appear to play a role in mushroom development. Proteins with a Jumonji (JMJ) domain have previously been shown to function as a transcription factor (Takeuchi *et al.*, 1995; Balciunas & Ronne, 2000).

Taken together, we have shown that the relative incidence of gene inactivation by homologous integration is drastically increased in a Δ*ku80* strain when compared to the wild type. This strain will therefore be highly instrumental in the functional analysis of genes in *S. commune* and, in this way, contribute to our understanding of the biology of mushroom-forming basidiomycetes.

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Chapter 4

The use of the upstream region of the *hsp3* gene as an inducible promoter system for *Schizophyllum commune*

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ABSTRACT

The basidiomycete *Schizophyllum commune* contains 7 genes of the Hsp26/Hsp42 small heat shock protein family. Four of these genes, called *hsp4-7*, were expressed at 25 °C in one or more stages of the life cycle of this mushroom-forming fungus. The other three genes, called *hsp1-3*, were not expressed. By using dTomato as a reporter protein it was shown that the promoters of *hsp1-3* were induced by a heat shock of 1 hour at 42 °C in both monokaryotic and dikaryotic mycelium. Activity of the *hsp3* promoter was studied in more detail. No activity was observed at 25 or 30 °C, weak activity at 37 °C and strong activity at 42 °C. Moreover, it could be locally induced in the mycelium simply by a 10 seconds contact with a metal needle that had been heated to 100 °C. Taken together, it is concluded that the *hsp3* promoter of *S. commune* represents the first reported inducible promoter system for a mushroom-forming fungus.

INTRODUCTION

The fungus *Schizophyllum commune* belongs to the phylum of Basidiomycota and is used as a model system to study mushroom formation. The organism completes its life cycle on synthetic medium in approximately 10 days. It can be transformed using one of the three available antibiotic resistance markers (phleomycin (Schuren & Wessels, 1994), hygromycin (Mooibroek *et al.*, 1990; Scholtmeijer *et al.*, 2001) and nourseothricin (van Peer *et al.*, 2009)). Genes can be efficiently inactivated by RNAi (de Jong *et al.*, 2006) or using a deletion construct (Chapter 2; Ohm *et al.*, 2010a) in combination with a strain in which the *ku80* gene has been deleted (Chapter 3; de Jong *et al.*, 2010). In addition, the genome sequence has recently become available (Chapter 5; Ohm *et al.*, 2010b).

So far, an inducible promoter system has neither been described for *S. commune* nor for any other mushroom-forming fungus. Inducible promoters can be used in biotechnological applications to produce proteins in a specific growth phase or in (part of) the vegetative or reproductive mycelium. Inducible promoters can also be used to study gene function at a particular condition, developmental stage or position in the colony. Many inducible promoters have been described. For instance, the promoter of *hsp30* of *Aspergillus oryzae*, which encodes a heat-shock protein, was found to be highly induced simply by a short heat-treatment at 40 °C (Matsushita *et al.*, 2009). Heat shock proteins are found throughout the domains of life. They function in protection of cells against stress such as that caused by high

temperature. At low temperatures the expression of the heat shock genes can be low, but upon heat stress these genes are rapidly activated by the transcription factor HSF (heat shock factor). As a result, expression of heat shock genes increases several orders of magnitude (Santoro, 2000).

Here we show that *S. commune* contains 7 genes of the small heat shock protein family hsp26/hsp42 that are homologous to *hsp30* of *A. oryzae*. Three of these genes, *hsp1-3*, were found not to be expressed at 25 °C. However, expression was detected at 42 °C. These promoters can thus be used as an inducible system in *S. commune*.

Table 1. Primers used in this study to amplify promoter and coding sequences.
Restriction enzyme recognition sites are underlined in the primer sequence.
[a] restriction with this enzyme results in sticky ends that are compatible with HindIII.
[b] restriction with this enzyme results in sticky ends that are compatible with NcoI.

Gene	Protein ID	Primer	Primer sequence	Restriction site	Fragment length (bp)
hsp1	234587	Hsp1Pr1000Fw	GGTCTCAAGCTTGTCAGGCCGATAAGC	Eco31I [a]	1036
		Hsp1PrRev	CCATGGTAGTAGGTCGAGAGATATG	NcoI	
hsp2	51554	Hsp2Pr1000Fw	GGTCTCAAGCTTCCCGCACTTCAAAATC	Eco31I [a]	1004
		Hsp2PrRev	GGTCTCCCATGGCTGTCGAGTAGAGTATAG	Eco31I [b]	
hsp3	51927	Hsp3Pr1000Fw	AAGCTTGGCCACAATATCTCCATC	HindIII	1001
		Hsp3PrRev	CCATGGTGGGTTAGGTGAG	NcoI	
dTomato	-	TomatoFw	CCATGGTGAGCAAGGGCGAGGAG	NcoI	434
		TomatoMutRev	CTCTTCCATCGTCTTCTTCTGCATTAC	EarI	
		TomatoMutFw	CTCTTCCGATGGGCTGGGAGGCCTCCAC	EarI	295
		TomatoRev	GGATCCTTACTTGTACAGCTCGTCCATGC	BamHI	

MATERIAL AND METHODS

Strains

S. commune strain H4-8 (FGSC #9210; Fowler *et al.*, 1999) was used for transformation. Dikaryons were obtained by crossing with the compatible isogenic strain H4-8b (Chapter 2; Ohm *et al.*, 2010a).

Genes in the genome of S. commune

Genes (indicated by ProteinID) of *S. commune* are available through the interactive JGI Genome Portal at http://jgi.doe.gov/Scommune (see Chapter 5; Ohm *et al.*, 2010b). The predicted gene models and annotations are also deposited at DDBJ/EMBL/GenBank under the project accession ADMJ00000000.

Promoter analysis

Promoters are defined as the 1000 bp upstream region of the translation start site of genes. This 1000 bp region is expected to contain a core promoter and upstream regulatory elements. The MEME Motif Discovery Tool (Bailey & Elkan, 1994) was used to identify similar regions in promoters. The TOMTOM Motif Comparison Tool (Gupta *et al.*, 2007) was used to compare these regions with known motifs in the TRANSFAC database (Wingender *et al.*, 2000).

Subcloning of dTomato

The gene encoding the red fluorescent protein dTomato (Shaner *et al.*, 2004) was mutagenised in order to incorporate a NcoI site and a BamHI site at the 5' and 3' end of the coding sequence, respectively. Moreover, the NcoI site within the coding sequence was removed by changing the cytosine at position 423 into a guanine. Mutagenesis did not affect the amino acid sequence of the reporter protein and was performed using the procedure described previously (Ko & Ma, 2005). Primers used for the mutagenesis are listed in Tabel 1. The 5' part of *dTomato* was amplified from the plasmid pRSET-B-dTomato (Shaner *et al.*, 2004) using primers TomatoFw and TomatoMutRev. The 3' part of the gene was amplified from the same plasmid using primers TomatoMutFw and TomatoRev. The fragments were digested with the restriction enzyme EarI, ligated, and cloned into pGEM®-T Easy (Promega). This resulted in plasmid pRO020. The coding sequence of *dTomato* was excised using NcoI and BamHI and ligated into plasmid pGDSi3 (van Peer, 2008), which had been cut with the same enzymes. The resulting plasmid pRO151 is a pUC vector containing the *gpd* promoter of *S. commune*, the *dTomato* coding sequence, an intron, the *sc3* terminator and the phleomycin resistance gene.

Table 2. Putative heat shock genes in the genome of *S. commune* and their expression in four developmental stages as determined by Massively Parallel Signature Sequencing (MPSS).

Expression values are in TPM (tags per million). Samples were grown at 25 °C. (For more details, see Ohm *et al.*, 2010b; Chapter 5).

Gene name	Protein ID	Monokaryon	Stage I aggregates	Stage II primordia	Mushrooms
hsp1	234587	0	0	0	0
hsp2	51554	0	0	0	0
hsp3	51927	0	0	0	0
hsp4	55038	8.9	7.6	0	9.2
hsp5	232651	9.7	14.6	0	0
hsp6	53457	17.0	0	0	0
hsp7	85119	685.6	9.3	0	5.8

Subcloning of heat shock gene promoters

The regions upstream of the predicted translation start site of the heat shock genes *hsp1* (proteinID 234587), *hsp2* (proteinID 51554), and *hsp3* (proteinID 51927) were amplified from *S. commune* chromosomal DNA using Phusion polymerase (Finnzymes, Finland). Primer pairs, product sizes and introduced restriction sites are presented in Table 1. The amplified fragments were cloned into the SmaI site of pUC20. The promoters were excised from pUC20 using the restriction enzymes indicated in Table 1 and ligated into pRO151 which had been cut with HindIII and NcoI. This resulted in plasmids pRO199, pRO200, and pRO201, which contain the promoter of *hsp1*, *hsp2*, and *hsp3*, respectively, in front of the coding sequence of dTomato.

Transformation of S. commune

Transformation of *S. commune* strain H4-8 was done as described previously (van Peer *et al.*, 2009). Regeneration medium and selection plates contained 5 and 25 µg ml^{-1} phleomycin, respectively. Plates were incubated at 30 ºC for 2 days and the resulting colonies were grown for 1 day on a second selection plate containing 25 µg ml^{-1} phleomycin.

RNA isolation and Northern hybrdization

Mycelium frozen in liquid nitrogen was homogenized using the TissueLyser II (Qiagen). After lyophilization, total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions and purified using the RNeasy Mini kit (Qiagen). 1 µg total RNA was used for a Northern analysis, which was performed as described previously (Schuren *et al.*, 1993). The 290 bp sequence upstream of the start codon of *hsp3* was used as a probe, which was cut from plasmid pRO197 with the restriction enzymes XhoI and NcoI**.** The fragment was purified using the NucleoSpin Extract II kit (Macherey Nagel) and labelled with α-^{32}P-dCTP using standard protocols (Sambrook *et al.*, 1989).

Fluorescence microscopy

Fluorescence of dTomato was visualised using the Leica MZ16F stereomicroscope with dsRED3 filters (Leica Microsystems, Germany).

RESULTS

Seven *S. commune* genes encoding putative heat shock proteins (Table 2) have been annotated to KOG-ID KOG0710 "Molecular chaperone (small heat-shock protein Hsp26/Hsp42)" on basis of sequence similarity (Koonin *et al.*,

2004; Ohm *et al.*, 2010b; Chapter 5). These genes are homologous to *hsp3*0 of *Aspergillus oryzae*. The promoters of these 7 genes, called *hsp1-7*, contain at least one consensus sequence nGAAnnTTCnnGAAn. These sequences that were found 100 - 350 bp upstream of the predicted translation start site (Figure 1) show significant similarity with the consensus sequence of the binding site of the heat shock factor (TRANSFAC motif M00169, p-value 7.8E-11). This suggests that these promoters are regulated by the *S. commune* homolog of this transcription factor (the genome of *S. commune* contains two putative homologs: ProteinIDs 114760 and 234774).

Expression analysis using MPSS showed that *hsp1*, *hsp2*, and *hsp3* were not active in any of 4 developmental stages when grown at 25 °C (Table 2; Ohm *et al.*, 2010b; Chapter 5). In contrast, *hsp4-7* were expressed under these conditions in at least 1 developmental stage. The 1000 bp promoter fragments of *hsp1-3* were cloned in front of the coding sequence of the red fluorescent protein dTomato and introduced in the monokaryotic strain H4-8 of *S. commune*. Fifty transformants of each construct were screened. More than 95% of the transformants were not fluorescent when grown at 25 °C. This was expected on basis of the MPSS analysis. After an incubation of 1 hour at 42 °C, about 75% of these colonies did show fluorescence of dTomato. This was irrespective of the promoter that was used (data not shown). Absence of fluorescence at 42 °C in 25% of the transformants can be explained by a single cross-over event in the *hsp3* promoter or the *dTomato* gene, which make up about 25% of the expression construct.

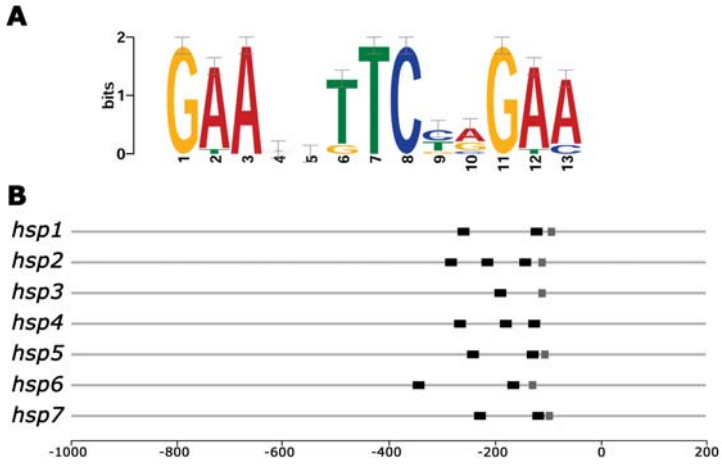

Figure 1. Promoter analysis of the putative heat shock genes *hsp1-7*.
A. Consensus sequence found in the promoters of *hsp1-7*. **B.** Locations of the consensus sequence (black box) in the promoters of *hsp1-7*, relative to the predicted translation start site. The putative TATA-box is depicted as a grey box.

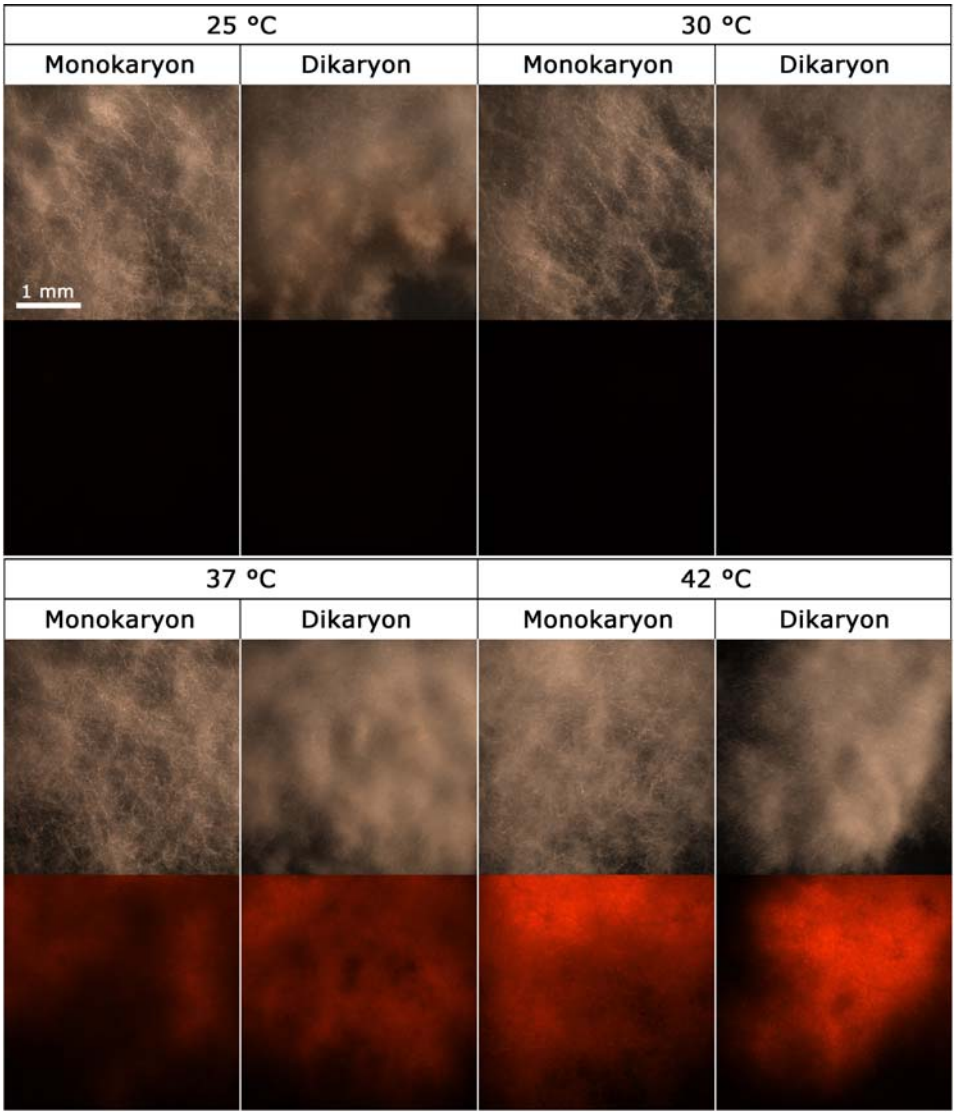

Figure 2. Fluorescence of dTomato resulting from activation of the *hsp3* promoter. Upper panels represent light microscopy images, whereas the bottom panels represent fluorescence microscopy images. At 25 °C and 30 °C no fluorescence was observed. In contrast, weak fluorescence was observed at 37 °C and strong fluorescence was detected at 42 °C. Monokaryotic and dikaryotic colonies showed similar behavior.

Expression of the *hsp3* promoter was studied in more detail in 1 of the transformants. 5 day old colonies of transformant scRO034 that had been grown at 25 °C were incubated for 1 hour at either 25 °C, 30 °C, 37 °C or 42 °C, after which they were placed back at 25 °C to allow folding of dTomato. After 1 hour, no fluorescence was observed in colonies incubated at 25 °C or

30 °C (Figure 2). However, weak fluorescence was observed after incubation at 37 °C and strong fluorescence at 42 °C. Monokaryotic and dikaryotic colonies gave an identical pattern of fluorescence. Wild type colonies showed no fluorescence in any of the conditions (data not shown). In the next experiment, a hot needle (1 mm in diameter; heated to 100 °C in boiling water) was used to locally heat the mycelium for 10 seconds. After 5 hours, fluorescence was only observed in a ring around the heated area (Figure 3). The wild type showed no fluorescence. This shows that the *hsp3* promoter can be used to locally induce gene expression.

A Northern analysis was performed using the region 290 bp upstream of the start codon of *hsp3* as a probe. By hybridizing to the non-translated part of the mRNA, the endogenous *hsp3* and *dTomato* transcripts can be detected with this probe simultaneously. No signal was observed in colonies incubated continuously at 25 °C, even after prolonged exposure of the film to the blot. After a 1 hour heat shock at 42 °C, a clear signal was observed for the endogenous *hsp3* transcripts. In the case of transformant scRO034 also a signal for *dTomato* was observed. This, however, was much weaker than that of *hsp3*. An even weaker signal was observed in the dikaryon that resulted from a cross between scRO034 and H4-8b. After a 1 hour recovery at 25 °C, the mRNA levels of endogenous *hsp3* had dropped sharply, whereas levels of *dTomato* mRNA had decreased only slightly.

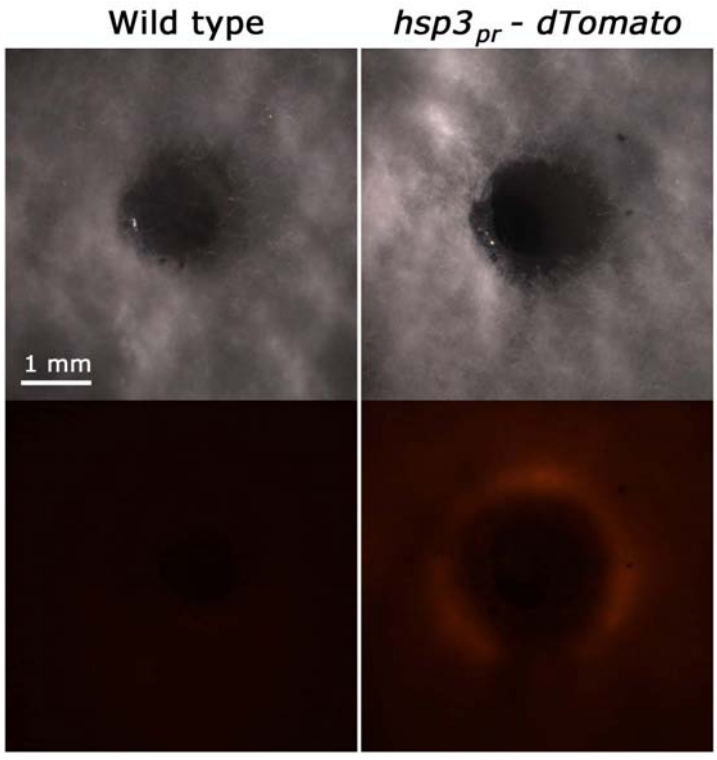

Figure 3. Local induction of *dTomato* expression from the *hsp3* promoter using a hot needle. Light (above) and fluorescence (below) microscopy of a wild type *S. commune* colony and a colony expressing *dTomato* from the *hsp3* promoter.

Importantly, the local or systemic heat shocks that were used in this study showed no effect on colony diameter, biomass and, in the case of a dikaryon, on number and shape of the mushrooms. Note that growth was very poor when *S. commune* was continuously grown at 42 °C.

DISCUSSION

Several inducible promoter systems have been described for fungi. Many of these promoters originate from genes that are involved in carbohydrate degradation. They can be repressed, uninduced or induced depending on the composition of the growth medium. For example, the promoters of the *GAL* genes of *Saccharomyces cerevisiae* are induced by GAL4 when galactose is present in the growth medium (Johnston, 1987). On the other hand, the promoters are repressed in the presence of glucose and uninduced on carbon sources other than glucose and galactose. In *Aspergillus niger*, the *suc1* promoter is induced in the presence of sucrose (Roth *et al.*, 2007), whereas the *glaA* promoter is induced in the presence of maltose or glucose (Ganzlin & Rinas, 2008). The disadvantage of promoters of metabolic genes is that they often still show some activity under repressing conditions. We here used the promoters of the *hsp1*, *hsp2* and *hsp3* genes as an inducible system for *S. commune.* They are inactive at 25 °C and highly expressed at 42 °C.

The genome of *S. commune* contains 7 predicted genes, called *hsp1-7*, that encode small heat shock proteins of the hsp26/42 family. MPSS analysis showed that *hsp4-7* were expressed at 25 °C in one or more stages in the life cycle of this basidiomycete. The other genes, *hsp1-3*, however did not show activity at 25 °C. In agreement, no fluorescence was observed at this temperature in transformants containing *dTomato* under regulation of one of these three promoters and no mRNA was detected for *hsp3* or *dTomato*. In contrast, after a heat shock at 42 °C they did show fluorescence and mRNA was shown to be present for *hsp3* and *dTomato*. From these data it is concluded that the *hsp1-3* promoters are not leaky. The *hsp3* promoter showed a dose dependent induction: weak fluorescence of dTomato was observed after a heat shock at 37 °C, whereas strong fluorescence was obtained after incubation at 42 °C. Notably, the *hsp3* promoter could be induced very locally in the mycelium simply by contact with a hot needle.

In the Northern analyses a probe was used that binds to the 5′ untranslated region of the mRNAs of the endogenous *hsp3* transcripts and the introduced *dTomato* gene. This strategy allowed for direct comparison of these transcripts. Remarkably, the *hsp3* levels were much higher than that of *dTomato.* Moreover, the mRNA levels of *hsp3* dropped rapidly when colonies

were placed back at 25 °C, whereas mRNA levels of *dTomato* remained almost constant for at least 1 hour. This shows that the *dTomato* mRNA is stable and that the low expression levels, as compared to endogenous *hsp3*, may be due to the integration site in the genome. The stability of the *dTomato* transcripts contrasts that of *hsp3.* Future studies should identify the sequence within the *hsp3* transcript that is responsible for this instability. This sequence could be inserted in the expression construct to analyze the effect of short term presence of mRNAs of a gene of interest in the mycelium. Alternatively, a protein fusion with the entire Hsp3 protein could be made.

Taken together, it is concluded that the *hsp3* promoter is an excellent inducible system for *S. commune.* It may also function in other basidiomycetes given the fact that *S. commune* promoters are expressed in other members of this phylum (Alves *et al.*, 2004).

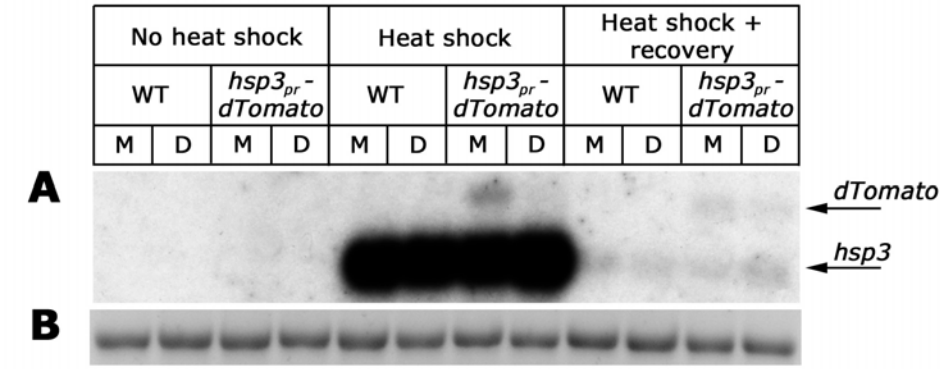

Figure 4. A. Expression of *hsp3* and *dTomato* during heat shock treatment in the wild-type strain H4-8 and the transformant scRO034 that expresses *dTomato* from the *hsp3* promoter. Both monokaryon (M) and dikaryon (D) were analysed. The 5' UTR of the *hsp3* transcript was used as a probe, allowing simultaneous detection of both transcripts. **B.** Ethidium bromide staining of 16S RNA was used as a loading control.

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This work was supported by the Dutch Technology Foundation STW, Applied Science division of NWO and the Technology Program of the Ministry of Economic Affairs.

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Chapter 5

Mushroom formation encoded in the genome sequence of *Schizophyllum commune*

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ABSTRACT

The wood degrading fungus *Schizophyllum commune* is a model system for mushroom development. Here, we describe the 38.5 Mb assembled genome of this basidiomycete and application of whole genome expression analysis to study the 13,210 predicted genes. Comparative analysis of the *S. commune* genome revealed mating type loci with the highest number of reported genes. Gene expression analyses revealed that one third of the 471 identified transcription factor genes were differentially expressed during sexual development. Two of these transcription factor genes were deleted. Inactivation of *fst4* resulted in the inability to form mushrooms, whereas inactivation of *fst3* resulted in more but smaller mushrooms than wild type. These data illustrate that mechanisms underlying mushroom formation can be dissected using *S. commune* as a model. This will impact commercial production of mushrooms and the industrial use of these fruiting bodies to produce enzymes and pharmaceuticals.

INTRODUCTION

The fungal kingdom comprises diverse and important organisms that impact agriculture, human health, carbon cycling, and biotechnology. The mushroom fruiting body is the most conspicuous form of the fungi and is found primarily in the basidiomycete group. Mushrooms produce anti-tumor and immuno-stimulatory molecules (Kües & Liu, 2000; Kothe, 2001) and enzymes that can be used for bioconversions (Lomascolo *et al.*, 1999). Moreover, they have been identified as promising cell factories for the production of pharmaceutical proteins (Berends *et al.*, 2009). The main economic value of mushrooms, however, is their use as food (Kües & Liu, 2000; Kothe, 2001). The world-wide production of edible mushrooms amounts approximately 2.5 million tons annually. Despite their economical interest, relatively little is known about how fruiting bodies are formed. Many mushroom-forming fungi cannot be cultured in the lab nor genetically modified. The basidiomycete *Schizophyllum commune* is one of the notable exceptions. It can be cultured on defined media and it completes its life cycle in approximately 10 days. Moreover, molecular tools to study growth and development of *S. commune* have been developed. In fact, it is the only mushroom-forming fungus in which genes have been inactivated by homologous recombination. The importance of *S. commune* as a model system is also exemplified by the fact that its

recombinant DNA constructs will express in other mushroom-forming fungi (Alves *et al.*, 2004).

S. commune is one of the most commonly found fungi and can be isolated from all continents except for Antarctica. *S. commune* has been reported to be a pathogen of humans and trees but it mainly adopts a saprobic life style by causing white rot (Schmidt & Liese, 1980). It is predominantly found on fallen branches and timber of deciduous trees. At least 150 genera of woody plants are substrates for *S. commune*, but it also colonizes softwood and grass silage (de Jong, 2006). The mushrooms of *S. commune* that are formed on these substrates are used as a food source in Africa and Asia.

In the life cycle of *S. commune* (Wösten & Wessels, 2006) meiospores germinate to form a sterile monokaryotic mycelium, in which each hyphal compartment contains one nucleus. This mycelium grows initially submerged but after a few days aerial hyphae are formed (Figure 1A, E). Monokaryons that are confronted with each other will fuse. A fertile dikaryon is formed when the alleles of the mating-type loci *matA* and *matB* of the partners differ. A short exposure to light is essential for fruiting, while a high concentration of carbon dioxide and high temperatures (30-37°C) are inhibitory. Mushroom formation is initiated with the aggregation of aerial dikaryotic hyphae. These stage I aggregates (Figure 1B, F) form stage II fruiting body primordia (Figure 1C, G), which further develop into mature fruiting bodies (Figure 1D, H). Karyogamy and meiosis take place in the basidia within the mature fruiting body. The resulting basidiospores can give rise to new monokaryotic mycelia.

Here, we report the genomic sequence of the monokaryotic *S. commune* strain H4-8 and demonstrate the role of this basidiomycete as a model system to study mushroom formation.

MATERIAL AND METHODS

Strains and culture conditions

S. commune was routinely grown at 25 °C on minimal medium (MM) with 1% glucose and with or without 1.5% agar (van Peer *et al.*, 2009). Liquid cultures were shaken at 225 rpm. Glucose was replaced with 4% glycerol for cultures used in the isolation of genomic DNA. All *S. commune* strains used were isogenic to strain 1-40 (Raper *et al.*, 1958). Strain H4-8 (*matA43matB41*; FGSC #9210) was used for sequencing. EST libraries were generated from H4-8 and from a dikaryon that resulted from a cross between H4-8 and strain H4-8b (*matA4 matB43*) (Chapter 2; Ohm *et al.*, 2010). Strains 4-39 (*matA41matB41;* CBS 341.81) and 4-40 (*matA43matB43;* CBS 340.81) were

used for MPSS analysis. These strains show a more synchronized fruiting compared to a cross between H4-8 and H4-8b. Partial sequencing of the haploid genome revealed that strains 4-40 and 4-39 have minor sequence differences (< 0.2%) with strain H4-8 (data not shown).

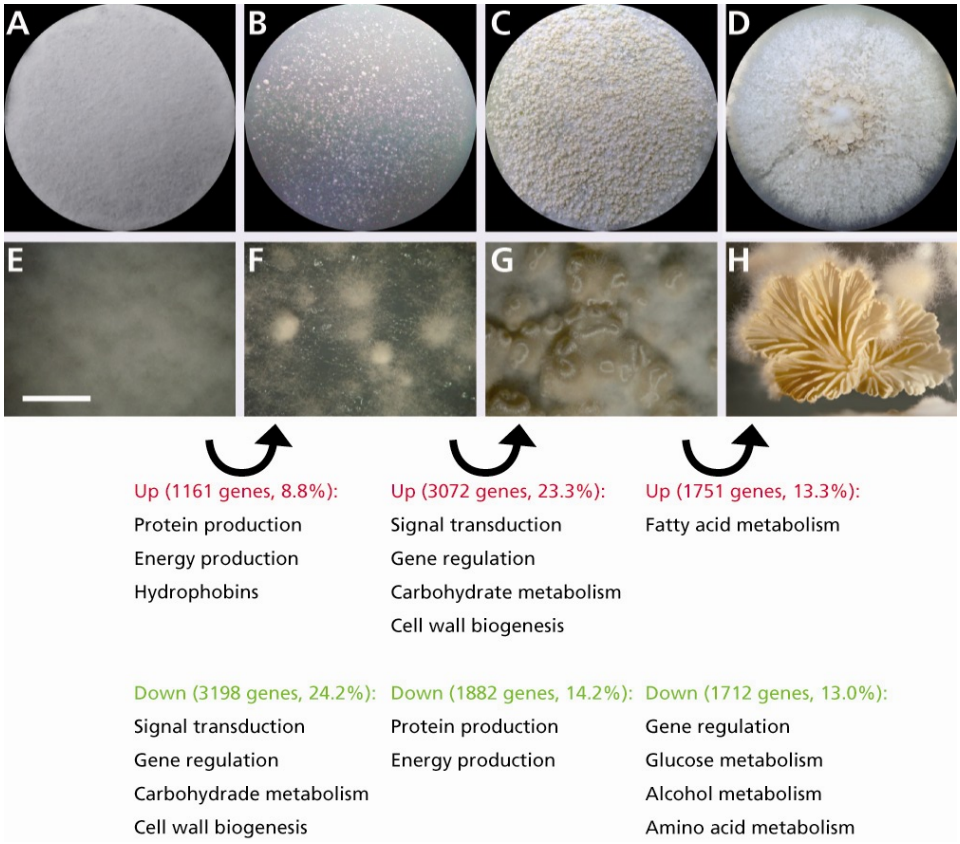

Figure 1. Development of *S. commune*. Four-day-old (A-C; E-G) and eight-day-old (D, H) colonies grown from homogenates showing typical developmental stages in the life-cycle of *S. commune*. A monokaryon forms sterile aerial hyphae that form a fluffy white layer on top of the vegetative mycelium (A, E). Aerial hyphae of a dikaryon interact with each other to form stage I aggregates (B, F) which, after a light stimulus, develop into stage II primordia (C, G). These primoridia further differentiate into sporulating mushrooms (D, H). An enrichment analysis shows that particular functional terms are over-represented in genes that are up- or down-regulated during a developmental transition. These terms are indicted below panels A-H. A-D represent cultures grown in 9 cm Petri-dishes, whereas E-H represent magnifications thereof. Bar represents 2.5 mm (E, F), 5 mm (G) and 1 cm (H).

Isolation of genomic DNA, genome sequencing and assembly

Genomic DNA of *S. commune* was isolated as described (van Peer *et al.*, 2009) and sequenced with the use of a whole-genome shotgun strategy. All data were generated by paired-end sequencing of cloned inserts with 6 different insert sizes using Sanger technology on ABI3730xl sequencers. The data were assembled using the whole-genome shotgun assembler Arachne (http://www.broad.mit.edu/wga/).

EST library construction and sequencing

Cultures were inoculated on MM plates with 1% glucose using mycelial plugs as an inoculum. Strain H4-8 was grown for 4 days in the light, whereas the dikaryon H4-8 x H4-8b was grown for 4 days in the dark and 8 days in the light. Mycelium of the dikaryotic stages was combined and RNA was isolated as described (van Peer *et al.*, 2009). The PolyA+ RNA fraction was obtained using the Absolutely mRNA Purification kit and manufacturer's instructions (Stratagene, La Jolla, CA). cDNA synthesis and cloning followed the SuperScript plasmid system procedure with Gateway technology for cDNA synthesis and cloning (Invitrogen, Carsbad, CA). For the monokaryon, two size ranges of cDNA were cut out of the gel to generate two cDNA libraries (JGI library codes CBXY for range 0.6k-2kb and CBXX for the range >2kb). For the dikaryon, cDNA was used in the range >2kb, resulting in library CBXZ. The cDNA inserts were directionally ligated into vector pCMVsport6 (Invitrogen) and introduced into ElectroMAX T1 DH10B cells (Invitrogen). Plasmid DNA for sequencing was produced by rolling circle amplification (Templiphi, GE Healthcare, Piscataway, NJ). Subclone inserts were sequenced from both ends using Big Dye terminator chemistry and ABI 3730 instruments (Applied Biosystems, Foster City, CA).

Annotation methods

Gene models in the genome of *S. commune* were predicted using Fgenesh (Salamov & Solovyev, 2000), Fgenesh+ (Salamov & Solovyev, 2000), Genewise (Birney & Durbin, 2000) and Augustus (Stanke & Waack, 2003). Fgenesh was trained for *S. commune* with a sensitivity of 72% and a specificity of 74%. Augustus *ab initio* gene predictions were generated with parameters based on *Coprinopsis cinerea* gene models (Stajich *et al.*, 2010). In addition, about 31,000 *S. commune* ESTs were clustered into nearly 9,000 groups. These groups were either directly mapped to the genomic sequence with a threshold of 80% coverage and 95% identity, included as putative full-length (FL) genes, or used to extend predicted gene models into FL genes by adding 5' and/or 3' UTRs. Since multiple gene models were generated for

each locus, a single representative model at each locus was computationally selected based on EST support and similarity to protein sequences in the NCBI non-redundant database. This resulted in a final set of 13,210 predicted genes, of which 1314 genes have been manually curated. In 66 cases, models were created or coordinates were changed.

All predicted gene models were functionally annotated by homology to annotated genes from NCBI non-redundant set and classified according to Gene Ontology (GO) (Ashburner *et al.*, 2000), eukaryotic orthologous groups (KOGs) (Koonin *et al.*, 2004), KEGG metabolic pathways (Kanehisa *et al.*, 2004), and Protein Family (PFAM) domains (Finn *et al.*, 2010).

Repeat content

RepeatModeler 1.0.3 (http://www.repeatmasker.org/RepeatModeler.html) was used to generate *de novo* repeat sequence predictions for *S. commune*. Repeats were classified by comparison to the RepBase database (Jurka *et al.*, 2005). RepeatModeler produced 76 families of repeats used as a search library in RepeatMasker (http://www.repeatmasker.org).

Data availability

S. commune assemblies, annotations, and analyses are available through the interactive JGI Genome Portal at http://jgi.doe.gov/Scommune. Genome assemblies together with predicted gene models and annotations were also deposited at DDBJ/EMBL/GenBank under the project accession ADMJ00000000.

Orthologs of* S. commune *proteins in the fungal kingdom

Proteins of *S. commune* were assigned to orthologous groups with OrthoMCL V2.0 (Li *et al.*, 2003) with an inflation value of 1.5. Members of such groups were assigned as orthologs (in the case of proteins from another species) or inparalogs (in the case of proteins from *S. commune*). Orthologs were determined in *Coprinopsis cinerea* (Stajich *et al.*, 2010)*, Laccaria bicolor* (Martin *et al.*, 2008)*, Postia placenta* (Martinez *et al.*, 2009)*, Phanerochaete chrysosporium* (Martinez *et al.*, 2004)*, Cryptococcus neoformans* (Loftus *et al.*, 2005)*, Ustilago maydis* (Kamper *et al.*, 2006)*, Saccharomyces cerevisiae* (Goffeau *et al.*, 1996)*, Aspergillus nidulans* (Galagan *et al.*, 2005) and *Neurospora crassa* (Galagan *et al.*, 2003). All versus all BLASTp analysis was performed using NCBI standalone BLAST v2.2.20 with an E-value of 1e-5 as a cut-off. Custom scripts were used to further analyse the orthologous groups resulting from the OrthoMCL analysis. The evolutionary conservation for each

orthologous group was expressed as the taxon this orthologous group was most specifically confined to (Figure 2).

Representation analysis

FuncAssociate 2.0 (Berriz *et al.*, 2009) was used to study over- and under-representation of taxon-specific genes and of functional annotation terms in sets of differentially regulated genes. Default settings were used with a p-value of 0.05 as the cut off.

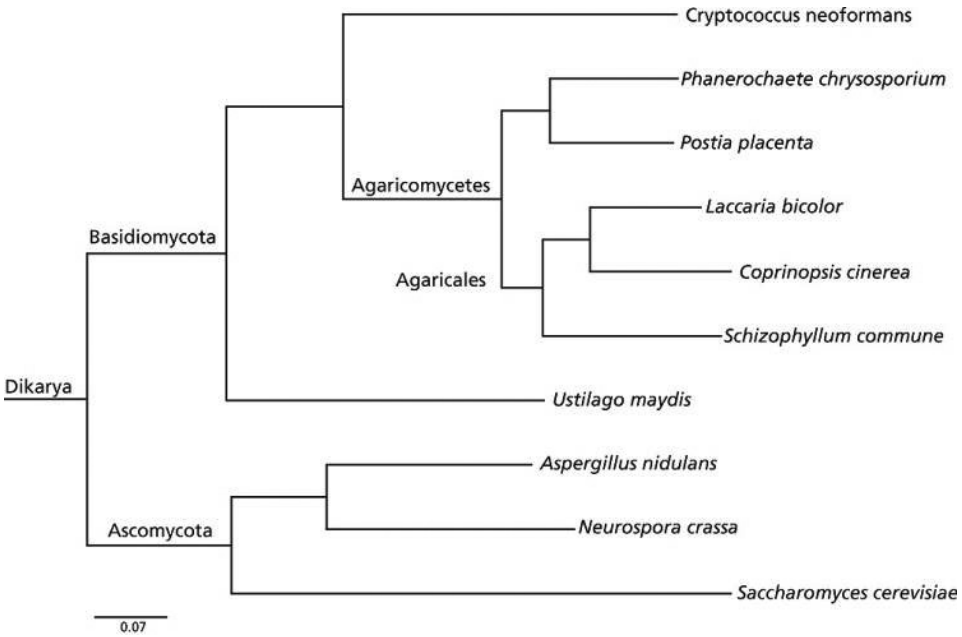

Figure 2. Phylogenetic tree of the fungal species used in comparison of *S. commune* proteins. The tree was built with MrBayes (Ronquist & Huelsenbeck, 2003) from 432 single-copy orthologs found in all genomes as identified with OrthoMCL. Bar represents 0.07 substitutions per site.

Protein families

The PFAM database version 24.0 (Finn *et al.*, 2010) was used to identify PFAM protein families. Custom scripts in Python were written to group genes on basis of their PFAM domains. Differences in the number of predicted proteins belonging to a PFAM family across the fungal domain was determined using the Student's t-test. When Agaricales were compared to the rest of the Dikarya or when *S. commune* was compared to the Agaricales, only groups with a minimum of 5 members in at least one of the fungi were analysed. In the case *S. commune* was compared to the rest of the Dikarya, only groups with a minimum of 5 members in at least four of the fungi were analysed. In

all cases, a p value of 0.05 was used as a cut off. Similar results were obtained using the non-parametric Mann-Whitney-U test.

MPSS expression analysis

Total RNA was isolated from the monokaryotic strain 4-40 and from the dikaryon resulting from a cross between 4-40 and 4-39. A 7-day-old colony grown on solid MM at 30 ^{o}C in the dark was homogenized in 200 ml MM using a Waring blender for 1 min at low speed. 2 ml of the homogenized mycelium was spread out over a polycarbonate membrane (Profiltra, pore size 0.1 µm and diameter 76 mm) that was placed on top of solidified MM. Vegetative monokaryotic mycelium was grown for 4 days in the light. The dikaryon was grown for 2 and 4 days in the light to isolate mycelium with stage I aggregates and stage II primordia, respectively. Mature mushrooms of 3 days old were picked from dikaryotic cultures that had grown for 8 days in the light. RNA was isolated as described (van Peer *et al.*, 2009). MPSS was performed essentially as described (Brenner *et al.*, 2000) except that after DpnII digestion MmeI was used to generate 20 bp tags. Tags were sequenced using the Clonal Single Molecule Array technique (Illumina, Hayward, CA, US). Between 4.2 and 7.6 million tags of 20 bp were obtained for each of the stages. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.*, 2002) and are accessible through GEO Series accession number GSE21265 (http://www.ncbi.nlm.nih.gov/geo/). Programs were developed in the programming language Python to analyze the data. Tag counts were normalized to tags per million (TPM). Those with a maximum of < 4 TPM in all developmental stages were removed from the data set. This data set consisted of a total of 40,791 unique tags. Of these tags, 61.7% and 58.6% could be mapped to the genome sequence and the predicted transcripts, respectively, using a perfect match as the criterion. The mapped tags accounted for 71.4% and 70.8% of the total number of tags, respectively. For comparison, 97.4% of the ESTs from *S. commune* strain H4-8 could be mapped to the assembly. Unmapped tags can be explained by sequencing errors in either tag or genomic DNA. Moreover, RNA editing may have altered the transcript sequencing to produce tags that do not match the genome perfectly. It may also be that the assigned UTR is incomplete or that the DpnII restriction site that defines the 5’ end of the tag is too close to the polyA-tail of the mRNA. TPM values of tags originating from the same transcript were summed to assess their expression levels. A transcript is defined as the predicted coding sequence (CDS) extended with 400 bp flanking regions at both sides.

Comparison of gene expression in L. bicolor *and* S. commune

Whole genome expression analysis of *L. bicolor* (Martin *et al.*, 2008) and *S. commune* was done essentially as described (McCarroll *et al.*, 2004). For *L. bicolor*, the microarray values from replicates were averaged. Expression values of genes were increased by one and the ratio between monokaryon and mushrooms (for *S. commune*) and free-living mycelium and mature fruiting bodies (for *L. bicolor*) was log-transformed. All expressed genes from *S. commune* that had at least 1 expressed ortholog in *L. bicolor* were taken into account, resulting in a total of 6751 orthologous pairs. These pairs were classified on basis of functional annotation terms. Correlation of changes in expression of these gene classes was expressed as the Pearson correlation coefficient. Only GO-terms with 10-200 pairs were used in the analysis. In the case of PFAM domains, a minimum number of 10 ortholog pairs were used.

Deletion of transcription factors fst3 *and* fst4

The transcription factor genes *fst3* (proteinID: 257422) and *fst4* (proteinID: 66861) were deleted using vector pDelcas (Chapter 2; Ohm *et al.*, 2010). Transformation of *S. commune* strain H4-8 was done as described (van Peer *et al.*, 2009). Regeneration medium contained no antibiotic, whereas selection plates contained 20 µg ml^{-1} nourseothricin. Deletion of the target gene was confirmed by PCR. Compatible monokaryons with a gene deletion were selected from spores originating from a cross of the mutant strains with wild type strain H4-8b.

RESULTS

Genome of S. commune

Sequencing genomic DNA of *S. commune* strain H4-8 with 8.29x coverage (Supplementary Table 1) revealed 38.5 Megabase genome assembly with 11.2% repeat content (Supplementary Text 1). The assembly is contained on 36 scaffolds (Supplementary Table 2), which represent 14 chromosomes (Ásgeirsdóttir *et al.*, 1994). A total of 13,210 gene models are predicted, with 42% supported by expressed sequenced tags (ESTs) and 69% being similar to proteins from other organisms (Supplementary Tables 3 and 4). Clustering of the proteins of *S. commune* with those of other sequenced fungi (a phylogenetic tree of the organisms used in the analysis is shown in Figure 2) resulted in 7055 groups containing at least one *S. commune* protein (Supplementary Table 5). Analysis of these clusters suggested that 39% of the *S. commune* proteins have orthologs in the Dikarya and are thus conserved in the Basidiomycota and Ascomycota (Supplementary Table 6).

Intriguingly, a similar percentage of proteins (36%) are unique to *S. commune.* Of these proteins, 46% have at least one inparalog (*i.e.* a gene resulting from a duplication within the genome) in *S. commune.* Predicted proteins of *S. commune, C. cinerea, L. bicolor, P. chrysosporium, P. placenta, C. neoformans, U. maydis, N. crassa, A. nidulans* and *S. cerevisiae* were analysed for the presence of protein family (PFAM) domains. We assessed which of these domains are over- and under-represented in the genomes of *S. commune* and the Agaricales (*i.e. L. bicolor*, *C. cinerea* and *S. commune*). 53 PFAMs are enriched in the Agaricales, whereas 4 protein families are under-represented (Supplementary Table 7). Over-represented PFAMs include the fungal hydrophobin domain, the transcription factor homeobox domain, the zf-MYND domain, and the fungal mating type pheromone. Within the Agaricales, *S. commune* contains 37 PFAM families with at least two-fold more members and 18 families with at least two-fold less members (Supplementary Table 8). For instance, *S. commune* has more members of several glycosyl hydrolase families and a decrease in several multicopper oxidase families when compared to *L. bicolor* and *C. cinerea*. Compared to all other fungi, *S. commune* has no members of the AIG1 family and an increased number of members from the thaumatin family, both of which have been implicated in defense against pathogens (Supplementary Table 9). The uniqueness of the *S. commune* proteome is also illustrated by the fact that only 43% of the predicted genes (5,703 out of the 13,210) could be annotated with a gene ontology (GO) term.

Global gene expression analysis

Whole genome expression was analysed in four developmental stages (monokaryon, stage I aggregates, stage II primordia, and mature fruiting bodies; see Figure 1) using Massively Parallel Signature Sequencing (MPSS). The majority of genes are either expressed in all stages (4859 genes) or not expressed (5308 genes) (Figure 3 A,D; Supplementary Table 10). 59.8% of the 13,210 predicted genes are expressed in at least 1 developmental stage. Fewer of the unique *S. commune* genes meet this threshold, whereas a higher percentage is observed for genes that share orthologs with Agaricomycetes or more distant fungi (Supplementary Table 6). This suggests that genes specific to *S. commune* are subject to a more stringent regulation. This is consistent with the observation that *S. commune* specific genes are over-represented in the pool of genes that are differentially expressed during the four developmental stages (Supplementary Tables 11 and 12).

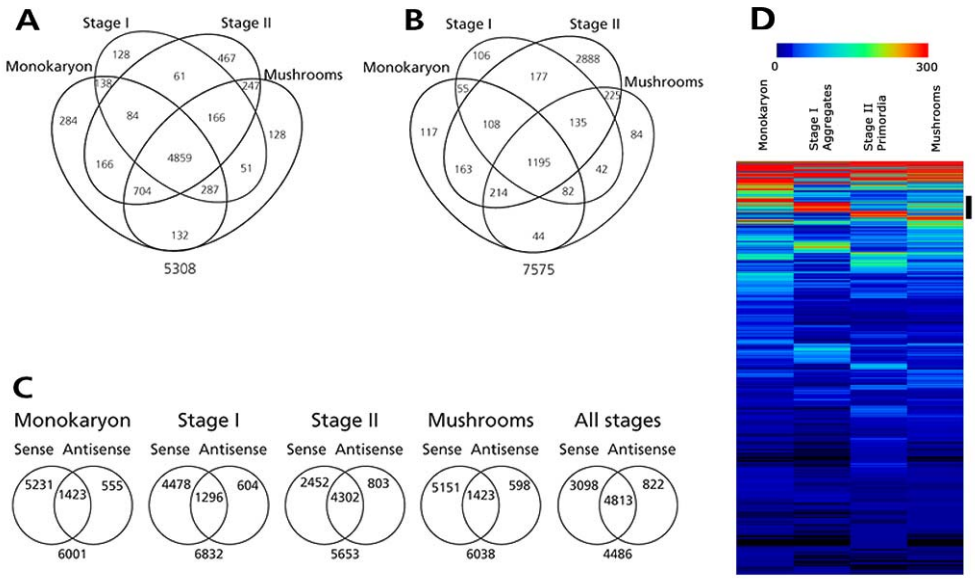

Figure 3. Gene expression in four developmental stages of *S. commune* illustrated by VENN diagrams (A-C) and a heat map (D). The cut-off for expression is 4 TPM (tags per million). VENN diagrams in (A) and (B) show the overlap of genes that are expressed in sense and anti-sense direction in the four developmental stages, respectively. As an example, in (A) 61 genes are expressed in sense direction in stage I and stage II, 4859 genes are expressed in all stages, 132 genes are expressed in the monokaryon and mature fruiting bodies and 5308 genes are not expressed in any of the stages. (C) VENN diagram of the overlap in genes that show sense and anti-sense expression in a particular developmental stage and with all stages combined. (D) Heat map of expression of expressed *S. commune* genes in the four developmental stages. Bar on top of the panel represents expression values between 0 and 300 TPM. Genes with expression values higher than 300 TPM are also indicated in red. The bar on the right indicates a cluster of 366 highly expressed and differentially regulated genes. Annotation information of the genes in this cluster is given in Supplementary Table 18.

Anti-sense transcription is a widespread phenomenon in *S. commune*. 18.7% of the tags that could be related to a gene model originate from an anti-sense transcript. 42.3% of the predicted genes have anti-sense expression in one or more developmental stages (Supplementary Tables 10 and 13). Northern hybridization with strand-specific probes confirmed the existence of anti-sense transcripts of *sc4* (Protein ID 73533) (data not shown). In the anti-sense direction, a relatively large number of genes are uniquely expressed in stage II (2888 genes) and relatively few genes are expressed in all stages (1195 genes) (Figure 3B). In stage II, 4302 genes are expressed in both sense and anti-sense direction (Figure 3C). This overlap is large compared to the other developmental stages.

Fruiting body development

An enrichment analysis of functional annotation was performed on the expression profiles of the four developmental stages (monokaryon, stage I aggregates, stage II primordia and mature fruiting bodies). Functional terms involved in protein production, energy production and hydrophobins are over-represented in genes that were up-regulated during formation of stage I aggregates (Figure 1 and Supplementary Table 12). Genes involved in signal transduction, regulation of gene expression, cell wall biogenesis and carbohydrate metabolism are enriched in the group of down-regulated genes during formation of stage I aggregates. These functional terms are enriched in the up-regulated genes during formation of stage II primordia, whereas terms involved in protein and energy production are enriched in the down-regulated genes (Figure 1 and Supplementary Table 12). During formation of mature fruiting bodies, genes encoding transcription factors, and genes involved in amino acid, glucose and alcohol metabolism are enriched in the group of down-regulated genes.

Whole genome expression analysis during mushroom formation has also been performed in *Laccaria bicolor* (Martin *et al.*, 2008). Regulation of orthologous gene pairs of *L. bicolor* and *S. commune* could therefore be correlated during fruiting. To this end, microarray expression profiles of free-living mycelium and mature fruiting bodies of *L. bicolor* were compared to the MPSS expression profiles of monokaryotic mycelium and mature fruiting bodies of *S. commune.* 6751 expressed genes from *S. commune* had at least 1 expressed ortholog in *L. bicolor.* The correlation of changes in expression of the functional annotation terms to which these orthologous pairs belong was determined. There were 15 GO terms, 2 KEGG terms, 4 KOG terms and 4 PFAM terms that showed a positive correlation in expression ($p < 0.01$) (Supplementary Table 14). These terms include metabolic pathways (such as valine, leucine and isoleucine biosynthesis) and regulatory mechanisms (such as transcriptional regulation by transcription factors and signal transduction by G-protein alpha subunit). This indicates that regulation of these processes during mushroom formation is conserved in *S. commune* and *L. bicolor*.

Analysis of specific gene groups

Formation of a fertile dikaryon is regulated by the *matA* and *matB* mating type loci. Proteins encoded in these loci activate signalling cascades (Supplementary Text 2), thus regulating target genes. These target genes include proteins that fulfil structural functions such as hydrophobins and enzymes. As a result, fruiting bodies are formed.

matA

The *matA* locus of strain H4-8 appears to have the highest homeodomain gene number in a fungal mating type locus described so far. This locus consists of two subloci, *Aα* and *Aß*, which are separated by 550 kb on chromosome I of strain H4-8. Annotation revealed that the *Aα* locus of H4-8 contains two divergently transcribed genes encoding Y and Z homeodomain proteins of the HD2 and HD1 class, respectively (Figure 4; Supplementary Table 15). These two genes, *aay4* and *aaz4*, have been previously described (Kothe, 2001). One homeodomain gene had also been identified in the *Aß* locus of H4-8 (Shen *et al.*, 2001). The genomic sequence revealed that this locus actually contains six predicted homeodomain genes *abq6* (HD1), *abr6* (HD2), *abs6* (HD1), *abt6* (HD1, but lacking the NLS), *abu6* (HD1) and *abv6* (HD2) (Figure 4; Supplementary Table 15).

matB

Annotation of the genomic sequence of *S. commune* has revealed that the *matB* system contains more genes than previously envisioned. The *matB* locus comprises two linked loci *Bα* and *Bß*, which encode pheromones and pheromone receptors (Figure 4; see Kothe, 2001). Previously, one pheromone receptor gene was identified both in *Bα3* and *Bß2* of strain H4-8 (called *bar3* and *bbr2,* respectively) (Fowler *et al.*, 2001). The genome sequence revealed four additional genes with high sequence similarity to these pheromone receptor genes, which we call *B* receptor-like genes 1-4 (*brl1-4*) (Figure 4). Three of these genes are located near *bar3* and *bbr2* on scaffold 10, whereas one (*brl4*) is located on scaffold 8. MPSS analysis showed that the *brl* genes are expressed (Supplementary Table 16). In fact, of all receptor and receptor-like genes, *brl3* shows the highest expression under the conditions tested.

Three and eight pheromone genes have been previously identified in the *Bα3* and *Bß2* loci, respectively (Fowler *et al.*, 2004). One additional pheromone gene, *bpl5* (*B* pheromone-like), has been identified in the *Bα3* locus. Moreover, four additional pheromone-like genes were detected in the *Bß2* locus, called *bpl1-4* (Figure 4). Based on the MPSS analysis, only *bpl2* failed to show expression (Supplementary Table 16).

Table 1. Predicted photoreceptors of *S. commune* and their expression (in tags per million, TPM) as analyzed by MPSS.

Protein ID	Name	Monokaryon	Stage I	Stage II	Mushrooms
78657	*wc-1*	0	0	0	0
13988	*wc-2*	18.9	18.3	32.3	42.4
76719	*cry*	0	0	0	0
49182	*phy*	140.8	46.4	55.5	59.7

Table 2. Predicted hydrophobin genes of *S. commune* and their expression (in tags per million, TPM) as analyzed by MPSS.

Protein ID	Name	Monokaryon	Stage I	Stage II	Mushrooms
77028	*sc3*	12151.5	669.9	1538.9	837.0
73533	*sc4*	25.8	4843.2	5696.2	4076.8
13677	*sc1*	5.6	2688.2	6002.7	630.1
13059	*sc6*	0.9	265.7	576.2	34.0
58269	*hyd1*	0	6.7	6.0	77.8
82440	*hyd2*	186.1	10.9	79.9	4.3
232866	*hyd3*	0	3.3	28.7	5.5
51576	*hyd4*	0	65.1	47.7	49.4
250297	*hyd5*	0	0	0	0
85429	*hyd6*	425.5	94.1	296.0	471.0
109698	*hyd7*	1.5	87.8	552.2	1074.8
105121	*hyd8*	0.9	688.1	2389.8	176.8
249002	*hyd9*	2.4	249.8	1.0	2.9

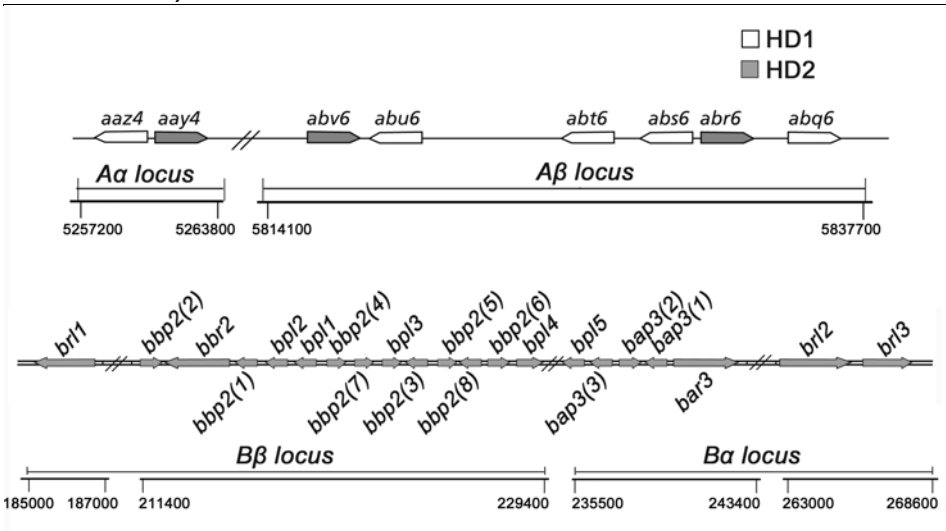

Figure 4. Distribution of genes encoding HD1 and HD2 homeodomain proteins in the *matA* locus (above) and genes encoding pheromone receptors and pheromones in the *matB* locus (below) of *S. commune* strain H4-8. The *matA* and *matB* loci are positioned on scaffold 1 and 10, respectively. One additional pheromone receptor gene, *brl4*, has been identified on scaffold 8.

Light signalling in S. commune

A short exposure to blue light (448 nm) is needed for development of aggregates into primordia (Perkins & Gordon, 1969). *S. commune* contains homologues of *wc-1* and *wc-2*, which encode central components in blue light signal transduction in *N. crassa* (Linden, 2002). The *wc-2* homologue is constitutively expressed, whereas the *wc-1* homologue was not shown to be transcribed during the stages tested (Table 1). The latter is remarkable considering the fact that the *wc-1* homologue of *C. cinerea*, called *dst1,* is involved in mushroom formation. Strains lacking *dst1* show rudimentary fruiting body development similar to dark-grown mushrooms (Terashima *et al.*, 2005). *S. commune* also contains a putative cryptochrome (Table 1), which would respond to blue light and a phytochrome which would be activated by red and far-red light. Only the latter was shown to be expressed, predominantly in the monokaryon. This phytochrome may modify the activity of WC-2, and possibly WC-1, as was suggested in *N. crassa* (Olmedo *et al.*, 2010).

Hydrophobins

The *S. commune* genome contains 13 class I hydrophobin genes (Table 2). Two of these have been studied previously. The SC3 hydrophobin enables hyphae to escape into the air by lowering the surface tension and provides aerial hyphae with a hydrophobic coating. The SC4 hydrophobin lines air channels in fruiting bodies preventing water entry by capillary force (Wösten, 2001). Expression of *sc3* and *sc4* transcripts peak in the monokaryon, and during formation of stage II primordia, respectively. Of the other hydrophobin genes, expression of one peaks in the monokaryon, two peak during formation of stage I aggregates, five during formation of stage II primordia and three in the mature fruiting bodies (Table 2). The presence of 13 hydrophobin genes allows not only temporal but also spatial regulation of hydrophobin production. Moreover, these proteins may have evolved to fulfil specific functions during mushroom development.

Transcription factors

Genes for 471 putative transcription factors have been identified in the genome of *S. commune*, of which 311 are expressed in at least one developmental stage (Supplementary Table 17). Of these genes, 56% are expressed in all developmental stages. 268, 200, 283 and 253 of these transcription factor genes were expressed, respectively, in the monokaryon, and during formation of stage I aggregates, stage II primordia and mushrooms. Interestingly, a cluster of monokaryotic specific transcription

factors and a cluster of transcription factors that are upregulated in stage II primordia and/or in mature mushrooms were identified (Figure 5). The latter cluster includes *fst3* (Protein ID: 257422) and *fst4* (Protein ID: 66861). These genes encode transcription factors that contain a fungal specific Zn(II)2Cys6 zinc finger DNA binding domain.

Genes *fst3* and *fst4* were inactivated by targeted gene deletions. The Δ*fst3* and Δ*fst4* monokaryons showed no phenotypic differences when compared to the wild type. In contrast, the Δ*fst4*Δ*fst4* dikaryon did not fruit but produced more aerial hyphae when compared to the wild type (Figure 6). Apparently, Fst4 is involved in the switch between the vegetative phase and the reproductive phase. The Δ*fst3*Δ*fst3* dikaryon did form fruiting bodies. In fact, the mutant formed more, but smaller reproductive structures than those of the wild type (Figure 6). Spatial and temporal regulation of fruiting body formation and sporulation was not altered in the Δ*fst3*Δ*fst3* strain. From these data we conclude that Fst3 inhibits formation of clusters of mushrooms.

DISCUSSION

The phylum Basidiomycota contains roughly 30,000 described species, accounting for 37% of the true fungi (Kirk *et al.*, 2001). The Basidiomycota comprises of two class level taxa (Wallemiomycetes, Entorrhizomycetes) and the subphyla Pucciniomycotina (rust), Ustilaginomycotina (smuts), and Agaricomycotina (Hibbett *et al.*, 2007). The Agaricomyoctina include the mushroom and puffball forming fungi, crust fungi, and jelly fungi. Currently five genomic sequences of Agaricomycotina are available: *P. chrysosporium* (Martinez *et al.*, 2004), *L. bicolor* (Martin *et al.*, 2008), *P. placenta* (Martinez *et al.*, 2009), *C. neoformans* (Loftus *et al.*, 2005) and *C. cinerea* (Stajich *et al.*, 2010). We here report the 38.5 Megabase genome assembly of *S. commune,* which represents the first genomic sequence of the family of the Schizophyllaceae. The genome of *S. commune* is predicted to have 13,210 genes. 36% of the encoded proteins have no ortholog in other fungi. The fact that much about the proteome of *S. commune* is still unknown is also illustrated by the fact that only 43% of the predicted genes could be annotated with a gene ontology (GO) term. This is a similar percentage as seen in other basidiomycetes: 30% in *L. bicolor* (Martin *et al.*, 2008), 48% for *P. placenta* (Martinez *et al.*, 2009) and 49% for *P. chrysosporium* (Martinez *et al.*, 2004).

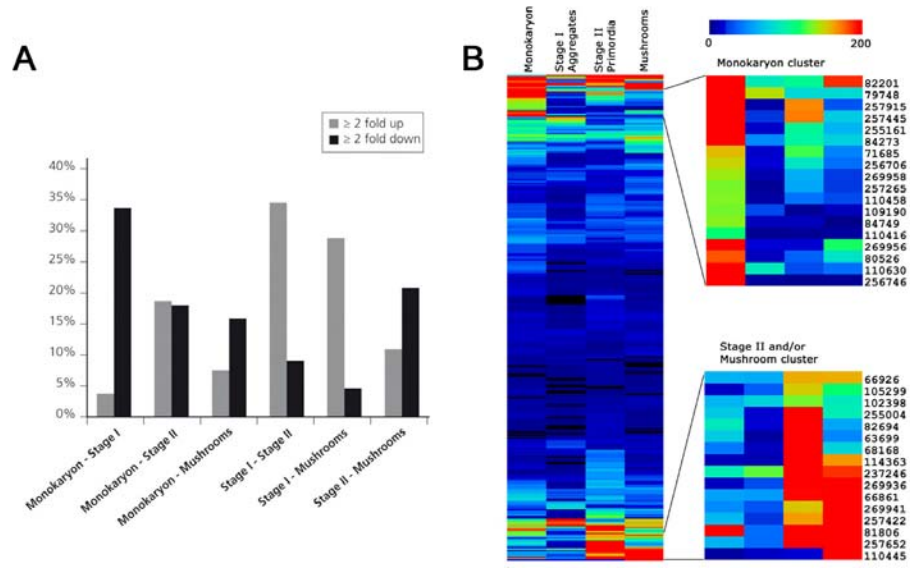

Figure 5. Histogram (A) and heat map (B) of expression of the 471 transcription factors in the genome of *S. commune*. The histogram (A) shows the percentage of transcription factor genes that are differentially expressed between stages of development. The heat map (B) shows a cluster containing predominantly monokaryon specific transcription factors and a cluster containing predominantly stage II and/or mushroom specific transcription factors. These clusters are enlarged on the right part of the heat map. The latter group contains two fungal specific transcription factor genes, called *fst3* and *fst4* (protein IDs 257422 and 66861, respectively).

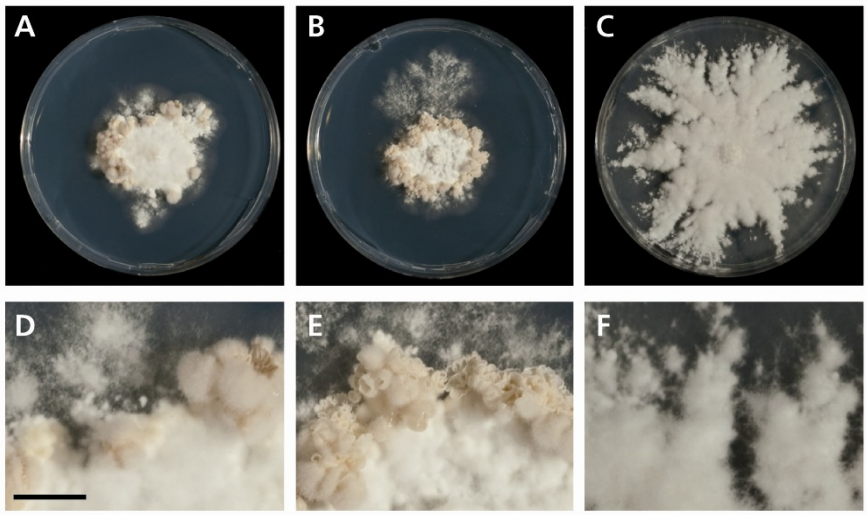

Figure 6. Fruiting body formation in a wild type dikaryon (A, D) and in dikaryons in which *fst3* (B, E) and *fst4* (C, F) have been inactivated.
D-F show a magnification of part of the colonies shown in A-C. Bar represents 5 mm (D-F). Colonies have been grown for 10 days in continuous light.

The mating type loci

Monokaryons of *S. commune* will fuse when they are confronted with each other. Formation of a fertile dikaryon is regulated by the *matA* and *matB* mating type loci. The genome sequence has revealed that *matA* and *matB* of *S. commune* represent the fungal mating type loci that contain the highest number of genes. The *matB* locus comprises two linked loci *Bα* and *Bß*, which encode pheromones and pheromone receptors (Kothe, 2001). Nine allelic specificities have been identified for both loci, resulting in 81 different mating types for *matB*. It was previously described that the *Bα3* and *Bß2* loci of H4-8 each contain one pheromone receptor gene and three and eight pheromone genes, respectively (Fowler *et al.*, 2001; Fowler *et al.*, 2004). Five additional pheromone genes and 4 pheromone receptor-like genes were identified in the genome of H4-8. These newly identified receptor-like genes are present in a *matB* deletion strain, which has no pheromone response with any mate (T. Fowler, unpublished results). This raises the question whether the four receptor genes are functional in *matB*-regulated development. MPSS analysis showed that they are all expressed, which suggests that they do not represent pseudogenes.

The *matA* locus consists of two subloci, *Aα* and *Aß*, of which 9 and 32 alellic specificities are expected to occur in nature (Kothe, 2001). These loci are separated by 550 kb on chromosome I of strain H4-8. This large distance has not been found in other fungi that have a tetrapolar mating system. The functionally well-characterized *Aα* locus showed no significant differences from the published descriptions (Kothe, 2001). It is composed of two genes encoding Y and Z homeodomain proteins of the HD2 and HD1 class, respectively. The Y and Z proteins, as in other basidiomycetes, interact in non-self combinations to activate the A-pathway of sexual development (Spit *et al.*, 1998; Kothe, 2001). Notably, a nuclear localization signal is present in Y but not in Z. This is consistent with non-self interaction of the two proteins taking place in the cytosol, which is followed by the translocation of the active protein complex into the nucleus (Kothe, 2001).

Aß of *S. commune* has been studied much less compared to the *Aα* locus. Interestingly, *Aß* reflects the highest homeodomain gene complexity of a fungal mating type locus described to date. It contains 4 homeodomain genes of the HD1 class and two of the HD2 class. The *Aß* locus of *S. commune* thus resembles that of *C. cinerea*, which consists of two pairs of functional HD1 and HD2 homeodomain genes (b and d) (Casselton & Olesnicky, 1998). The large number of genes in *matAß* would explain why recombination analyses predict as many as 32 mating specificities for this locus (Raper, 1966).

Whole genome expression

Little is known about molecular processes that control formation of fruiting bodies in basidiomycetes other than the role of the mating type loci (Wösten & Wessels, 2006). Therefore, we performed a whole genome expression analysis in four developmental stages (*i.e.* in the sterile monokaryon, and in stage I aggregates, stage II primordia, and mature fruiting bodies of the dikaryon; see Figure 1). MPSS showed that relatively few genes were specifically expressed in the monokaryon (284 genes) and in stage I aggregates and the mature mushrooms (128 genes in both cases). Interestingly, 467 genes were specifically expressed in stage II primordia. This suggests that this stage represents a major developmental switch. This is supported by the fact that genes involved in signal transduction and regulation of gene expression are enriched in the group of up-regulated genes during formation of stage II primordia. Of interest, expression of these gene groups, among others, show a positive correlation during mushroom formation of *S. commune* and *L. bicolor.* This suggests that regulation of mushroom formation is a conserved process in the Agaricales.

Anti-sense expression was found to occur widely in *S. commune*. About 20% of all sequenced mRNA tags originated from an anti-sense transcript and more than 5600 of the predicted genes showed anti-sense expression in one or more developmental stages. Anti-sense transcription was most pronounced in stage II primordia. In this stage, more than 4300 genes were expressed in both sense and anti-sense direction and more than 800 genes were expressed in the anti-sense direction only. Previously, MPSS showed anti-sense transcripts in *Magnaporthe grisea* (Gowda *et al.*, 2006). Little is known about the function of these transcripts in fungi. The circadian clock of *N. crassa* is entrained in part by the action of an anti-sense transcript of a clock component locus (Kramer *et al.*, 2003), possible by RNA interference. It is tempting to speculate that anti-sense transcripts also regulate mRNA levels in *S. commune*. Natural anti-sense transcripts in eukaryotes have also been implicated in other processes such as translational regulation, alternative splicing and RNA editing (Lavorgna *et al.*, 2004). The anti-sense transcripts of *S. commune* may also have such functions. In all these cases, the anti-sense transcripts could be functional in the developmental switch that would occur when stage II primordia are formed.

Transcriptional regulators

The fact that gene regulation seems to be conserved in the Agaricales made us decide to study the transcriptional regulators in more detail. 471 genes were identified that are predicted to encode a transcription factor. 268 of

these transcription factors were expressed in the monokaryon, whereas 200, 283 and 253 were expressed during formation of stage I aggregates, stage II primordia and mushrooms, respectively. The relatively high number of transcription factors that are expressed during formation of stage II primordia again point to a major switch that takes place during this developmental stage.

A cluster of monokaryotic specific transcription factors and a cluster of transcription factors that are up-regulated in stage II primordia and/or in mature mushrooms were identified. Genes *fst3* and *fst4* that encode transcriptional regulators with a fungal specific Zn(II)2Cys6 zinc finger DNA binding domain belong to the latter cluster and were inactivated by targeted gene deletion. Growth and development of monokaryotic strains in which *fst3* or *fst4* were inactivated were not affected. Phenotypic differences, however, were observed in the dikaryon. The Δ*fst4*Δ*fst4* dikaryon did not fruit but produced more aerial hyphae when compared to the wild type. On the other hand, the Δ*fst3*Δ*fst3* dikaryon formed more, but smaller, fruiting bodies than the wild type. Taken together, we conclude that Fst4 is involved in the switch between the vegetative and the reproductive phase and that Fst3 inhibits formation of clusters of mushrooms. Inhibition of such clusters could be important in a natural environment to ensure sufficient energy is available for full fruiting body development. Interestingly, *fst3* and *fst4* have homologs in other mushroom forming fungi and it is tempting to speculate that they have a similar function in these organisms. This is supported by the observation that the homologs of *fst3* and *fst4* are up-regulated in young fruiting bodies of *L. bicolor* compared to free-living mycelium (Martin *et al.*, 2008). In mature fruiting bodies of *L. bicolor*, the expression level of the homolog of *fst3* remains constant compared to young fruiting bodies, whereas the *fst4* homolog returns to the level of expression in the free-living mycelium.

SUMMARY

The genomic sequence of *S. commune* will be an essential tool to unravel mechanisms by which mushroom forming form their reproductive structures. The deep capture of gene expression via MPSS has provided us with leads on how mushroom formation is regulated. In addition to certain transcription factors, anti-sense transcription may play an important role in this process. Without doubt, the understanding of physiology and sexual reproduction of *S. commune* will have an impact on the commercial production of edible mushrooms and the use of mushrooms as a cell factory.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY DATA

All supplementary data can be found at http://tinyurl.com/robinohmthesis or obtained from the author.

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Chapter 6

Inactivation of transcription factor genes reveals a regulatory model for mushroom formation in *Schizophyllum commune*

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ABSTRACT

Mushrooms are among the most complex structures of fungi. They are an important food source and are of industrial and medical interest. Despite this, little is known about the regulation of mushroom formation. Previously, we showed that the genome of the mushroom-forming basidiomycete *Schizophyllum commune* contains 472 genes encoding predicted transcription factors. Of these, *fst3* and *fst4* were shown to inhibit and induce mushroom development, respectively. Here, we inactivated 5 additional transcription factor genes. This resulted in the absence of mushroom development (in the case of Δ*bri1*Δ*bri1* and Δ*hom2*Δ*hom2* dikaryons), in arrested development in stage I aggregates (in the case of the Δ*c2h2*Δ*c2h2* dikaryon) and in the formation of more but smaller mushrooms (in the case of Δ*hom1*Δ*hom1* and Δ*gat1*Δ*gat1* dikaryons). Moreover, it was shown that colony morphology was affected in the Δ*hom2*Δ*hom2* and Δ*bri1*Δ*bri1* dikaryons. The wild type dikaryons formed irregular colonies, whereas the shape of the Δ*hom2*Δ*hom2* and Δ*bri1*Δ*bri1* colonies was more symmetrical. A genome-wide expression analysis identified several gene classes that are differentially expressed in the Δ*hom2*Δ*hom2* and the Δ*fst4*Δ*fst4* dikaryons. Among the genes that were down regulated in these strains were *c2h2* and *hom1.* Based on these results, a regulatory model of mushroom development is proposed.

INTRODUCTION

The mushroom fruiting body is the most conspicuous structure of fungi. Mushrooms represent an important food source (Kües & Liu, 2000; Kothe, 2001). Their world-wide production amounts approximately 2.5 million tons annually. Mushrooms are also of interest for the industry because they produce molecules with therapeutic activities (Kües & Liu, 2000; Kothe, 2001) and enzymes that can be used for bioconversions (Lomascolo *et al.*, 1999). Moreover, they have been identified as promising cell factories for the production of pharmaceutical proteins (Berends *et al.*, 2009).

Relatively little is known about mushroom development (Kües, 2000; Wösten & Wessels, 2006). Many mushroom-forming fungi cannot be cultured in the lab nor genetically modified. The basidiomycete *Schizophyllum commune* is one of the notable exceptions. It completes its life cycle in approximately 10 days on defined media. Moreover, the genome of *S. commune* has been sequenced and molecular tools have been developed to

study growth and development of this basidiomycete (e.g. Chapters 2, 3, 4 and 5; Munoz-Rivas *et al.*, 1986; Kothe *et al.*, 1993; Schuren & Wessels, 1994; Scholtmeijer *et al.*, 2001; de Jong *et al.*, 2006; van Peer *et al.*, 2009; de Jong *et al.*, 2010; Ohm *et al.*, 2010a; Ohm *et al.*, 2010b). In fact, it is the only mushroom-forming fungus in which genes have been inactivated by homologous recombination.

Germination of a basidiospore of *S. commune* results in a sterile monokaryotic mycelium that colonizes the substrate and that forms aerial hyphae. Monokaryons fuse with each other to form dikaryons. A fertile dikaryon is formed when the alleles of the mating-type loci *matA* and *matB* of the partners differ. A short exposure to light is essential to initiate mushroom formation, whereas a high concentration of carbon dioxide and high temperatures (30-37 °C) are inhibitory (Niederpruem, 1963). Mushroom formation starts with the aggregation of aerial dikaryotic hyphae. These aggregates develop into fruiting body primordia, which further differentiate into mature fruiting bodies. Karyogamy and meiosis take place in the basidia within the mature fruiting body. This results in haploid monokaryotic basidiospores (Wösten & Wessels, 2006).

Table 1. Expression and evolutionary conservation of the transcription factor genes *hom1, hom2, c2h2*, *gat1* and *bri1* of *S. commune*.
Expression values are in TPM (tags per million) and were determined by massively parallel signature sequencing (MPSS). The conservation is expressed as the taxon the protein is most specifically confined to. For more details see Chapter 5; Ohm *et al.*, 2010b.

Name	ProteinID	Monokaryon	Aggregates	Primordia	Mushrooms	Conservation
hom1	257652	63.96	38.79	281.92	540.52	Agaricomycetes
hom2	257987	36.82	1.23	59.01	15.42	Agaricales
c2h2	114363	11.20	10.05	246.52	165.96	Agaricomycetes
gat1	255004	73.00	17.10	327.81	94.66	Agaricomycetes
bri1	255701	15.72	8.99	55.86	22.88	Basidiomycetes

Recently, 472 genes were identified in the genome of *S. commune* that are predicted to encode transcription factors. The expression profile of these genes was determined in a monokaryotic mycelium, in a mycelium forming aggregates, in a mycelium forming primordia and in mature fruiting bodies (Chapter 5; Ohm *et al.*, 2010b). 311 of the transcription factor genes were shown to be expressed in at least one developmental stage. Of these genes, 56% were expressed in all developmental stages. 268, 200, 283 and 253 of these transcription factors were expressed, respectively, in the monokaryon, and during formation of stage I aggregates, stage II primordia and mushrooms. Interestingly, a group of 16 transcription factor genes was up-

regulated during formation of primordia and/or mature mushrooms. This group includes *fst3* and *fst4*, which encode transcription factors that contain a fungal specific Zn(II)2Cys6 zinc finger DNA binding domain. Monokaryons in which either *fst3* or *fst4* were inactivated showed no phenotypic differences when compared to the wild type. However, the Δ*fst3*Δ*fst3* dikaryon formed more, but smaller reproductive structures than those of the wild type, whereas the Δ*fst4*Δ*fst4* dikaryon did not fruit at all. From these data it was proposed that *fst3* inhibits formation of clusters of mushrooms, whereas *fst4* is involved in the switch between the vegetative and the reproductive phase.

Here we show by targeted gene inactivation that the predicted transcription factor genes *hom1*, *hom2*, *c2h2*, *gat1* and *briI* are also involved in fruiting body formation in *S. commune.* Gene *bri1* encodes a protein with a DNA-binding BRIGHT domain (Herrscher *et al.*, 1995). It is conserved in the basidiomycetes and is up-regulated during formation of primordia (Table 1; Chapter 5; Ohm *et al.*, 2010b). Genes *hom1* and *hom2* encode homeodomain proteins. Gene *hom1* is up-regulated during formation of primordia and mushrooms, whereas *hom2* is down-regulated in stage I primordia. Their conservation is restricted to the Agaricomycetes and Agaricales, respectively (Table 1; Chapter 5; Ohm *et al.*, 2010b). Genes *c2h2* and *gat1* are conserved in the Agaricomycetes and encode proteins with a zinc finger domain of the C2H2 and GATA type, respectively (Yamamoto *et al.*, 1990; Wolfe *et al.*, 2000). Gene *c2h2* is up-regulated during formation of primordia and mushrooms, whereas *gat1* is up-regulated during development of primordia (Table 1; Chapter 5; Ohm *et al.*, 2010b).

MATERIAL AND METHODS

Culture conditions and strains

S. commune was routinely grown from a point inoculum at 25 °C in the light on minimal medium (MM) with 1% glucose and with 1.5% agar (van Peer *et al.*, 2009). All *S. commune* strains used were isogenic to strain 1-40 (Raper *et al.*, 1958). The sequenced strain H4-8 (FGSC #9210; Chapter 5; Ohm *et al.*, 2010b) and the isogenic compatible strain H4-8b (Chapter 2; Ohm *et al.*, 2010a) were used as wild type strains. The Δ*ku80* H4-8 strain (Chapter 3; de Jong *et al.*, 2010) was used to inactivate genes.

Deletion constructs of putative transcription factors genes

The putative transcription factors *hom1* (proteinID 257652), *hom2* (proteinID 257987), *gat1* (proteinID 255004), *c2h2* (proteinID 114363) and *bri1* (proteinID 255701) were deleted using vector pDelcas (Chapter 2; Ohm *et*

al., 2010a). Upstream and downstream flanks were amplified by PCR using Phusion polymerase (Finnzymes, Finland) and *S. commune* H4-8 chromosomal DNA.

The 1050 bp upstream flank of *hom1* was amplified with primers dHom1UpFw2 and dHom1UpRev2 (Table 2), whereas the 999 bp downstream flank was amplified with primers dHom1DwFw and dHom1DwRev. These flanks were cloned into the SmaI site of pUC19, resulting in plasmids pRO091 and pRO090, respectively. The downstream flank was excised from pRO090 using SfiI and introduced in the SfiI site of pDelcas, resulting in pRO092. The upstream flank was excised from pRO091 using SfiI and cloned into pRO092 that had been cut with Van91I. This resulted in the gene deletion construct pRO093.

The 1163 bp upstream flank of *gat1* was amplified with primers dGATA1UpFw and dGATA1UpRev, whereas the 1189 bp downstream flank was amplified with primers dGATA1DwFw and dGATA1DwRev (Table 2). These flanks were cloned into the SmaI site of pUC20, resulting in plasmids pRO180 and pRO181, respectively. The upstream flank was excised from pRO180 using SfiI and introduced in the Van91I site of pDelcas, resulting in pRO186. The downstream flank was excised from pRO181 using SfiI and cloned into pRO186 that had been cut with the same enzyme. This resulted in the gene deletion construct pRO190.

The 1040 bp upstream flank of *c2h2* was amplified with primers dC2H2UpFw and dC2H2UpRev, whereas the 1043 bp downstream flank was amplified with primers dC2H2DwFw and dC2H2DwRev (Table 2). These flanks were cloned into the SmaI site of pUC19, resulting in plasmids pRO100 and pRO101, respectively. The upstream flank was excised from pRO100 using SfiI and introduced in the Van91I site of pDelcas, resulting in pRO102. The downstream flank was excised from pRO101 using SfiI and cloned into pRO102 that had been cut with the same enzyme. This resulted in the gene deletion construct pRO103.

The 1167 bp upstream flank of *hom2* was amplified with primers dHom2UpFw and dHom2UpRev, whereas the 1146 bp downstream flank was amplified with primers dHom2DwFw and dHom2DwRev (Table 2). These flanks were cloned into the SmaI site of pUC20, resulting in plasmids pRO178 and pRO179, respectively. The upstream flank was excised from pRO178 using SfiI and introduced in the Van91I site of pDelcas, resulting in pRO185. The downstream flank was excised from pRO179 using SfiI and cloned into pRO185 that had been cut with the same enzyme. This resulted in the gene deletion construct pRO189.

Table 2. Primers used in this study.

Primer name	Sequence
dC2H2UpFw	GGCCTAATAGGCCCGGATGCTTTCTCGGAGAGG
dC2H2UpRev	GGCCTCGCAGGCCGAGCAGATGCTTCGCTCCGG
dC2H2DwFw	GGCCTGCGAGGCCCCAGTCGACCTCAATTAGCC
dC2H2DwRev	GGCCTATTAGGCCGCCCCTCACCCGTGTACCCG
dC2H2ChkA	GCCTTCCTTTCGTGCGAGAC
dC2H2ChkB	GACGGTCGAGTTTCGACTAC
dC2H2ChkC	ATGGGTATCGGCCAGTATG
dC2H2ChkD	CACTGTAGGCGGACAATTTC
dGATA1UpFw	GGCCTAATAGGCCTGGTCAAGGCATCCCGCAG
dGATA1UpRev	GGCCTCGCAGGCCCTTCTTCTCAAGCCCAAATG
dGATA1DwFw	GGCCTGCGAGGCCTACTCTCATGCGAGACCCAC
dGATA1DwRev	GGCCTATTAGGCCCGTGGGTTGTTGAACTTACC
dGATA1ChkA2	CCAATGTCGCTGGTAACG
dGATA1ChkB2	GAATGGAAGTGGGGATGTC
dGATA1ChkC	GAAGTGGACGAGGTGTTG
dGATA1ChkD	GCCTGGTCTTCTTCATAC
dHom2UpFw	GGCCTAATAGGCCTTGAGATGTTGCCTTGTCG
dHom2UpRev	GGCCTCGCAGGCCCAAGAGCAAGCGTTGAG
dHom2DwFw	GGCCTGCGAGGCCCACGATCTACCCAAACAG
dHom2DwRev	GGCCTATTAGGCCAGATCCAACGTGAGAGCCAG
dHom2ChkA2	AAGAGCCCCATGTTCAAG
dHom2ChkB2	CAGCTATCGAATCCCATTCC
dHom2ChkC	TGGGTGGGAGAAGGAATG
dHom2ChkD	CGCGGATAGCAGTTTATCG
dHom1UpFw2	GGCCTAATAGGCCAGTGCTGGTGAGACTCACG
dHom1UpRev2	GGCCTCGCAGGCCCGATTGGTACGAGCTGGATG
dHom1DwFw	GGCCTGCGAGGCCCATTCTCATATGCCTCAAAC
dHom1DwRev	GGCCTATTAGGCCTCGTCTCTATTCACAACCGC
dHom1ChkA	GCATCTGTTCCGCTCTGTTG
dHom1ChkB	CAAGAGACTGGAAACCTCCG
dHom1ChkC	AAAGGATGGGAGGTCCTAC
dHom1ChkD	GCCTATGCTTCGACTCTTC
BRIGHTufw	GGCCGAATGGGCCGTATGAAGGAAG
BRIGHTuprev	GGCCCCGCTGGCCCTGCAAACGAAC
BRIGHTdfw28-01	GGCCAGCGAGGCCAGGTCCGTGATCCTTTGTG
BRIGHTdrev28-01	GGCCTATTAGGCCTGAAGGGCGGTAATGCTG
BRIGHTfwcon	TTCGCCAGAGCGACGTATTC
BRIGHTrevcon	GGAAGAGGCGGAGATGAAAC
BRIGHTwtrev	GGGTGCTGGAAGAGGACAAG
BRIGHToutfw	GCTCGAGATACGCGGACAAG
Nourdelrev	TTGGTGACCTCCAGCCAGAG
sc3tersqf	GCCTCAGGTCCCGAAGTAAG

The 1369 bp upstream flank of *bri1* was amplified with primers BRIGHTufw and BRIGHTuprev, whereas the 1077 bp downstream flank was amplified with primers BRIGHTdfw28-01 and BRIGHTdrev28-01 (Table 2). These flanks were cloned into the SmaI site of pUC19, resulting in plasmids BRIGHTup and BRIGHTdw, respectively. The upstream flank was excised from BRIGHTup using SfiI and introduced in the Van91I site of pDelcas, resulting in pDelcasBRIGHTup. The downstream flank was excised from BRIGHTdw using SfiI and cloned into pDelcasBRIGHTup that had been cut with the same enzyme. This resulted in the gene deletion construct pDelcasBRIGHT.

Transformation and selection

The Δ*ku80* H4-8 strain was transformed with 20 µg of deletion construct using $5{\cdot}10^7$ protoplasts (van Peer *et al.*, 2009). Transformants were selected on plates containing 20 µg ml^{-1} nourseothricin after regeneration in medium without antibiotic. Nourseothricin resistant colonies were screened on plates containing 25 µg ml^{-1} phleomycin. Nourseothricin resistant but phleomycin sensitive transformants are potential deletion strains, whereas nourseothricin and phleomycin resistant strains are the result of a single cross-over event. Deletion of genes was confirmed by colony PCR (Chapter 2; Ohm *et al.*, 2010a). in the case of *hom1* primers dHom1ChkD (anneals upstream of the up-flank) and nourdelrev (anneals within the nourseothricin resistance cassette) and primers sc3tersqf (anneals within the nourseothricin resistance cassette) and dHom1ChkA (anneals downstream of the down-flank) (Table 2) formed a product in the knock-out strain but not in the wild type. In contrast, primers dHom1ChkD (anneals upstream of the up-flank) and dHom1ChkC (anneals in the deleted gene) and dHom1ChkB (anneals within the deleted gene) and dHom1ChkA (anneals downstream of the down-flank) did not form a product in the knock-out strain, but did so in the wild type. Deletion of *gat1* was confirmed using primers dGATA1ChkD (anneals upstream of the up-flank) and nourdelrev (anneals within the nourseothricin resistance cassette) and sc3tersqf (anneals within the nourseothricin resistance cassette) and dGATA1ChkA2 (anneals downstream of the down-flank) (Table 2). These primer pairs formed a product in the knock-out strain but not in the wild type. In contrast, primers dGATA1ChkD (anneals upstream of the up-flank) and dGATA1ChkC (anneals within the deleted gene) and dGATA1ChkB2 (anneals within the deleted gene) and dGATA1ChkA2 (anneals downstream of the down-flank) did not form a product in the knock-out strain, but did so in the wild type. Deletion of *c2h2* was confirmed using primers dC2H2ChkD (anneals upstream of the up-flank) and nourdelrev (anneals within the nourseothricin resistance cassette) and sc3tersqf (anneals within the nourseothricin

resistance cassette) and dC2H2ChkA (anneals downstream of the down-flank) (Table 2). These primer pairs formed a product in the knock-out strain but not in the wild type. In contrast, primers dC2H2ChkD (anneals upstream of the up-flank) and dC2H2ChkC (anneals within the deleted gene) and dC2H2ChkB (anneals within the deleted gene) and dC2H2ChkA (anneals downstream of the down-flank) did not form a product in the knock-out strain, but did so in the wild type. Deletion of *hom2* was confirmed using primers dHom2ChkD (anneals upstream of the up-flank) and nourdelrev (anneals within the nourseothricin resistance cassette) and sc3tersqf (anneals within the nourseothricin resistance cassette) and dHom2ChkA2 (anneals downstream of the down-flank) (Table 2). These primer pairs formed a product in the knock-out strain but not in the wild type. In contrast, primers dHom2ChkD (anneals upstream of the up-flank) and dHom2ChkC (anneals within the deleted gene) and dHom2ChkB2 (anneals within the deleted gene) and dHom2ChkA2 (anneals downstream of the down-flank) did not form a product in the knock-out strain, but did so in the wild type. Deletion of *bri1* was confirmed using primers BRIGHToutfw (anneals upstream of the up-flank) and nourdelrev (anneals within the nourseothricin resistance cassette) and sc3tersqf (anneals within the nourseothricin resistance cassette) and BRIGHTfwcon (anneals downstream of the down-flank) (Table 2). These primer pairs formed a product in the knock-out strain but not in the wild type. In contrast, BRIGHToutfw (anneals upstream of the up-flank) and BRIGHTwtrev (anneals within the deleted gene) and BRIGHTrevcon (anneals with in the deleted gene) and BRIGHTfwcon (anneals downstream of the down-flank) did not form a product in the knock-out strain, but did so in the wild type.

The Δ*ku80* H4-8 strains in which one of the predicted transcription factor genes had been inactivated (which are hygromycin resistant due to the *ku80* deletion (Chapter 3; de Jong *et al.*, 2010) and nourseothricin resistant due to the deletion vector (Chapter 2; Ohm *et al.*, 2010a) were crossed with the wild type strain H4-8b. After 10 days of growth, spores were collected from the lids of the plates (plates had been placed inverted in the growth chamber) and counted with a haemocytometer. 500 spores were plated out on complete medium (glucose 20 g l^{-1}; $MgSO_4$ 2mM; KH_2PO_4 3.4 mM; K_2HPO_4 5.7 mM; pepton 2 g l^{-1}; yeast extract 2 g l^{-1}, 1.5 % (wt/vol) agar) to assess whether nourseothricin resistance and sensitivity segregated 1:1 as an evidence for a single integration event of the deletion vector. To this end, 100 1-day-old colonies were transferred to MM plates and grown for two days. The colonies were replicated on plates containing 20 µg ml^{-1} nourseothricin and growth was monitored after two days. In the next step, nourseothricin resistant siblings were selected that were hygromycin sensitive. This

screening allowed the selection of strains with an intact copy of *ku80.* In the final step, strains were selected that had compatible mating types. To this end, siblings were crossed with wild type strains H4-8 and H4-8b and clamp formation was monitored.

Phenotypic analysis

Morphology of the monokaryotic strains in which one of the transcription factor genes was inactivated was compared with the wild type after 6 days of growth on MM plates. Growth and fruiting body formation of homo- and heterozygous dikaryons was followed for 10 days on MM plates and compared to that of a wild type dikaryon. Spore formation was assessed by growing the dikaryons on plates that had been placed inverted in the growth chamber.

RNAseq gene expression analysis

A whole genome gene expression analysis was performed on dikaryotic colonies (wild type strain, Δ*hom2*Δ*hom2* strain, Δ*fst4*Δ*fst4* strain) that were grown from plug inocula. Colonies were grown at 25 ºC in the light for 6 days. RNA was isolated as described (van Peer *et al.*, 2009) from 3 biological replicates. The quality of the RNA was analysed on a RNA 6000 Lab-on-a-Chip using the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The Illumina mRNA-Seq Sample Prep Kit was used to process the samples. The sample preparation was performed according the Illumina protocol "Preparing Samples for Sequencing of mRNA" (1004898 Rev. D). Briefly, mRNA was isolated from total RNA using the poly-T-oligo-attached magnetic beads. After fragmentation of the mRNA, cDNA was synthesized. This was used for ligation with the sequencing adapters and PCR amplification, resulting in fragments of approximately 275 bp. A total of 8 pmol DNA was used. The samples were sequenced on the Illumina Genome Analyzer IIx, according to the manufacturer's protocols. Two sequencing reads of 51 cycles each using the Read 1 sequencing and Read 2 sequencing primers were performed with the flow cell. Image analysis, base-calling, and quality check was performed with the Illumina Genome Analyzer data analysis pipeline v1.5.1. The sequence data has been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.*, 2002) and is accessible through GEO Series accession number GSE23594 (http://www.ncbi.nlm.nih.gov/geo/).

The sequenced reads were mapped to the genome sequence using the program Tophat 1.0.13 (Trapnell *et al.*, 2009) and Bowtie 0.12.5 (Langmead *et al.*, 2009), using default settings with the exception of the minimum and maximum intron length (10 and 400 bp, respectively). The program Cufflinks 0.8.2 (Trapnell *et al.*, 2010) was used to identify reads overlapping with

predicted genes (described in Chapter 5; Ohm *et al.*, 2010b) and to determine the expression levels of these predicted genes for each sample. Expression levels of genes are indicated in FPKM (mapped Fragments Per Kilobase of exon model per Million mapped fragments). Custom scripts were developed to collect the data in 1 file and to identify differentially expressed genes based on a 2 fold change in expression and an absolute change in expression of at least 2 FPKM.

Representation analysis

FuncAssociate 2.0 (Berriz *et al.*, 2009) was used to study over- and under-representation of GO-terms in sets of differentially regulated genes. Default settings were used with a p-value of 0.05 as the cut off.

Hierarchical clustering analysis

For hierarchical clustering analysis, genes were used that were expressed in at least 1 of the 5 samples. Expression values were log2-transformed after the values were increased with 1 in order to prevent negative log2 values. Hierarchical clustering of genes and samples and the subsequent analysis of the results were performed with the program MeV, which is part of the TM4 Software Suite (Saeed *et al.*, 2006). Euclidian distance was used as a distance metric and average linkage clustering as linkage method.

RESULTS

Deletion of transcription factor genes

Genes *bri1*, *hom1*, *hom2*, *gat1*, and *c2h2* encode predicted transcription factors. Gene *hom2* is down-regulated during formation of aggregates, *gat1* and *bri1* are up-regulated during formation of primordia and *c2h2* and *hom1* are up-regulated during formation of primordia and mature mushrooms (Table 1; Chapter 5; Ohm *et al.*, 2010b). These genes were inactivated to assess their role in development. To this end, the Δ*ku80* strain of H4-8 was transformed with deletion constructs that are based on the pDelcas vector (the advantage of the Δ*ku80* strain in which the non-homologous end joining pathway is inactivated is that it has a relative high homologous integration frequency). Phleomycin sensitive and nourseothricin resistant transformants were candidates to have a gene deletion (see Material and methods). Inactivation of one of the transcription factor genes was confirmed in 7 out of 8 candidate strains by colony PCR (Table 3). One knock out strain of each of the predicted transcription factor genes was crossed with strain H4-8b to select for strains with an intact *ku80* gene and with either *bri1*, *hom1*, *hom2*,

gat1, and *c2h2* being inactivated. In the next step, siblings were selected that had compatible mating type loci, thus allowing crossings to obtain hetero- and homozygous mutant strains. The crossings did not reveal strains that had an inactivated *c2h2* gene and an active copy of the *ku80* gene. This is explained by the fact that these genes are separated by less than 27 kb on scaffold 15 (Chapter 5; Ohm *et al.*, 2010b). Therefore, compatible Δ*c2h2*Δ*ku80* strains were used for phenotypic analysis. Note that the analysis of the siblings of the crossings revealed that nourseothricin resistance strictly correlated with the phenotypes described below. Therefore, the phenotypes can be attributed to the gene deletions.

Table 3. Inactivation of the transcription factor genes *hom1, hom2, c2h2*, *gat1* and *bri1* of *S. commune*. A Δ*ku80* strain was transformed with deletion constructs that are based on the pDelcas plasmid. The first selection was on the antibiotic nourseothricin, the second selection on phleomycin. Gene deletion was confirmed by colony PCR in nourseothricin resistant and phleomycin sensitive colonies. Antibiotic resistance is indicated with (+) and sensitivity with (-). For more details, see Chapter 2; Chapter 3; de Jong *et al.*, 2010; Ohm *et al.*, 2010a.

Name	Protein ID	Nourseothricin(+) transformants	Nourseothricin(+) and phleomycin(-) transformants	Transformants with confirmed gene deletion
hom1	257652	3	3	3
hom2	257987	2	1	1
c2h2	114363	1	1	1
gat1	255004	6	2	1
bri1	255701	1	1	1

Formation of the vegetative mycelium and of aerial hyphae was not affected in 6 day-old colonies of the Δ*hom1*, Δ*bri1*, Δ*gat1* and Δ*c2h2* monokaryons. The Δ*hom2* strain grew 30% faster than the wild type (data not shown). Growth and development of mushrooms were not affected in the heterozygous mutants strains. However, this was the case for the homozygous mutant dikaryons. Wild type (Figure 1A) and heterozygous mutant dikaryons formed asymmetrical colonies when they were grown in the light and mature sporulating mushrooms had formed after 10 days of growth. In contrast, 10 days-old Δ*hom2*Δ*hom2* dikaryons had formed a symmetrical colony and no mushrooms had developed (Figure 1B). The Δ*bri1*Δ*bri1* dikaryon had also not formed mushrooms and the diameter of the colonies was smaller than that of a wild type and the Δ*hom2*Δ*hom2* dikaryon (Figure 1C). Moreover, the colonies of the Δ*bri1*Δ*bri1* dikaryon were more regular than the wild type. Development of the Δ*c2h2*Δ*c2h2* dikaryon had been arrested in the aggregate stage (Figure 1D). In contrast, the Δ*hom1*Δ*hom1* and Δ*gat1*Δ*gat1* dikaryons formed more but smaller mushrooms than the wild type dikaryon (Figure 1E and F). These mutant fruiting bodies were more and

less pigmented in the Δ*hom1*Δ*hom1* and the Δ*gat1*Δ*gat1* dikaryons, respectively, when compared to the wild type. Moreover, both mutant strains formed fruiting bodies with an enlarged surface of the hymenium (the tissue where the spore-forming basidia are situated). Both strains formed viable spores and the morphology and diameter of the colonies were not affected.

Genome-wide expression analysis

A genome-wide expression analysis was performed on 6 days-old dikaryotic colonies of the wild type, and the Δ*hom2*Δ*hom2* and Δ*fst4*Δ*fst4* strains. By then, wild type colonies had formed stage I aggregates, while no fruiting structures had formed in Δ*hom2*Δ*hom2* and Δ*fst4*Δ*fst4* dikaryons. In general, expression in Δ*hom2*Δ*hom2* and Δ*fst4*Δ*fst4* dikaryons was more similar to each other than to the wild type (Figure 2).

Compared to the wild type, 861 and 845 genes were up- and down-regulated, respectively (≥ 2-fold), in the Δ*hom2*Δ*hom2* dikaryon. Functional categories that were over-represented in the up-regulated genes include terms related to metabolism and cytochrome P450 (Supplementary Table 1). Under-represented were genes involved in regulation of transcription, such as transcription factors. In the down-regulated genes, functional categories comprising hydrophobins and multicopper oxidases were over-represented.

Compared to the wild type, 486 and 634 genes were up- and down-regulated, respectively (≥ 2-fold), in the Δ*fst4*Δ*fst4* dikaryon. Functional categories that were over-represented in the up-regulated genes included terms related to metabolism (Supplementary Table 1). In the down-regulated genes, the functional categories comprising hydrophobins and cytochrome P450 were over-represented.

In the next step, expression of specific gene groups was analyzed in the Δ*hom2*Δ*hom2* and Δ*fst4*Δ*fst4* dikaryons. Genes contained in the mating type A and B loci were generally not differentially expressed (Table 4). As an exception, the pheromone encoding genes *bbp2(3)* and *bbp2(5)* were down-regulated in the Δ*hom2*Δ*hom2* strain. Genes encoding predicted components of the RAS and pheromone signalling pathways were also not differentially expressed (except for *cry1* in Δ*hom2*Δ*hom2*). Notably, *hom1* and *c2h2* were down-regulated in the Δ*hom2*Δ*hom2* and Δ*fst4*Δ*fst4* dikaryons. Moreover, most hydrophobins were down-regulated in these strains. Several dikaryon-specific genes predicted to encode structural cell wall proteins were also down-regulated, e.g. *sc7* and *sc14* (Schuren *et al.*, 1993) and the lectin gene *agg1* (Ohm *et al.*, 2010b). In contrast, the monokaryon-specific hydrophobin-like gene *sc15* (Lugones *et al.*, 2004) was up-regulated in Δ*hom2*Δ*hom2*.

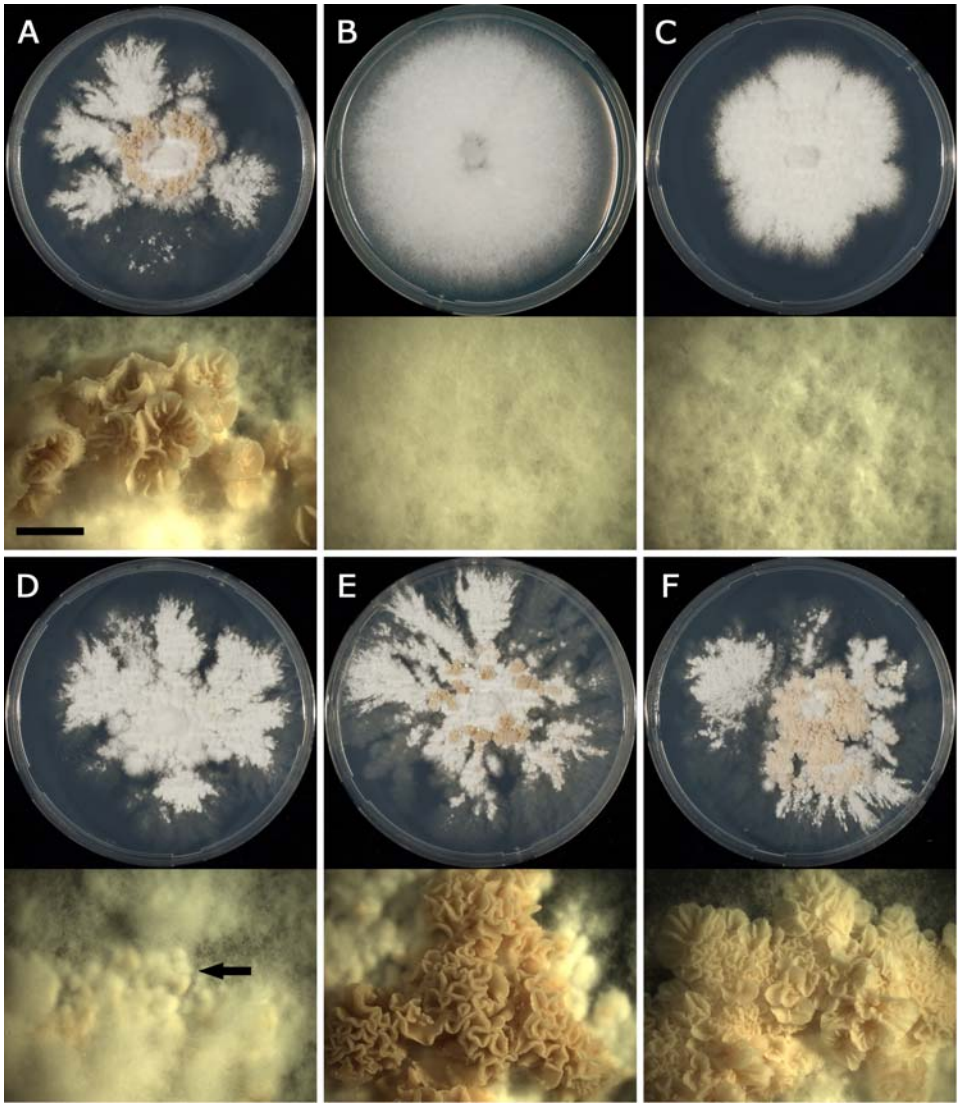


Figure 1. Mushroom formation is affected in dikaryotic *S. commune* strains in which the transcription factor genes *hom1, hom2, c2h2*, *gat1* or *bri1* are inactivated. Moreover, colony morphology is changed in the Δ*hom2*Δ*hom2* and Δ*bri1*Δ*bri1* dikaryons. Colonies were grown for 10 days from a point inoculum at 25 °C. Lower panels represent a magnification of the upper panels. Bar represents 5 mm. Arrow indicates a region with aggregates. **A.** Wild type dikaryon. **B.** Δ*hom2*Δ*hom2* dikaryon. **C.** Δ*bri1*Δ*bri1* dikaryon. **D.** Δ*c2h2*Δ*c2h2* dikaryon. **E.** Δ*hom1*Δ*hom1* dikaryon. **F.** Δ*gat1*Δ*gat1* dikaryon.

DISCUSSION

So far, little is known about transcription factors that are involved in mushroom development. The well studied homeodomain proteins encoded in the A mating type locus play a crucial role in the formation of a fertile dikaryon (Kües *et al.*, 1992; Stankis *et al.*, 1992). Two other transcription factors involved in fruiting body formation have been identified in *Coprinopsis cinerea*. The *pcc1* gene functions in A regulated development and encodes a putative DNA binding protein (Murata *et al.*, 1998; Murata & Kamada, 2009). Mutations in the gene resulted in a complete program of sexual differentiation independent of the mating type genes. The *exp1* gene encodes a transcription factor and is involved in cap expansion and autolysis (Muraguchi *et al.*, 2008). Clearly, these data do not present a complete picture how fruiting body formation is regulated at the molecular level. We have taken the approach to systematically study the role of transcription factor genes of *S. commune* that are differentially expressed during mushroom development. Recently, 472 transcription factors have been identified in the genome of this organism (Chapter 5; Ohm *et al.*, 2010b). A group of these genes were differentially expressed during formation of primordia and/or during formation of mature mushrooms. Genes *fst3* and *fst4*, which are part of this group, were inactivated. It was shown that a Δ*fst3*Δ*fst3* dikaryon formed more but smaller mushrooms than the wild type, whereas the Δ*fst4*Δ*fst4* dikaryon formed no mushrooms at all (Chapter 5; Ohm *et al.*, 2010b). Here, it is shown that the transcription factor genes *hom1*, *hom2*, *gat1*, *c2h2* and *bri1* also play a role in mushroom development.

Monokaryons form symmetrical colonies when they are grown in the light. In contrast, when a dikaryon is grown under this condition it forms an asymmetrical colony with patches of leading hyphae that explore the substrate. The asymmetry in dikaryotic colonies has been attributed to the secretion of an auto-inhibitor (Klein *et al.*, 1997). In contrast to the wild type, a Δ*hom2*Δ*hom2* dikaryon formed a symmetrical colony in the light. A similar but less pronounced phenotype was observed for the Δ*bri1*Δ*bri1* strain. Our results indicate that Hom2 and Bri1 affect colony morphology by (in)direct regulation of the formation of the auto-inhibitor.

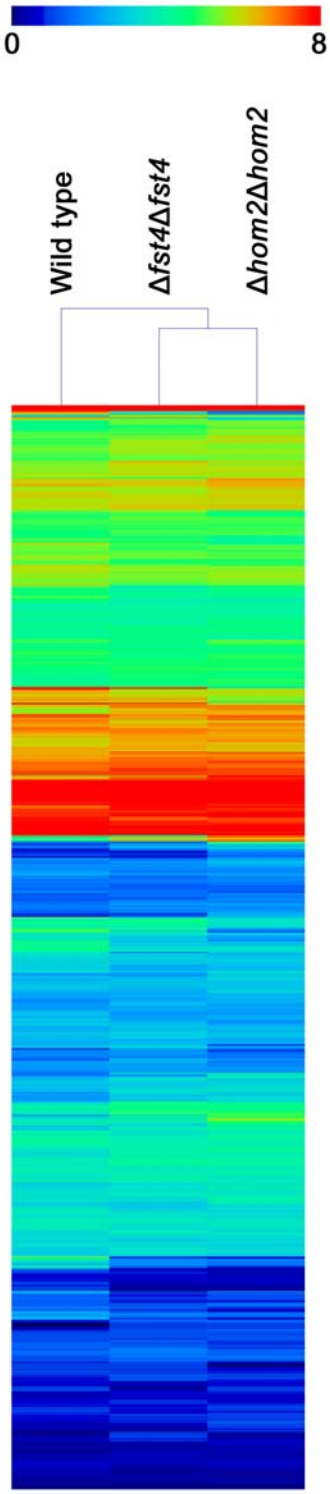
The asymmetry of the dikaryotic wild type colony correlates with the development of mushrooms. These mushrooms are formed in a ring around the centre of the colony. The Δ*hom2*Δ*hom2* and the Δ*bri1*Δ*bri1* dikaryons did not form mushrooms, strengthening the relatedness between colony asymmetry and mushroom development. Of interest, Δ*fst4*Δ*fst4* dikaryons did form asymmetrical colonies but they did not form any aggregates, primordia

Table 4. Expression of genes in wild type, Δ*fst4*Δ*fst4* and Δ*hom2*Δ*hom2* dikaryons. Expression values are in FPKM. For more details, see Chapter 5; Ohm *et al.*, 2010b.

ProteinID	Name	Wild type	Δ*fst4* Δ*fst4*	Δ*hom2* Δ*hom2*
Genes on the MatA locus				
80879	*aay4*	8.19	6.54	7.59
231556	*aaz4*	6.54	5.78	6.75
269864	*abq6*	3.20	6.06	4.70
104345	*abr6*	3.10	2.27	2.52
269862	*abs6*	3.46	4.00	2.82
269861	*abt6*	2.54	1.68	1.37
269858	*abu6*	2.66	2.36	1.75
269851	*abv6*	2.46	2.79	2.58
Genes on the MatB locus (Pheromone receptors)				
258340	*bar3*	7.05	7.33	7.97
85947	*bbr2*	9.06	6.99	7.74
112464	*brl1*	22.31	19.10	18.02
112482	*brl2*	10.23	23.11	12.95
258344	*brl3*	41.99	45.07	29.28
111749	*brl4*	13.48	14.19	8.74
Genes on the MatB locus (Pheromones)				
83222	*bbp2(1)*	55.75	73.06	58.48
251504	*bbp2(2)*	41.31	22.63	23.99
60499	*bbp2(3)*	0.92	0.92	4.47
60526	*bbp2(4)*	29.55	26.31	30.64
112472	*bbp2(5)*	0.22	0.43	3.74
60621	*bbp2(6)*	1.29	0.64	0.46
17248	*bbp2(7)*	0.19	0.00	0.41
17250	*bbp2(8)*	20.06	19.64	16.70
60117	*bap3(1)*	29.54	24.07	39.24
270001	*bap3(2)*	1.84	2.03	2.80
60520	*bap3(3)*	1.73	0.87	0.31
251507	*bpl1*	10.48	12.50	12.91
270005	*bpl2*	0.00	0.00	0.00
270006	*bpl3*	0.18	0.74	0.99
270007	*bpl4*	9.97	12.35	5.71
270009	*bpl5*	0.00	0.00	0.00
Predicted components of the pheromone signalling pathway				
54965	*ste20*	18.17	29.34	32.49
233977	*ste50*	10.76	12.75	12.58
74823	*ste11*	29.43	31.92	38.00
70675	*fus3*	48.62	51.18	50.66

Table 4 continued

ProteinID	Name	Wild type	Δ*fst4* Δ*fst4*	Δ*hom2* Δ*hom2*
Predicted components of the Ras signalling pathway				
45883	*ras1*	210.00	292.15	343.71
67931	*ras2*	480.14	487.09	439.79
63024	*gap1*	15.95	21.93	21.17
77962	*Adenylate cyclase, cry1*	16.50	11.05	7.90
258790	*Catalytical subunit of PkA, tpk1*	9.34	9.35	10.49
16601	*Catalytical subunit of PkA, tpk2*	51.45	40.84	44.11
237359	*Catalytical subunit of PkA, tpk3*	0.31	0.31	0.38
80341	*Regulatory subunit of PkA, bcy1*	130.43	117.82	128.08
Transcription factors				
257987	*hom2*	62.57	44.58	0.06
66861	*fst4*	52.00	3.13	43.73
114363	*c2h2*	17.82	8.61	5.41
257422	*fst3*	25.90	19.23	27.21
255004	*gat1*	20.48	13.68	19.15
257652	*hom1*	119.78	45.65	49.76
255701	*bri1*	15.05	11.65	9.89
Hydrophobins				
77028	*sc3*	29213.40	17886.60	10198.60
73533	*sc4*	12039.50	1241.74	25.18
13677	*sc1*	1081.63	79.55	4.16
13059	*sc6*	115.11	8.44	0.85
58269	*hyd1*	212.15	7.22	2.07
82440	*hyd2*	97.80	11.74	89.71
232866	*hyd3*	30.98	0.65	0.00
51576	*hyd4*	266.92	10.24	2.17
250297	*hyd5*	2.03	1.01	1.31
85429	*hyd6*	5801.93	2239.41	1430.60
109698	*hyd7*	37.29	0.95	0.17
105121	*hyd8*	198.02	3.64	0.43
249002	*hyd9*	80.54	17.57	0.57
Putative structural genes				
269921	*lccA*	29.32	11.38	0.38
85445	*sc7*	988.12	36.73	9.61
16267	*sc14*	8.26	0.94	0.55
109852	*agg1*	6341.25	885.79	24.06
82353	*sc15*	11019.50	18109.70	29294.90



or mushrooms. From this it is concluded that Fst4 functions downstream of Hom2 and Bri1 (Figure 3). Whole genome expression analysis of the Δ*hom2*Δ*hom2* and the Δ*fst4*Δ*fst4* dikaryons showed that many hydrophobin genes are down-regulated in these mutant strains. Previously, it has been shown that the SC3 hydrophobin is involved in the escape of hyphae into the air (Wösten *et al.*, 1999) and in lining aerial hyphae with a hydrophobic coat (Wösten *et al.*, 1993; Wösten *et al.*, 1994; van Wetter *et al.*, 1996; van Wetter *et al.*, 2000). SC3 fulfils this function both in the monokaryon and in the dikaryon but is considered to be a "monokaryotic" gene. This was explained by the fact that dikaryotic hyphae that express monokaryotic specific genes exhibit a large distance between the nuclei in each compartment. These nuclei were proposed to experience a monokaryotic-like environment. In contrast, nuclei are in close proximity in dikaryotic hyphae that express "dikaryon-specific genes" (Ásgeirsdóttir *et al.*, 1995; Schuurs *et al.*, 1998). One dikaryon-specific hydrophobin gene is *sc4*. The hydrophobin that is encoded by this gene lines air channels within fruiting bodies (Lugones *et al.*, 1999; van Wetter *et al.*, 2000). This lining prevents water to enter these air channels by capillary force. Also *sc1* and *sc6* have been shown to be dikaryon-specific (Wessels *et al.*, 1995). In contrast to *sc3*, expression of *sc1*, *sc4* and *sc6* (as well as many of the other

Figure 2. Heat map depicting hierarchically clustered gene expression data. Only expressed genes are shown. Given values are the log2-transformed FPKM expression values (mapped Fragments Per Kilobase of exon model per Million mapped fragments, see Material and methods for more details). In general, expression in Δ*hom2*Δ*hom2* and Δ*fst4*Δ*fst4* dikaryons was more similar to each other than to the wild type.

recently identified hydrophobin genes; Chapter 5; Ohm *et al.*, 2010b) is highly affected in the Δ*hom2*Δ*hom2* and the Δ*fst4*Δ*fst4* dikaryons. This may well explain why these strains do form aerial hyphae but do not form aggregates. It should be noted that we could not identify conserved sequences in the promoters of the genes encoding the dikaryon-specific hydrophobins (data not shown). This indicates that Hom2 and Fst4 may regulate different transcription factor genes that each affect one or more hydrophobin genes. Indeed, the expression of *c2h2* and *hom1* (which both play a role downstream of *hom2* and *fst4*, see below) is lower in the Δ*hom2*Δ*hom2* and the Δ*fst4*Δ*fst4* dikaryons, showing that Hom2 and Fst4 (in)directly regulate transcription factor genes. Hom2 and Fst4 do not exert their role by regulating genes involved in RAS or pheromone signalling, as expression of these genes was generally not affected in the mutant strains.

In wild type colonies aggregates develop into primordia. During this process different tissues are formed and this is accompanied by an increase in antisense expression of genes (Chapter 5; Ohm *et al.*, 2010b). The Δ*c2h2*Δ*c2h2* colonies were asymmetrical when grown in the light and they did form aggregates. These aggregates, however, did not develop into primordia. Whether C2h2 exerts its role by regulating antisense expression is unknown, but it is an interesting topic for future research. It is clear that C2h2 functions downstream of Fst4 (Figure 3).

Strains in which *fst3* (Chapter 5; Ohm *et al.*, 2010b), *hom1* or *gat1* were inactivated formed more but smaller mushrooms than the wild type. The mushrooms of the Δ*fst3*Δ*fst3* dikaryon were smaller but otherwise similar to the wild type (Chapter 5; Ohm *et al.*, 2010b). From this it was proposed that Fst3 is involved in the local inhibition of primordia formation. This repressor may be important in the natural environment to ensure that mushrooms can fully develop with the limited resources that are available. Gene *fst3* may become activated by a signal from a developing mushroom. This would prevent outgrowth of neighbouring aggregates, thereby preventing competition between developing mushrooms for the limiting resources. The mushrooms produced by the Δ*hom1*Δ*hom1* and the Δ*gat1*Δ*gat1* dikaryon were not only smaller than those of the wild type but their morphology was also affected. The hymenium (i.e. the spore producing tissue) was enlarged in these mutant stains when compared to the wild-type. From this we propose that *hom1* and *gat1* have a function in tissue development in *S. commune.* The smaller size of the mushrooms in the absence of *hom1* or *gat1* would result in a reduced flow of nutrients to the developing mushroom. This would enable more primordia to grow out, which would explain the higher number of mushrooms that develop in the Δ*hom1*Δ*hom1* and the Δ*gat1*Δ*gat1* dikaryon.

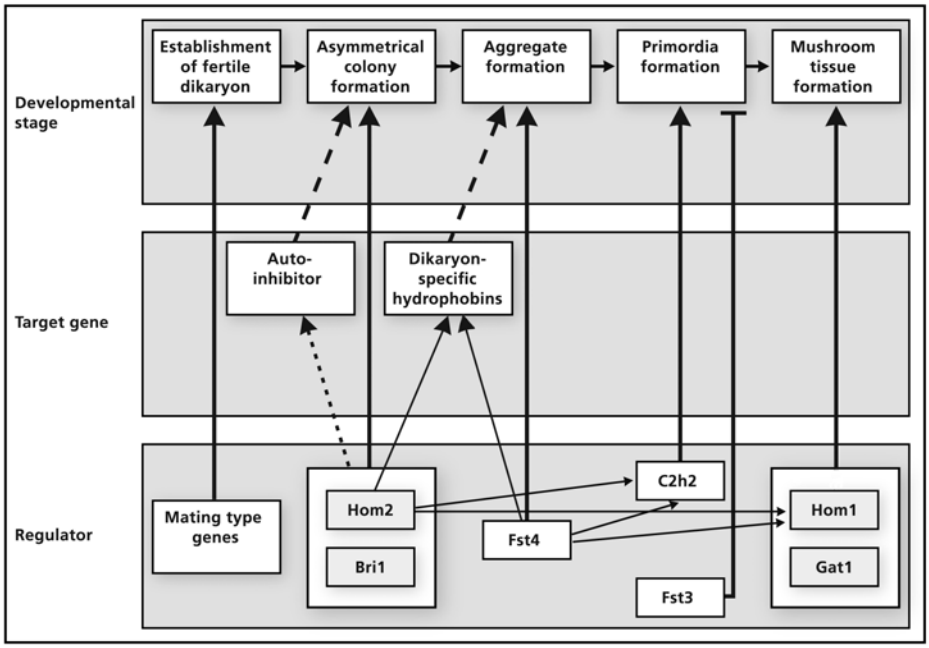


Figure 3. Model for the regulation of mushroom development in continuous light at 25 °C. Thick lines represent a regulatory function in a developmental process. Thin arrows represent (in)direct transcriptional activation of target genes. The continuous lines are supported by experimental evidence, which is not the case for the dotted lines. The boxed transcription factors have a similar role in the process of mushroom formation.

The regulatory model of mushroom formation in *S. commune* may well apply to other mushroom-forming fungi. This is supported by the observation that the homologs of *fst3* and *fst4* are also up-regulated in young fruiting bodies of *Laccaria bicolor* compared to free-living mycelium. Furthermore, we previously showed that the regulation of transcription factor genes positively correlates between *S. commune* and *L. bicolor* during mushroom formation (Chapter 5; Martin *et al.*, 2008; Ohm *et al.*, 2010b). The regulatory model of mushroom formation may be instrumental to explain why certain mushrooms can not be cultivated commercially and also represents an excellent lead to improve production of commercial mushrooms.

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SUPPLEMENTARY DATA

All supplementary data can be found at http://tinyurl.com/robinohmthesis or obtained from the author.

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Chapter 7

The blue light receptor WC-2 is involved in mushroom formation in *Schizophyllum commune*

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ABSTRACT

Blue light is necessary for the initiation of mushroom formation in *Schizophyllum commune*. The genome of this basidiomycete contains 4 genes encoding putative light receptors, of which *wc-1* and *wc-2* encode homologues of blue light receptor genes of *Neurospora crassa*. Here, it is shown that targeted gene deletion of *wc-2* results in a blind phenotype. This was manifested by the absence of mushroom formation. Moreover, the mutant did not form asymmetrical colonies in the light. A genome-wide expression analysis of colonies that had been grown in the light showed that the transcription factor genes *c2h2* and *hom1* as well as many hydrophobin genes are down-regulated in the Δ*wc-2*Δ*wc-2* mutant strain when compared to the wild type. Mushroom formation in the Δ*wc-2*Δ*wc-2* mutant strain was restored by introduction of a construct encompassing *wc-2* under control of the heat inducible *hsp3* promoter and by applying a heat shock at 42 °C. Genome-wide expression analysis of the wild type strain, the Δ*wc-2*Δ*wc-2* dikaryon and the Δ*wc-2*Δ*wc-2* $hsp3_{pr}$-*wc-2* dikaryon led to the identification of several putative downstream target genes and promoter binding sites of WC-2. Based on the results a model for mushroom formation is proposed.

INTRODUCTION

Light plays an important role in growth and development of fungi. Most studies on the effects of light and the underlying mechanisms in fungi have been performed in the ascomycete *Neurospora crassa*. The development of the sexual fruiting bodies called protoperithecia (Innocenti *et al.*, 1983) and the entrainment (resetting) of the circadian clock (Crosthwaite *et al.*, 1995) are affected by blue light in this organism. Blue light is detected by the white collar (WC) complex, which is a protein complex consisting of White Collar 1 and White Collar 2 (WC-1 and WC-2) (Ballario *et al.*, 1996; Linden & Macino, 1997; Linden *et al.*, 1997; Chen *et al.*, 2009). PAS (Per-Arnt-Sim) domains in WC-1 and WC-2 mediate the interaction between these proteins (Cheng *et al.*, 2002). The Light, Oxygen, Voltage (LOV) domain of WC-1 binds a flavin chromophore FAD (flavin adenine dinucleotide), and as such the complex can detect light (Froehlich *et al.*, 2002). Upon light activation, the WC complex induces transcription of genes by binding to their promoter via the zinc finger DNA binding domains of WC-1 and WC-2. The light induced activation of gene expression by the WC complex is transient. Light-exposed WC-1 becomes

phosphorylated, which leads to inactivation of the complex and subsequent degradation of WC-1. Light stimulated development proceeds via newly translated WC-1 (Schwerdtfeger & Linden, 2000). The genome of *N. crassa* also contains the *vivid* (*vvd)* gene, which encodes a protein that is homologous to WC-1 and WC-2. The VIVID protein has also been shown to be involved in the photo activation of genes (Heintzen *et al.*, 2001; Schwerdtfeger & Linden, 2001; Schwerdtfeger & Linden, 2003).

Phytochromes, cryptochromes and rhodopsins are three other classes of photoreceptors (reviewed in Corrochano, 2007). Phytochromes detect red and far red light and are found in fungi, plants and bacteria. Deletion of the two phytochrome genes in *N. crassa* did not result in a clear phenotype (Froehlich *et al.*, 2005). However, inactivation of the phytochrome encoding gene *fphA* in *Aspergillus nidulans* affected conidiation. This agrees with the fact that formation of these a-sexual spores is activated by red light (Blumenstein *et al.*, 2005). Little is known about the function of cryptochromes and rhodopsins in fungi. In plants, cryptochromes are blue light receptors and they show high sequence similarity to DNA photolyases, which are involved in light-dependent DNA repair (Lin & Todo, 2005). In the genome of *N. crassa* one cryptochrome gene was identified (*cry*), which was shown to play a role in circadian entrainment (Froehlich *et al.*, 2010). Rhodopsins are membrane embedded proteins and are composed of a retinal chromophore bound to an opsin apoprotein. The rhodopsin of *N. crassa* (*nop-1*) encodes a putative green light receptor and is involved in repression of conidiation-specific gene expression (Bieszke *et al.*, 1999; Bieszke *et al.*, 2007).

Like *N. crassa*, *Schizophyllum commune* needs exposure to light for proper development of sexual fruiting bodies. These fruiting bodies are formed by fertile dikaryons that result from fusion of monokaryons that have different *matA* and *matB* mating type loci. In the dark, the fertile dikaryon forms symmetrical colonies and it is unable to form mushrooms. In contrast, the asymmetrical colonies that are formed in the light can form fruiting bodies. The fertile dikaryon is most sensitive to light in the blue part of the spectrum, which induces development of stage I aggregates into stage II primordia (Perkins & Gordon, 1969; Perkins, 1969). These primordia develop further into mature fruiting bodies. Here, the role of the *wc-2* homologue of *S. commune* was studied in fruiting body development. Moreover, the effect of light on genome-wide expression was studied.

MATERIAL AND METHODS

Culture conditions and strains

S. commune was routinely grown from a point inoculum at 25 °C on minimal medium (MM) with 1% glucose and 1.5% agar (van Peer *et al.*, 2009). All *S. commune* strains used were isogenic to strain 1-40 (Raper *et al.*, 1958). The compatible strains 4-40 (CBS 340.81) and 4-39 (CBS 341.81) were used for MPSS analysis. These strains, which are at least 99.8% identical to the sequenced strain H4-8 (FGSC #9210; Chapter 5; Ohm *et al.*, 2010b), show a more synchronized fruiting when compared to a cross between H4-8 and its compatible strain H4-8b (Chapter 2; Ohm *et al.*, 2010a). Strain H4-8 in which *ku80* is inactivated (Chapter 3; de Jong *et al.*, 2010) was used for transformation with the *wc-2* inactivation construct.

Genes in the genome of S. commune

Genes (indicated by ProteinID) of *S. commune* are available through the interactive JGI Genome Portal at http://jgi.doe.gov/Scommune (see Chapter 5; Ohm *et al.*, 2010b). The predicted gene models and annotations are also deposited at DDBJ/EMBL/GenBank under the project accession ADMJ00000000.

Deletion of wc-2

The putative blue light receptor gene *wc-2* (proteinID 13988; Chapter 5; Ohm *et al.*, 2010b) was deleted using vector pDelcas (Chapter 2; Ohm *et al.*, 2010a). Upstream and downstream flanks were amplified by PCR using Phusion polymerase (Finnzymes, Finland) and *S. commune* H4-8 chromosomal DNA. The 1256 bp upstream flank of *wc-2* was amplified with primers dWC2UpFw2 and dWC2UpRev2, whereas the 1515 bp downstream flank was amplified with primers dWC2DwFw and dWC2DwRev (Table 1). The upflank of *wc-2* was cloned into the SmaI site of pUC20, and the downflank into the SmaI site of pUC19, resulting in plasmids pRO184 and pRO112, respectively. The upstream flank was excised from pRO184 using SfiI and introduced in the Van91I site of pDelcas, resulting in pRO188. The downstream flank was excised from pRO112 using SfiI and cloned into pRO188 that had been cut with the same enzyme. This resulted in the gene deletion construct pRO192. The Δ*ku80* strain (Chapter 3; de Jong *et al.*, 2010) was transformed with pRO192 as described (van Peer *et al.*, 2009). $4{\cdot}10^7$ protoplasts were incubated with 80 µg of vector DNA. Regeneration medium and selection plates contained 20 µg ml^{-1} nourseothricin. Plates were incubated at 30 ºC for 3 days and resulting colonies were grown on a second

selection plate containing 20 µg ml^{-1} nourseothricin. Transformants were screened on plates containing 25 µg ml^{-1} phleomycin. Phleomycin sensitive transformants are potential deletion strains, whereas phleomycin resistant strains are the result of a single cross-over event.

Deletion of *wc-2* was confirmed by colony PCR (Chapter 2; Ohm *et al.*, 2010a) using the primer pair dWC2ChkD (upstream of the up-flank) and nourdelrev (downstream of the up-flank, in the nourseothricin resistance cassette) and the primer pair sc3tersqf (upstream of the down-flank, in the nourseothricin resistance cassette) and dWC2ChkA2 (downstream of the down-flank) (Table 1). These primer pairs form a product in the knock-out strain but not in the wild type. On the other hand, the primer pair dWC2ChkD (upstream of the up-flank) and dWC2ChkC (downstream of the up-flank, in the deleted gene) and the primer pair dWC2ChkB2 (upstream of the down-flank, in the deleted gene) and dWC2ChkA2 (downstream of the down-flank) do not form a product in the knock-out strain, but do so in the wild type.

Table 1. Primers used in this study.

Primer name	Sequence
dWC2UpFw2	GGCCTAATAGGCCACCGTCACGTCCATGTTCG
dWC2UpRev2	GGCCTCGCAGGCCCGAAACAACAATGATTG
dWC2DwFw	GGCCTGCGAGGCCCTAGATGTTCGGTAATTGCC
dWC2DwRev	GGCCTATTAGGCCCAGCCACCCATCTCGACTTG
dWC2ChkA2	GCCAGCATCGACTAAAGAC
dWC2ChkB2	GTCATGCTCTACGCATCAG
dWC2ChkC	GCAAGCTCAGTGACAAGTAG
dWC2ChkD	GGTGTCGAGTGTGATGTTTC
nourdelrev	TTGGTGACCTCCAGCCAGAG
sc3tersqf	GCCTCAGGTCCCGAAGTAAG
WC2CodingFw	CCATGGCTACAGTCCACGGCC
WC2CodingRev	GGATCCCAAAAGCTAGAGAAGG

Inducible mushroom formation

The coding sequence and terminator of *wc-2* were amplified by PCR using Phusion polymerase (Finnzymes, Finland) and *S. commune* H4-8 chromosomal DNA. The 1755 bp fragment was amplified with primers WC2CodingFw and WC2CodingRev (Table 1), which contain an NcoI and BamHI site, respectively. The fragment was cloned into the SmaI site of pUC20, which resulted in plasmid pRO203. The coding sequence and terminator of *wc-2* were excised using the restriction enzymes NcoI and BamHI and ligated into the plasmid pRO201, which had been opened with the same enzymes. The resulting plasmid pRO205 encompasses the *wc-2* fragment behind the promoter of the gene encoding the heat shock protein *hsp3* and contains the phleomycin resistance cassette (Chapter 4). Plasmid

pRO205 was introduced in the Δ*wc-2* H4-8 strain as described (van Peer *et al.*, 2009) using 25 µg ml^{-1} phleomycin to select the transformants.

MPSS gene expression analysis

Colonies were pre-grown for 7 days on solid MM at 30 °C in the dark and were homogenized in 200 ml MM using a Waring blender for 1 min at low speed. 2 ml of the homogenized mycelium was spread out over a polycarbonate (PC) membrane (Profiltra, pore size 0.1 µm and diameter 76 mm) that was placed on top of solidified MM. The resulting synchronised colonies were grown for 4 days, either in light or in darkness. RNA was isolated (van Peer *et al.*, 2009) and purified with an RNA clean-up kit (Macherey-Nagel, Germany). Quality of the RNA was analyzed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). Massively Parallel Signature Sequencing (MPSS) was performed essentially as described (Brenner *et al.*, 2000) except that after DpnII digestion MmeI was used to generate 20 bp tags. Tags were sequenced using the Clonal Single Molecule Array technique (Illumina, Hayward, CA, US). The MPSS procedure for the monokaryon and the dikaryon grown in the light have been previously described (Chapter 5; Ohm *et al.*, 2010b). MPSS analysis on the monokaryon and the dikaryon grown in the dark resulted in 2.4 and 5.5 million 20 bp-tags, respectively. All the described tags have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.*, 2002) and are accessible through GEO Series accession number GSE23533 (http://www.ncbi.nlm.nih.gov/geo/). Programs were developed in the programming language Python to analyze the data. Tag counts were normalized to transcripts per million (TPM). Tags with a maximum of < 4 TPM were removed from the data set. TPM values of tags originating from the same transcript (Chapter 5; Ohm *et al.*, 2010b) were summed to assess their expression levels. A transcript is defined as the predicted coding sequence (CDS) extended with 400 bp flanking regions at both sides.

RNAseq gene expression analysis

A whole genome gene expression analysis was performed on dikaryotic colonies (wild type strain, Δ*wc-2*Δ*wc-2* strain, Δ*wc-2*Δ*wc-2* $hsp3_{pr}$-*wc-2* strain) that were grown from a plug inoculum. Colonies were grown for 6 days in the light. This was either or not followed by a heat shock by placing the colony at 42 ºC for 1 hour, followed by a regeneration at 25 ºC in the light for 5 hours. RNA was isolated as described (van Peer *et al.*, 2009) from 3 biological replicates. The quality of the RNA was analysed on a RNA 6000 Lab-on-a-Chip using the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The Illumina mRNA-Seq Sample Prep Kit was used to process the

samples. The sample preparation was performed according the Illumina protocol "Preparing Samples for Sequencing of mRNA" (1004898 Rev. D). Briefly, mRNA was isolated from total RNA using the poly-T-oligo-attached magnetic beads. After fragmentation of the mRNA, cDNA was synthesised. This was used for ligation with the sequencing adapters and PCR amplification, resulting in fragments of approximately 275 bp. A total of 8 pmol DNA was used. The samples were sequenced on the Illumina Genome Analyzer IIx, according to the manufacturer's protocols. Two sequencing reads of 51 cycles each using the Read 1 sequencing and Read 2 sequencing primers were performed within the flow cell. Image analysis, base calling and quality check was performed with the Illumina Genome Analyzer data analysis pipeline v1.5.1. The sequence data has been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.*, 2002) and are accessible through GEO Series accession number GSE24007 (http://www.ncbi.nlm.nih.gov/geo/).

The sequenced reads were mapped to the genome sequence using the program Tophat 1.0.13 (Trapnell *et al.*, 2009) and Bowtie 0.12.5 (Langmead *et al.*, 2009), using default settings with the exception of the minimum and maximum intron length (10 and 400 bp, respectively). The program Cufflinks 0.8.2 (Trapnell *et al.*, 2010) was used to identify reads overlapping with predicted genes (Chapter 5; Ohm *et al.*, 2010b) and to determine the expression levels of these predicted genes for each sample. Expression levels of genes are indicated in FPKM (mapped Fragments Per Kilobase of exon model per Million mapped fragments). Custom scripts were developed to collect the data in 1 file and to identify differentially expressed genes based on a 2 fold change in expression and an absolute change in expression of at least 2 FPKM.

Representation analysis

FuncAssociate 2.0 (Berriz et al., 2009) was used to study over- and under-representation of GO-terms in sets of differentially regulated genes. Default settings were used with a p-value of 0.05 as the cut off.

Hierarchical clustering analysis

For hierarchical clustering analysis, only genes were used that were expressed in at least 1 of the 5 samples. Expression values were log2-transformed, after the values were increased with 1 in order to prevent negative log2 values. Hierarchical clustering of genes and samples and the subsequent analysis of the results were performed with the program MeV, which is part of the TM4 Software Suite (Saeed *et al.*, 2006). Euclidian distance was used as distance metric and average linkage clustering as linkage method.

Promoter analysis

Here, promoters are defined as the 750 bp upstream region of the translation start site of genes. This 750 bp region is expected to contain a core promoter and upstream regulatory elements. The MEME Motif Discovery Tool (Bailey & Elkan, 1994) was used to identify similar regions in promoters. The TOMTOM Motif Comparison Tool (Gupta *et al.*, 2007) was used to compare these regions with known motifs in the TRANSFAC database (Wingender *et al.*, 2000). The RSAT regulatory sequence analysis tools suite (Thomas-Chollier *et al.*, 2008) was used to count the occurrence of putative regulatory elements in various sets of promoters. Significance of over-representation of these putative regulatory elements in a subset of promoters compared to all the other promoters was calculated using the Fisher's exact test.

RESULTS

Gene expression in light and darkness

Wild type monokaryotic and dikaryotic cultures were grown for four days from a homogenate. In this way, development proceeds in a synchronized way throughout the agar plate. The monokaryotic cultures showed no phenotypic differences when cultures were grown in the light or dark. In both cases, the cultures consisted of a vegetative mycelium on top of which a layer of aerial hyphae had formed (Figure 1). The 4-days-old light-grown dikaryotic culture had formed stage II primordia. In contrast, development had stopped in dark grown cultures after formation of stage I aggregates (Figure 1). A genome-wide expression analysis was performed on these cultures using MPSS. In all cases, approximately 50% of the genes were expressed in sense direction (Table 2). Antisense transcripts were found in the case of 15% of the genes in the monokaryotic culture grown in the light. This number was more than 2-fold higher in the other cultures. Antisense transcripts were particularly abundant in the dikaryotic cultures that had been grown in the light.

28% of the genes showed a fold change in expression of two or more when light and dark grown monokaryons were compared. This was 12% in the case of the dikaryon (Table 3). Only 2.3% of the genes were similarly regulated in the monokaryon and the dikaryon. These genes are expected to be regulated by light independent of the developmental stage of the colony. An over-representation of GO-terms, KEGG-terms, KOG-terms and PFAM-terms was performed on these light-regulated genes. The genes that were upregulated in darkness showed no over-representation of any of the terms. On the other hand, there were several functional annotation terms that were over-represented in the genes that were up-regulated in light (Supplementary

Table 1). Genes encoding proteins involved in metabolism are more active in the light as well as genes encoding chitinases and cytochrome P450.

Six transcription factor genes were up-regulated in the light in both the mono- and dikaryon. Five of these genes encode predicted transcription factors with a zinc-finger domain (protein IDs 106014, 52392, 110310, 78566 and 54394), whereas one of the genes encodes a homeobox transcription factor (protein ID 237403).

Deletion of wc-2

Several classes of photoreceptor genes have been identified in the genome of *S. commune* (Figure 2; Chapter 5; Ohm *et al.*, 2010b). *S. commune* contains homologues of *wc-1* and *wc-2*, which encode central components in blue light signal transduction in *N. crassa*. *S. commune* also contains a putative cryptochrome and a phytochrome. No homologues of *vvd* or rhodopsins could be identified.

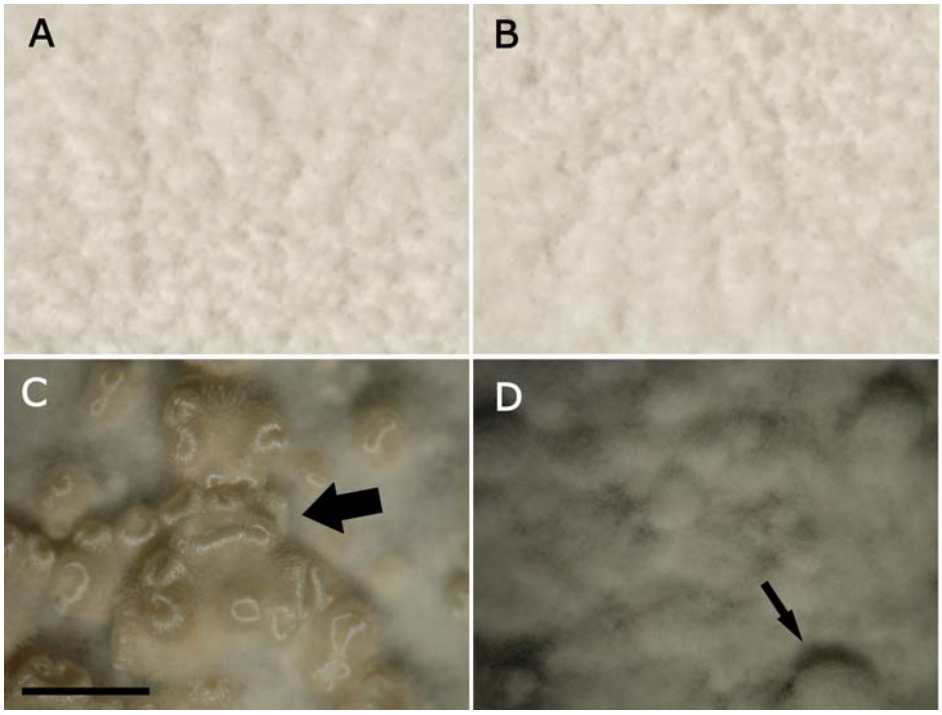

Figure 1. Monokaryotic and dikaryotic cultures of *S. commune* grown for 4 days in either continuous light or in darkness from a mycelial homogenate. In monokaryotic colonies grown in the light (**A**) or in the dark (**B**) only substrate hyphae and aerial hyphae had been formed. In contrast, stage II primordia had developed in light grown dikaryotic colonies (**C**), whereas development had stopped in the dark at the stage of stage I aggregates (**D**). The bar represents 5 mm. The thin arrow indicates a stage I aggregate, the thick arrow a stage II primordium.

Table 2. Gene expression in wild type monokaryotic and dikaryotic cultures of *S. commune* grown in continuous light or darkness as assessed by MPSS analysis. Cultures had been inoculated with mycelial homogenates allowing synchronized development throughout the agar plate.

Developmental stage	Percentage of genes expressed		Level of gene expression	
	Sense	Antisense	Sense (% of total)	Antisense (% of total)
Monokaryon - Light grown	50.6	15.3	86.3	13.7
Monokaryon – Dark grown	50.3	33.5	75.9	24.1
Dikaryon – Light grown	51.5	39.3	66.9	33.1
Dikaryon – Dark grown	51.9	33.3	76.4	23.6

Table 3. Differential gene expression (≥ 2 fold change in expression) in light and dark grown monokaryotic and dikaryotic wild type cultures of *S. commune*. Cultures had been inoculated with mycelial homogenates allowing synchronized development throughout the agar plate.

Sample	Regulation (≥ 2 fold difference)	Number of genes (% of total)
Monokaryon	High in darkness	1564 (11.8%)
	High in light	2103 (15.9%)
	Total	3667 (27.7%)
Dikaryon	High in darkness	631 (4.8%)
	High in light	990 (7.5%)
	Total	1621 (12.3%)
Monokaryon and dikaryon	High in darkness	181 (1.4%)
	High in light	119 (0.9%)
	Total	300 (2.3%)

The Δ*ku80* strain of H4-8 was transformed with the *wc-2* deletion construct pRO192 to assess the role of this gene in growth and development of *S. commune.* Three out of four transformants were phleomycin resistant, which is indicative for a single cross-over event (Chapter 2; Ohm *et al.*, 2010a). One of the transformants was phleomycin-sensitive, which is indicative for a double cross-over event and therefore for inactivation of the *wc-2* gene. Deletion of this gene was confirmed by colony PCR (for details see Material and methods). The Δ*ku80*Δ*wc-2* strain (which is hygromycin and nourseothricin resistant) was crossed with the wild type strain H4-8b to select for Δ*wc-2* strains (nourseothricin resistant and hygromycin sensitive) with mating types of strains H4-8 and H4-8b and with an intact copy of *ku80*. To determine the phenotype of these strains, cultures were grown from a point inoculum. This was done because strain H4-8, in contrast to 4-40, does not form fruiting structures when grown from a homogenate.

There was no detectable difference in the phenotype of wild type and Δ*wc-2* monokaryons (data not shown). In contrast, phenotypic differences were observed in the dikaryotic strains. A 7-day-old culture of the wild type dikaryon that had been grown in the light showed an irregular shape at the

periphery and had formed stage I aggregates, stage II primordia and mature mushrooms (Figure 3A, left panel). 7-day old colonies of the Δ*wc-2*Δ*wc-2* dikaryon, however, had formed a symmetrical colony in the light and no aggregates, primordia or mushrooms were observed (Figure 3B, left panel). This phenotype resembled that of a wild type and a Δ*wc-2*Δ*wc-2* dikaryon that had been grown in the dark (Figure 3A and B, right panels).

A genome-wide expression analysis was performed on 6-day-old wild type and Δ*wc-2*Δ*wc-2* dikaryons using RNAseq (Figure 4). Compared to the wild type, 641 and 844 genes were up- and down-regulated, respectively (≥ 2-fold), in the Δ*wc-2*Δ*wc-2* dikaryon. Functional categories that are over-represented in the up-regulated genes include terms related to metabolism of carbohydrates and secondary metabolites (Supplementary Table 2). In the down-regulated genes, mainly genes encoding hydrophobins and multicopper oxidases are over-represented. In the next step, expression was analyzed of selected individual genes. Expression of *c2h2* and *hom1* was more than two-fold decreased in the Δ*wc-2*Δ*wc-2* dikaryon when compared to the wild type (Table 4). The other transcription factor genes that have been shown to be involved in mushroom formation showed a fold change in expression of less than 2. Ten out of the 13 hydrophobin genes were down-regulated at least 2-fold in the Δ*wc-2*Δ*wc-2* dikaryon (Table 4). Genes *lccA*, *sc7* and *sc14*, which have previously been shown to be up-regulated during mushroom formation, also showed at least a 2-fold reduction in expression in the Δ*wc-2*Δ*wc-2* dikaryon (Table 4).

An inducible functional WCC

The Δ*wc-2* H4-8 strain (which is nourseothricin resistant) was complemented by transforming it with vector pRO205, which encompasses a phleomycin selection cassette and the coding sequence of *wc-2* under the control of the heat inducible promoter of *hsp3.* Transformants were selected on medium containing phleomycin. Twenty of such transformants were crossed with a compatible Δ*wc-2* strain, resulting in dikaryotic Δ*wc-2*Δ*wc-2* $hsp3_{pr}$–*wc-2* strains. When grown at 25 °C, the phenotype of these strains was identical to that of the Δ*wc-2*Δ*wc-2* dikaryon (Figure 5). However, when colonies were incubated at 42 °C for 1 hour every day (thereby activating the *hsp3* promoter), the shape of the Δ*wc-2*Δ*wc-2* $hsp3_{pr}$–*wc-2* colonies became irregular and mushrooms had formed after 10 days. These results show that the phenotype of the Δ*wc-2* strain is indeed caused by the absence of WC-2, and that mushroom formation can be induced using a heat shock inducible promoter fused to the coding sequence of *wc-2*.

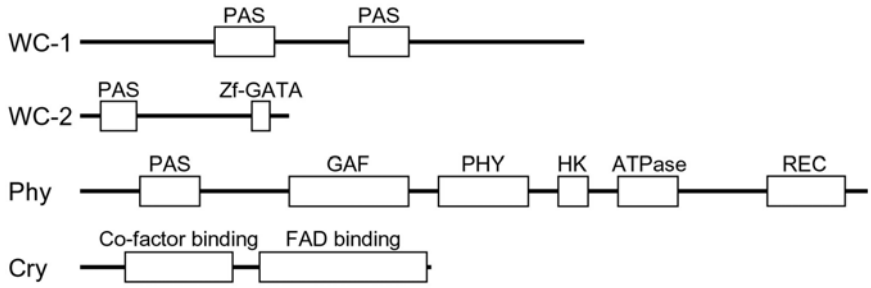

Figure 2. Predicted photoreceptor proteins in *S. commune*. Genes encoding homologues of WC-1 (proteinID 78657), WC-2 (proteinID 13988), phytochrome (Phy, proteinID 76719) and cryptochrome (Cry, proteinID 49182) have been identified in the genome of *S. commune*. WC-1 and WC-2 of *S. commune* contain protein interaction PAS (Per-Arnt-Sim) domains. Moreover, WC-2 is predicted to have a zinc finger (Zf) DNA binding domain of the GATA type. The predicted phytochrome of *S. commune* has a sensory part, which is composed of the PAS, GAF and PHY domains and has a binding site for the bilin chromophore. The effector part of phytochrome is composed of the HK (histidine kinase), ATPase (ATP binding domain) and REC (response regulator receiver) domains. The predicted cryptochrome of *S. commune* has an antenna co-factor binding domain and a FAD (flavin adenine dinucleotide) binding domain. Domains were identified using the Conserved Domain Database (Marchler-Bauer *et al.*, 2009) and are discussed in Corrochano, 2007.

A genome-wide expression analysis was performed on colonies of the Δ*wc-2*Δ*wc-2* dikaryon and the Δ*wc-2*Δ*wc-2 hsp3*$_{pr}$–*wc-2* dikaryon that had been grown for 6 days in the light and that were either or not subjected to a heat shock at 42 °C for 1 hour and a 5 h regeneration at 25 °C in the light. In general, the expression profiles of the Δ*wc-2*Δ*wc-2* dikaryon and the Δ*wc-2*Δ*wc-2 hsp3*$_{pr}$–*wc-2* were more similar to each other than to a wild type dikaryon grown for 6 days at 25 °C (Figure 4). Moreover, the profiles of cultures that did receive a heat shock cluster together, as well as the samples that did not receive such a treatment. From this it is concluded that the heat shock has a larger impact on gene expression than the induction of *wc-2.*

Genes were identified that were differentially expressed in the Δ*wc-2*Δ*wc-2 hsp3*$_{pr}$–*wc-2* dikaryon after heat shock when compared to the Δ*wc-2*Δ*wc-2* dikaryon before and after heat shock and the Δ*wc-2*Δ*wc-2 hsp3*$_{pr}$–*wc-2* dikaryon before heat shock. By making this comparison, genes induced by the heat stress response are ruled out (for these genes see Supplementary Text 4) and only genes are identified that are up- or down-regulated as a result of the induced expression of *wc-2*. 21 genes were found to be up-regulated (≥ 2-fold) after exposure of the Δ*wc-2*Δ*wc-2 hsp3*$_{pr}$–*wc-2* strain at 42 °C and 7 genes were found to be down-regulated (Table 5). Up-regulated genes included previously described genes *sc7* and *bap3-3* (B alpha 3 mating pheromone precursor). In addition, the putative cryptochrome and a predicted

signaling protein containing a TspO/MBR-related protein domain were up-regulated.

Of the 21 up-regulated genes, 5 were also higher expressed in the light in both the wild type monokaryon and the dikaryon, as determined by MPSS. In the promoters of these 5 genes, 3 conserved motifs were identified that contain a GAT subsequence, which is a zinc finger GATA transcription factor binding sequence (motifs 1–3 in Table 6). All these putative regulatory elements were over-represented in the promoters of the 21 up-regulated genes compared to all the other promoters. Interestingly, motif 1 shows similarity to part of the distal Light Responsive Element (LRE) in the *frq* promoter of *Neurospora crassa* (CGATGACGCT), which was shown to bind the WCC complex upon light induction (Froehlich *et al.*, 2002). Additionally, a conserved motif was found in the promoters of the down-regulated genes (motif 4 in Table 6).

As expected, expression of *wc-2* is absent in the Δ*wc-2*Δ*wc-2* dikaryon (Table 4). Low expression of *wc-2* is detected in the Δ*wc-2*Δ*wc-2* $hsp3_{pr}$–*wc-2* dikaryon without the heat shock treatment and this level almost doubles after heat shock. This level may have peaked at a higher level, however, before the mycelium was harvested. Expression of *wc-1* is lower in the Δ*wc-2*Δ*wc-2* dikaryon, but does not significantly increase upon induction of *wc-2* expression. None of the transcription factors described in Chapter 5 and 6 are induced (≥ 2 fold) upon *wc-2* induction (Table 6), but it should be noted that *hom2* is up-regulated 1.7 fold. The promoter of this gene does not contain any of the conserved motifs listed in Table 6.

Effect of light on transcription factor gene deletion strains

We have previously described the role of the transcription factor genes *hom2*, *bri1*, *fst4*, *c2h2*, *hom1*, *gat1* and *fst3* in mushroom development (Chapter 5; Chapter 6; Ohm *et al.*, 2010b). Dikaryotic cultures of these deletion strains were grown in continuous light or in continuous darkness. There was no or little difference in phenotype when Δ*hom2*Δ*hom2* or Δ*bri1*Δ*bri1* dikaryons where grown either in light or in darkness (Figure 3C and D). On the other hand, dark-grown colonies of Δ*fst4*Δ*fst4*, Δ*c2h2*Δ*c2h2* Δ*fst3*Δ*fst3*, Δ*hom1*Δ*hom1* or Δ*gat1*Δ*gat1* dikaryons were more regular than the light-grown colonies (Figure 3E, F, G, H and I). Furthermore, they did not form fruiting bodies. This phenotype strongly resembles the dark-grown wild type dikaryon (Figure 3A, right panel).

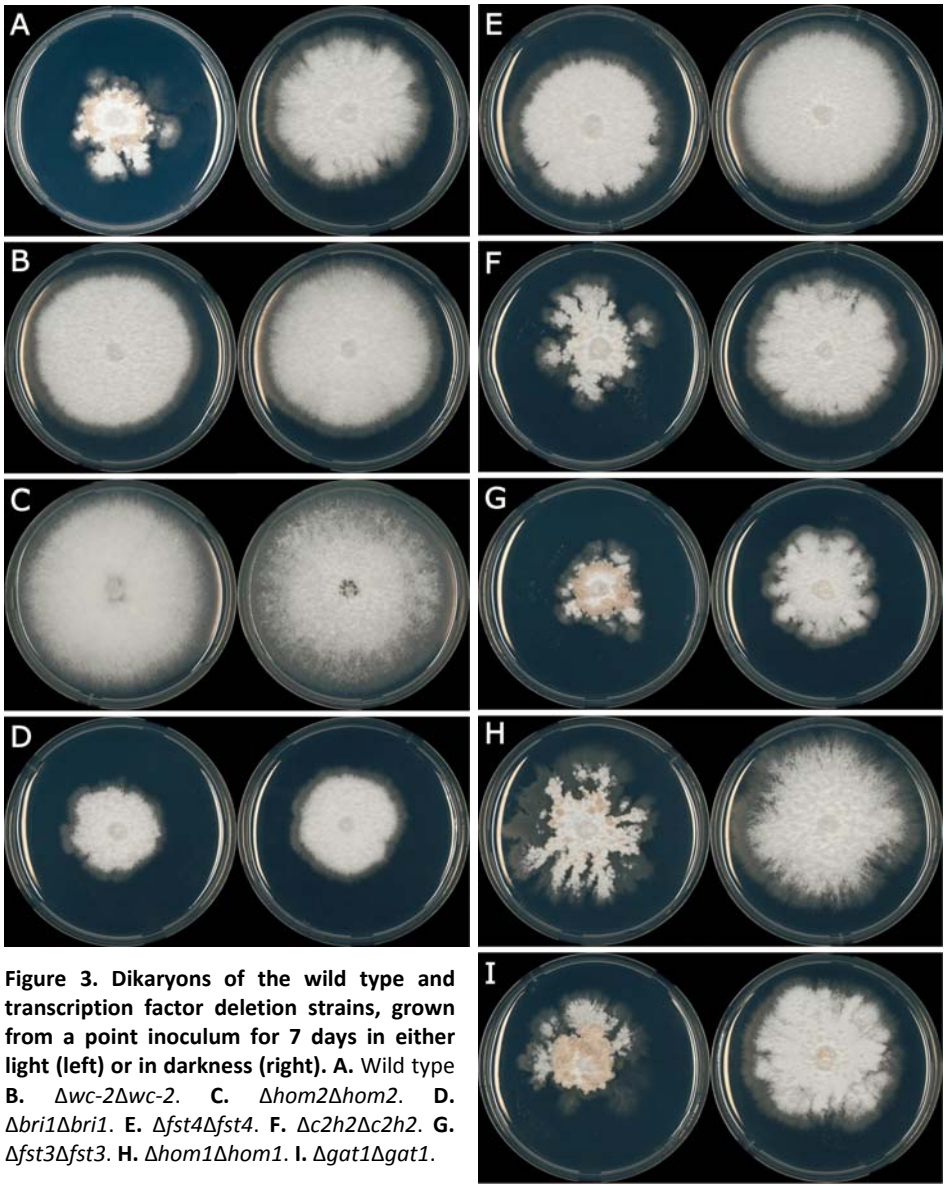

Figure 3. Dikaryons of the wild type and transcription factor deletion strains, grown from a point inoculum for 7 days in either light (left) or in darkness (right). A. Wild type **B.** Δ*wc-2*Δ*wc-2*. **C.** Δ*hom2*Δ*hom2*. **D.** Δ*bri1*Δ*bri1*. **E.** Δ*fst4*Δ*fst4*. **F.** Δ*c2h2*Δ*c2h2*. **G.** Δ*fst3*Δ*fst3*. **H.** Δ*hom1*Δ*hom1*. **I.** Δ*gat1*Δ*gat1*.

DISCUSSION

Recently, 7 transcription factor genes have been identified in *S. commune* that play a role in the formation of fruiting bodies (Chapter 5; Chapter 6; Ohm *et al.*, 2010b). In a Δ*hom2*Δ*hom2* dikaryon the colony grew symmetrically and no mushrooms developed. A similar phenotype was observed for a Δ*bri1*Δ*bri1* dikaryon, although the colony was less symmetrical. In a Δ*fst4*Δ*fst4* dikaryon also no mushrooms developed, but, like

in the wild type, the shape of the colony was very irregular. This was also the case in a Δ*c2h2*Δ*c2h2* dikaryon, but development stopped at stage I aggregates. In dikaryons of Δ*hom1*Δ*hom1*, Δ*fst3*Δfst3 and Δ*gat1*Δ*gat1*, more but smaller mushrooms were formed.

Here, the role of the putative transcription factor gene *wc-2* was studied. This gene is homologous to the *wc-2* gene of *N. crassa.* The WC-2 and WC-1 proteins *of N. crassa* are part of the white collar complex (WCC), which is involved in blue light mediated sexual development. Blue light is also most effective in inducing mushroom development in *S. commune* (Perkins & Gordon, 1969), suggesting a role for a white collar-like complex in this process. The presence of such a complex in *S. commune* is indicated by the presence of homologues of both *wc-1* and *wc-2.* In contrast to WC-1 of *N. crassa* (Ballario *et al.*, 1996), the *S. commune* homologue has no apparent DNA binding zinc finger domain. This was previously also shown for Dst-1, the *Coprinopsis cinerea* homologue of WC-1 (Terashima *et al.*, 2005).

Deletion of *wc-2* in *N. crassa* resulted in a blind phenotype (Linden & Macino, 1997). Here, we describe a similar phenotype for *S. commune.* The Δ*wc-2*Δ*wc-2* dikaryon grew in the light or in the dark similar to dark-grown wild type dikaryons. Colonies resulting from a point inoculum grew symmetrically and did not from mushrooms. Mutants with a blind phenotype have also been isolated in *C. cinerea*. One of them could be complemented with gene *dst1*, which is the *wc-1* homologue (Terashima *et al.*, 2005). Another strain with a blind phenotype could be complemented with *dst2*. This gene encodes a protein with a flavin adenine dinucleotide (FAD) binding domain. This domain is also found in WC-1 (Kuratani *et al.*, 2010). *S. commune* has a clear homologue of *dst2* (protein ID 69239), which is strongly up-regulated during development of stage II primordia (Chapter 5; Ohm *et al.*, 2010b). It is, however, similarly expressed in a Δ*wc-2*Δ*wc-2* dikaryon and wild type (data not shown). The exact role of this protein in blue light sensing remains to be elucidated.

Up-regulation of 6 selected dikaryon-specific genes after a light pulse has previously been shown in *S. commune* (Yli-Mattila *et al.*, 1989). Here, we show that 119 genes were up-regulated in the light in both a wild type mono- and dikaryon. Among these were six putative transcription factor genes, five of which are predicted to encode a zinc-finger domain and one a homeobox transcription factor. Expression of these six genes was not affected in the Δ*wc-2*Δ*wc-2* strain, however. This difference may be explained by the fact that growth conditions that were used for the expression profiling were different. Alternatively, these genes are regulated by another photoreceptor. In both cases these genes are unlikely to be involved in mushroom formation.

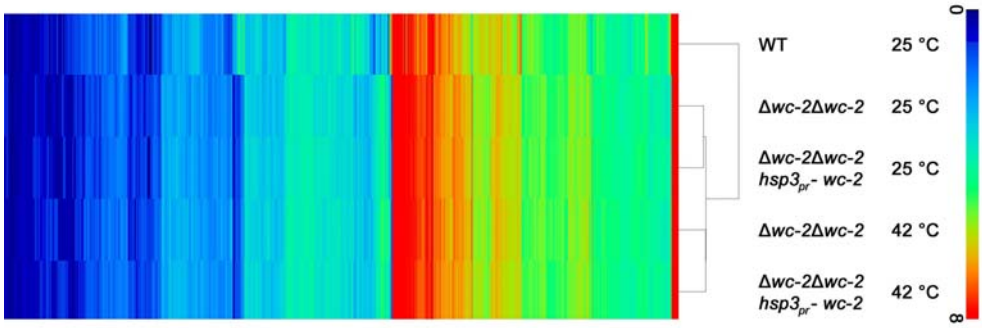

Figure 4. Heat map depicting hierarchically clustered gene expression data. The samples were grown for 6 days at 25 °C and had either or not received a heat shock of 1 hour at 42 °C. Only expressed genes are shown. Given values are the log2-transformed FPKM expression values (mapped Fragments Per Kilobase of exon model per Million mapped fragments) and range from 0 to 8.

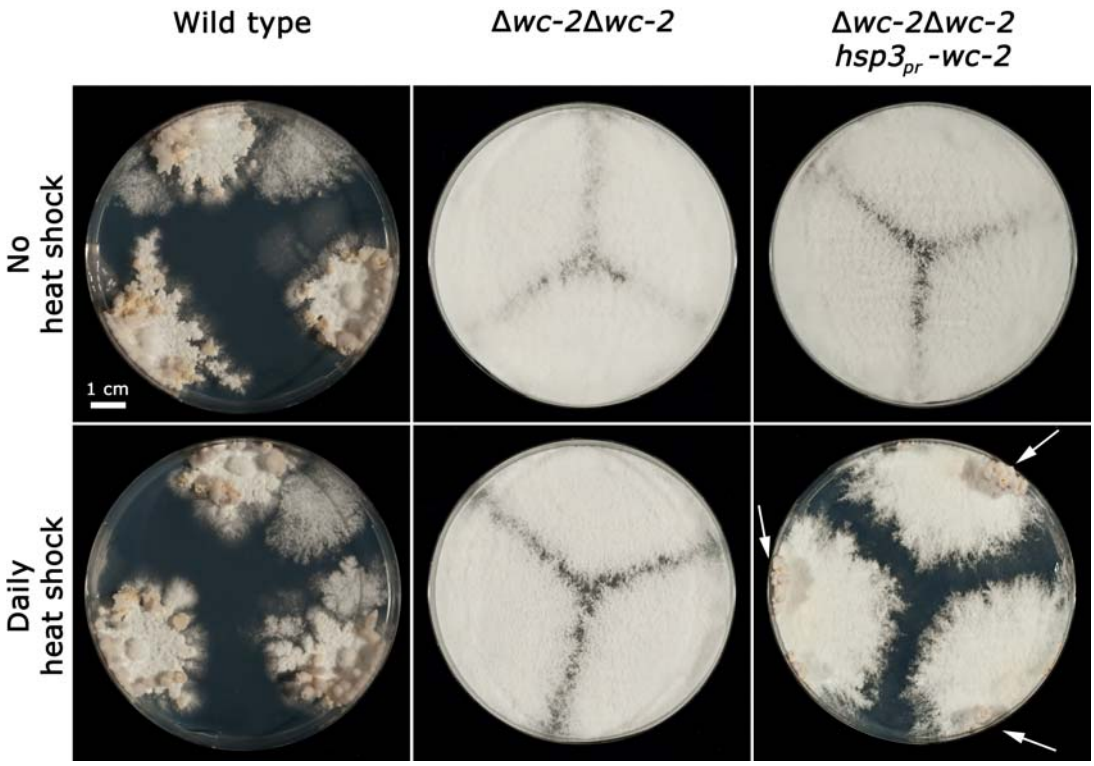

Figure 5. Induction of mushroom formation by induced expression of *wc-2*. Mushroom formation can be induced in a Δ*wc-2*Δ*wc-2 hsp3*$_{pr}$*–wc-2* dikaryon of *S. commune* by exposing these colonies every day for 1 h at 42 °C. Colonies that are depicted have been grown for 10 days. Induced mushrooms in the lower right panel are indicated with white arrows.

Table 4. Expression of genes in dikaryons of wild type, Δ*wc-2*Δ*wc-2* and Δ*wc-2*Δ*wc-2* *hsp3*$_{pr}$ – *wc-2*. The samples were grown for 6 days at 25 °C and had either or not received a heat shock of 1 hour at 42 °C. Expression values are in FPKM (mapped Fragments Per Kilobase of exon model per Million mapped fragments). For more details on these genes, see Chapter 5 and Ohm *et al.*, 2010b.

ProteinID	Name	Wild type 25 °C	Δ*wc-2* Δ*wc-2* 25 °C	Δ*wc-2* Δ*wc-2* 42 °C	Δ*wc-2* Δ*wc-2* *hsp3*$_{pr}$ – *wc-2* 25 °C	Δ*wc-2* Δ*wc-2* *hsp3*$_{pr}$ – *wc-2* 42 °C
Hydrophobin genes						
77028	*sc3*	29213.4	17326.60	22657.70	22942.80	24705.50
73533	*sc4*	12039.5	30.09	44.61	31.08	42.65
13677	*sc1*	1081.63	0.56	0.46	1.22	0.83
13059	*sc6*	115.11	0.22	0.14	0.00	0.82
58269	*hyd1*	212.15	0.18	0.22	0.17	0.00
82440	*hyd2*	97.8	410.20	425.56	460.38	431.42
232866	*hyd3*	30.98	0.00	0.00	0.00	0.00
51576	*hyd4*	266.92	3.16	2.08	3.82	4.44
250297	*hyd5*	2.03	0.67	1.12	1.68	0.00
85429	*hyd6*	5801.93	3374.77	3295.50	3510.61	3017.45
109698	*hyd7*	37.29	0.00	0.00	0.00	0.00
105121	*hyd8*	198.02	0.22	0.18	0.14	0.33
249002	*hyd9*	80.54	0.00	0.00	0.18	0.29
Genes encoding putative structural proteins						
269921	*lccA*	29.32	1.97	5.33	2.25	2.12
85445	*sc7*	988.12	0.45	1.44	0.83	8.37
16267	*sc14*	8.26	0.19	1.06	0.36	1.42
109852	*agg1*	6341.25	5005.22	2934.37	3946.64	2689.30
82353	*sc15*	11019.5	13040.00	12649.70	13095.90	19354.60
Transcription factor genes						
78657	*wc-1*	22.79	13.92	13.96	14.99	15.86
13988	*wc-2*	10.22	0	0	0.38	0.61
257987	*hom2*	62.57	34.45	39.01	38.79	66.97
66861	*fst4*	52	31.75	30.37	40.4	41.23
114363	*c2h2*	17.82	6.25	5.36	7.54	5.59
257422	*fst3*	25.9	27.66	31.23	36.23	34.76
255004	*gat1*	20.48	14.29	16.62	17.06	17.16
257652	*hom1*	119.78	33.28	25.07	35.29	29.57
255701	*bri1*	15.05	8.54	10.3	10.57	11.09

Previously, a regulatory model of mushroom formation has been proposed (Chapter 6). Here, this model is extended with the role of WC-2 (Figure 6). Phenotypic analysis of the Δ*wc-2*Δ*wc-2* dikaryon showed that WC-2 affects the shape of the colony and fruiting body formation, possibly by an effect on

the production of an auto-inhibitor (Klein *et al.*, 1997). A similar phenotype was observed for Δ*hom2*Δ*hom2* and Δ*bri1*Δ*bri1* dikaryons. This and the fact that the Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2* and Δ*bri1*Δ*bri1* dikaryons showed a similar phenotype, irrespective of the fact whether they had been grown in the light or in the dark indicates that *wc2* functions at the same level (Figure 6) or down-stream of *hom2* and *bri1.* The evidence that indicates that *wc-2*, *hom2* and *bri1* belong to the same functional group is supported by gene expression in the Δ*wc-2*Δ*wc-2* and Δ*hom2*Δ*hom2* strains. In both strains *c2h2* and *hom1* showed a reduced expression when compared to the wild-type strain. Moreover, expression of the dikaryon-specific hydrophobin genes is affected in the Δ*wc-2*Δ*wc-2* and Δ*hom2*Δ*hom2* strains. Notably, the expression of the monokaryotic hydrophobin gene *sc3* is not significantly decreased, which would explain why aerial hyphae are formed while mushrooms do not develop.

Mushroom formation could be induced in a Δ*wc-2*Δ*wc-2* strain by transforming this strain with a construct encompassing the coding sequence of *wc-2* behind the heat inducible *hsp3* promoter and by applying a daily heat shock at 42 °C. It was shown that the heat shock had no adverse effects on growth or development in the wild type. Whole genome expression analysis of this strain and the Δ*wc-2*Δ*wc-2* strain revealed that 21 genes were up-regulated 5 hours after the induced expression of *wc-2*. These genes are likely to be regulated by the white collar complex or its associated signal transduction pathway. Interestingly, a conserved element in the promoters of these genes was identified, which is very similar to a previously reported light responsive element (LRE) in the promoter of *frq* in *N. crassa* (Froehlich *et al.*, 2002). This strengthens the conclusion that these genes are indeed regulated by the white collar complex. A conserved element was also found in 5 of the 7 promoters of genes that are down-regulated after the induced expression of *wc-2*. This suggests that these genes are also co-regulated. Whether this is a direct or indirect effect of the induction of *wc-2* is a topic of future study.

None of the 7 transcription factor genes that have been shown to be involved in mushroom formation (Table 4 and Chapters 5 and 6) were up-regulated at least two-fold after a heat shock in the Δ*wc-2*Δ*wc-2* $hsp3_{pr}$–*wc-2* strain. However, a gene (protein ID 63148) encoding a protein containing a TspO/MBR-related domain, was much lower expressed in the Δ*wc-2*Δ*wc-2* dikaryon than in the wild type, and its expression was strongly up-regulated after induced expression of *wc-2*. Homologues of this gene have been shown to be involved in transmembrane signaling in prokaryotes and mitochondria (Yeliseev & Kaplan, 1995) and could have a role in light signaling in *S. commune*. The putative cryptochrome light receptor of *S. commune* (Chapter

Table 5. Continued on next page.

Up-regulated genes

Protein ID	Wild type 25 °C	Δ*wc-2* Δ*wc-2* 25 °C	Δ*wc-2* Δ*wc-2* 42 °C	Δ*wc-2* Δ*wc-2* *hsp3*$_{pr}$ – *wc-2* 25 °C	Δ*wc-2* Δ*wc-2* *hsp3*$_{pr}$ – *wc-2* 42 °C	Annotation	Occurences of motif (see table 6)			
							# 1	# 2	# 3	# 4
71602	1030	391	432	348	1020	Lectin	0	0	0	0
62971	16	4	4	5	12	Predicted E3 ubiquitin ligase	0	0	0	0
49182	13	1	1	1	6	Putative cryptochrome	0	0	0	0
63148	302	12	12	24	192	TspO/MBR-related protein	0	0	0	0
49478	7	2	1	1	6	Unknown	1	1	1	0
72453	858	181	167	153	398	Uroporphyrinogen decarboxylase	0	0	0	0
231454	16	5	5	6	12	Unknown	0	0	0	0
10237	118	32	26	36	113	Glutathione S-transferase	0	0	0	0
84309	2	1	1	1	3	Protein binding BTB/POZ	0	0	0	0
60520	2	0	0	0	2	Bap3-3, B alpha 3 mating pheromone precursor	0	0	0	0
70398	217	11	13	13	37	Candidate a-amylase; GPI anchor, Glycoside Hydrolase Family 13 protein	0	0	0	0
237570	2	0	1	1	3	Unknown	1	1	1	0
42355	2	2	2	1	4	Unknown	0	0	0	0
42397	3	3	5	5	10	6-O-methylguanine-DNA methyltransferase MGMT/MGT1, involved in DNA repair	0	0	0	0
66245	16	49	86	63	190	Catalase	1	0	0	1
53048	23	2	2	2	9	Cyclopropane fatty acid synthase	1	1	1	0
233627	430	7	10	10	23	Unknown	0	0	0	0
85445	988	0	1	1	8	SC7	0	0	0	0
111177	1	0	0	0	3	Unknown	0	0	0	0
58381	78	28	31	23	75	Protoheme ferro-lyase (ferrochelatase)	1	0	0	0
111500	42	2	3	2	10	Unknown	1	1	1	0

Table 5 (continued). Differentially expressed genes as a result of the induced expression of *wc-2*. The samples were grown for 6 days at 25 °C and had either or not received a heat shock of 1 hour at 42 °C. Expression values are in FPKM (mapped Fragments Per Kilobase of exon model per Million mapped fragments) and are rounded to the nearest number for display purposes. The number of occurrences of the 4 putative regulatory motifs (see Table 6) in the promoters of these genes are given. Up-regulated genes are described on the previous page, down-regulated genes are described on this page.

Down-regulated genes										
Protein ID	Wild type 25 °C	Δ*wc-2* Δ*wc-2* 25 °C	Δ*wc-2* Δ*wc-2* 42 °C	Δ*wc-2* Δ*wc-2* $hsp3_{pr}$ – *wc-2* 25 °C	Δ*wc-2* Δ*wc-2* $hsp3_{pr}$ – *wc-2* 42 °C	Annotation	Occurences of motif (see table 6)			
							# 1	# 2	# 3	# 4
113727	4	4	4	3	2	Unknown	0	0	0	0
66416	22	3	2	5	1	Unknown	1	0	0	0
54466	5	28	22	23	9	Glycoside Hydrolase Family 61 protein	0	0	0	1
84994	1905	929	266	747	127	Unknown	0	0	0	1
110393	1	2	2	2	0	Unknown	0	0	0	1
111587	4	6	5	7	2	Galectin, galactose-binding lectin	0	0	0	1
258159	3	4	5	3	1	Unknown	0	0	0	1

Table 6. Conserved motifs in the promoters of putative *wc-2* regulated genes. All motifs contain a GAT subsequence and are significantly over-represented in the promoters of a subset of either up- or down-regulated genes.

Motif	Consensus sequence	Found in promoters of up- or down-regulated genes	# promoters of regulated genes with motif / promoters in subset	# promoters of all other genes with motif / promoters in subset	Significance of over-representation
1	GAT[GT]ACGC	Up	6 / 21	693 / 13189	$p < 10^{-3}$
2	C[AT]ACGAT[CA][CT]A	Up	4 / 21	83 / 13189	$p < 10^{-5}$
3	A[CG]G[CT]G[GACT][AT] [GC]GATG[AT][GTA]G	Up	4 / 21	37 / 13189	$p < 10^{-6}$
4	C[AT]GAGGAT	Down	5 / 7	718 / 13203	$p < 10^{-5}$

5; Ohm *et al.*, 2010b) is also up-regulated after induced expression of *wc-2*. This indicates the existence of a cross-talk between the white collar complex and the cryptochrome. In *N. crassa*, the cryptochrome gene *cry* was recently also shown to be up-regulated by the white collar complex (Froehlich *et al.*, 2010).

314 genes (amounting to 5.6% of all detectable transcripts) were shown to be regulated by light in a genome-wide expression study in *N. crassa* (Chen *et al.*, 2009). These genes were divided in early and late light-responsive genes, peaking earlier or later than approximately 30 minutes after light induction, respectively. After 4 hours, the majority of these genes had returned to un-induced levels. In this study, the mycelium was harvested 5 hours after the heat shock treatment. During this time, the *wc-2* transcript is produced and translated into the WC-2 protein, which is activated by the light and in turn activates its target genes. The lower number of genes that are up-regulated in this study compared to that in *N. crassa* can at least partially be explained by the fact that most genes will have returned to un-induced levels by the time of harvesting. The genes that remain up-regulated may therefore play a role in the initiation of mushroom formation.

In *N. crassa*, WC-1 and WC-2 play an important role in the entrainment of the circadian clock (Dunlap & Loros, 2006). Together they form the white collar complex, which activates expression of the *frequency* (*frq*) gene (Merrow *et al.*, 2001; Froehlich *et al.*, 2003). The FRQ protein, in turn, represses the activity of WC-1 and WC-2 in a negative feedback loop. This results in an oscillation of WC-1 and WC-2 on the one hand and FRQ on the other hand. The output of the system is the rhythmic expression of the clock-controlled genes (Loros *et al.*, 1989). In *S. commune*, there are no indications of a circadian clock. For instance, a light-darkness cycle is not necessary for mushroom development. The absence of a circadian clock in *S. commune*, however, would make this basidiomycete an exception in the fungal kingdom (Greene *et al.*, 2003; Tan *et al.*, 2004; Liu & Bell-Pedersen, 2006; Eelderink-Chen *et al.*, 2010). Although homologues of the *frq* gene are found in many ascomycetes, no homologues can be found in the genomes of *S. commune* and several other basidiomycetes (Dunlap & Loros, 2006). This would support the hypothesis of the absence of a circadian clock in *S. commune*. It should be noted that *N. crassa* has another circadian oscillation, which is independent of FRQ (de Paula *et al.*, 2006; Brody *et al.*, 2010) and this may also be the case in *S. commune.*

Taken together, this study has shown that the *wc-2* gene of *S. commune* is involved in mushroom formation. Expression of *wc-2* from the *hsp3* promoter resulted in mushroom formation after a daily heat shock. To

our knowledge, this is the first report of mushroom formation that is induced by the use of an inducible promoter. These results may pave the way to improve control of production of commercial mushrooms.

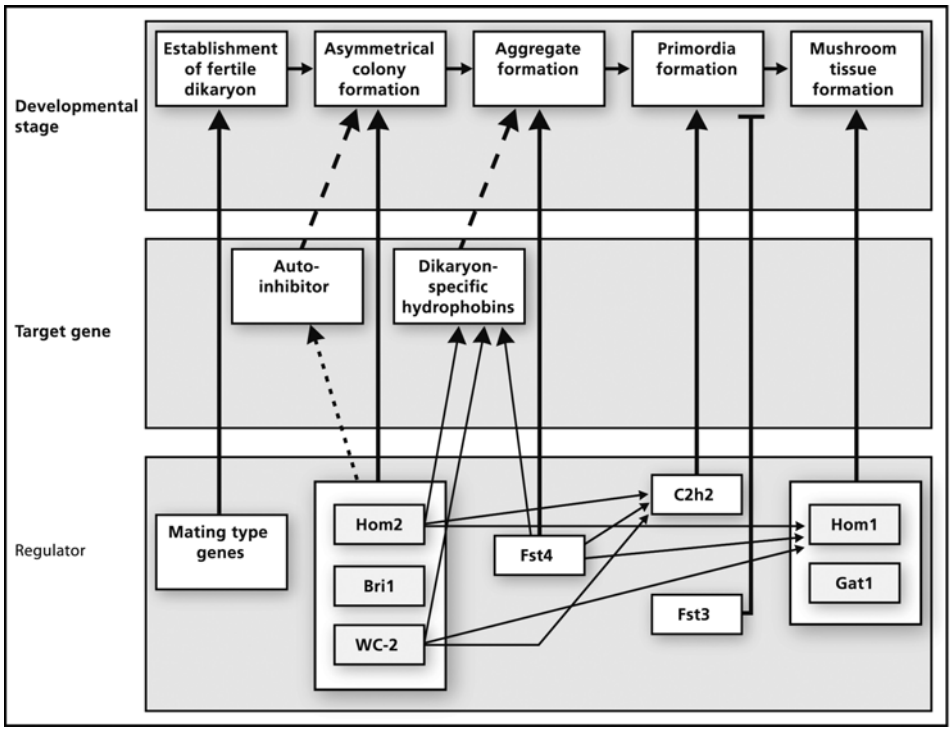

Figure 6. Model for the regulation of mushroom development in continuous light at 25 °C. Thick lines represent a regulatory function in a developmental process. Thin arrows represent (in)direct transcriptional activation of target genes. The continuous lines are supported by experimental evidence, which is not the case for the dotted lines. The boxed transcription factors have a similar role in the process of mushroom formation.

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SUPPLEMENTARY DATA

All supplementary data can be found at http://tinyurl.com/robinohmthesis or obtained from the author.

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Chapter 8

Absence of *sc3* and *sc4* hydrophobin gene expression in a dikaryon reverts the genome-wide expression profile to that of a monokaryon

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ABSTRACT

Monokaryons of *Schizophyllum commune* are sterile. They form aerial hyphae after a period of vegetative growth. Monokaryons with different *matA* and *matB* mating type loci can form a fertile dikaryon by fusion. These fertile dikaryons form aerial hyphae and fruiting bodies that form sexual spores. A dikaryon in which the *sc3* and *sc4* hydrophobin genes have been inactivated (a Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon) only forms few aerial hyphae. This phenotype is due to the failure of the fungus to escape the aqueous medium by the inability to reduce the water surface tension. The structural role of the hydrophobins in mediating aerial growth provides a tool to study the impact of aerial growth on gene expression. Here, it is shown that the genome-wide expression profile of a four-day-old Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon is more similar to a wild type monokaryon than to full grown mushrooms or a wild type dikaryon forming aggregates or primordia. However, the expression profile of 8 transcription factor genes that are involved in mushroom formation was similar in the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon and a wild type dikaryon forming primordia. These results indicate that the program for fruiting body development is in a stand-by mode in the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon. It is proposed that aerial hyphae generate a signal that is needed for full activation of fruiting body development.

INTRODUCTION

S. commune is one of the most common mushroom-forming fungi in the world. It normally feeds on fallen branches and timber of deciduous trees but it can also be a pathogen of humans and trees. Germination of basidiospores of *S. commune* results in a sterile monokaryotic mycelium. Each hyphal compartment within this mycelium contains one nucleus. A monokaryotic mycelium initially grows submerged, but after a few days sterile aerial hyphae are formed (Figure 1A, E of Chapter 5). Monokaryons that are confronted with each other can fuse. However, a fertile dikaryon is only formed when the alleles of the mating-type loci *matA* and *matB* of both partners differ. Light is essential to induce fruiting body formation, while a high concentration of carbon dioxide and high temperatures (30-37°C) are inhibitory. Fruiting body formation starts with the aggregation of aerial hyphae of the dikaryon. These stage I aggregates differentiate into stage II fruiting body primordia (Figure 1B, C, F, G of Chapter 5), which further develop into mature fruiting bodies (Figure 1D, H of Chapter 5). Nuclei of the dikaryotic compartments fuse in the

basidia within the fruiting body, which is followed by meiosis. The resulting haploid basidiospores are dispersed into the environment and can give rise to new monokaryotic mycelia.

The SC3 and SC4 hydrophobins play an important role in the life cycle of *S. commune.* The *sc3* gene is expressed in both mono- and dikaryons after a few days of vegetative growth (Wessels *et al.*, 1991). The hydrophobin that is encoded by this gene is secreted into the cell wall and the substrate. SC3 affects the cell wall composition in the water soluble form (van Wetter *et al.*, 2000b). This form, which is also released into the medium, assembles into an amphipathic membrane at the interface between the moist (or aqueous) substrate and the air (Wösten *et al.*, 1993; 1994; 1999). Assembly of SC3 is accompanied by conformational changes. In the stable end form, SC3 adopts an amyloid-like structure (de Vocht *et al.*, 1998; 2000; 2002; Wösten & de Vocht, 2000; Butko *et al.*, 2001; Scholtmeijer *et al.*, 2009). The amphipathic amyloid film of SC3 is highly surface active (van der Vegt *et al.*, 1996; Wösten *et al.*, 1999). As a result of SC3 assembly at the interface between the aqueous substrate and the air, hyphae can breach the surface tension of the aqueous environment to grow into the air. Not only substrate hyphae, but also aerial hyphae secrete the SC3 hydrophobin. In this case, the hydrophobin can not diffuse into the medium but rather assembles at the interface between the cell wall and the air (Wösten *et al.*, 1994; 1999). The hydrophilic side of the amphipathic SC3 membrane orients itself to the cell wall, whereas the hydrophobic side is exposed to the air. As a result, aerial hyphae become hydrophobic. This prevents hyphae to grow back into the aqueous substrate or to fall back when it rains or in the presence of dew.

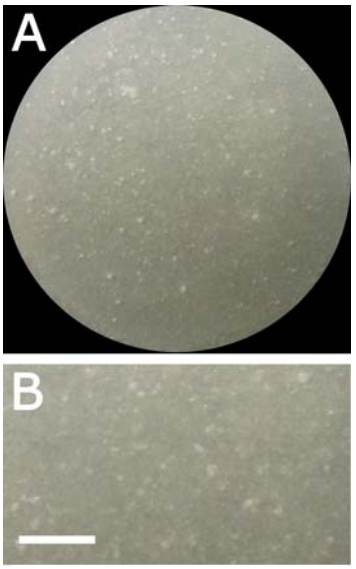

Figure 1. The dikaryotic strain Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* of *S. commune* forms few aerial hyphae and no fruiting bodies.
A. The culture was grown from a homogenate for 4 days in light at 25 °C. **B.** Magnification of (A). Bar represents 2.5 mm. The developmental stages during mushroom formation are depicted in Figure 2 of Chapter 5.

A Δ*sc3* monokaryon or a Δ*sc3*Δ*sc3* dikaryon forms few aerial hyphae (van Wetter *et al.*, 1996; 2000a). Formation of aerial hyphae can be further decreased by inactivation of the *sc4* hydrophobin gene (van Wetter *et al.*, 2000a) or the hydrophobin-like *sc15* gene (Lugones *et al.*, 2004). Apparently, the proteins encoded by these genes can partly take over the function of the

SC3 hydrophobin. The *sc4* gene also has a function in the fruiting bodies. The encoded hydrophobin self-assembles at the surface of air channels within the fruiting bodies (Lugones *et al.*, 1999). The resulting hydrophobicity prevents that these channels fill with water when it rains or when dew is present. As a consequence, gas exchange is not affected under these conditions (van Wetter *et al.*, 2000a).

The fact that the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon hardly forms aerial structures provides a possibility to assess the importance of physical growth into the air for the expression of genes involved in fruiting body formation. Here, it is shown that the expression profile of a 4-day-old culture of the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon is more similar to a 4-day-old culture of a wild type monokaryon that forms aerial hyphae than to full grown fruiting bodies or a 4-day-old culture of a wild type dikaryon that forms stage I or stage II primordia. Interestingly, expression of transcription factors involved in mushroom formation (Chapter 5; Chapter 6; Chapter 7; Ohm *et al.*, 2010) was not affected in the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon. This suggests that the regulatory machinery of mushroom formation is in a stand-by mode waiting for a signal of aerial growth to become activated.

MATERIAL AND METHODS

Strains and culture conditions

A dikaryotic *S. commune* strain was used that resulted from a cross of the 4-40 (CBS 340.81) and 4-39 (CBS 341.81) strains, in which the *sc3* and *sc4* genes have been deleted (van Wetter *et al.*, 2000a). For expression analysis, colonies were grown from plug inocula for 7 days at 30 ^{o}C in the dark on minimal medium (MM) with 1% glucose and 1.5% agar (Dons *et al.*, 1979). The resulting mycelium was homogenized in 200 ml MM in a Waring blender for 1 min at low speed. 2 ml of the homogenate was spread out over a polycarbonate membrane (Profiltra, diameter 76 mm, pore size 1 µm) that was placed on top of solidified MM. The resulting mycelium was isolated after 4 days of growth in the light at 25 °C.

Genes in the genome of S. commune

Genes (indicated by ProteinID) of *S. commune* are available through the interactive JGI Genome Portal at http://jgi.doe.gov/Scommune (see Chapter 5; Ohm *et al.*, 2010). The predicted gene models and annotations are also deposited at DDBJ/EMBL/GenBank under the project accession ADMJ00000000.

MPSS expression analysis

Total RNA was isolated as described previously (van Peer *et al.*, 2009). MPSS was performed essentially as described (Chapter 5; Brenner *et al.*, 2000; Ohm *et al.*, 2010) except that after DpnII digestion MmeI was used to generate 20 bp tags. Tags were sequenced using the Clonal Single Molecule Array technique (Illumina, Hayward, CA, US). A total of 5.2 million 20 bp-tags were obtained. These tags have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.*, 2002) and are accessible through GEO Series accession number GSE23531 (http://www.ncbi.nlm.nih.gov/geo/). Programs were developed in the programming language Python to analyze the data. Tag counts were normalized to tags per million (TPM). Those with a maximum of 4 TPM in all developmental stages were removed from the data set. TPM values of tags originating from the same transcript (Chapter 5; Ohm *et al.*, 2010) were summed to assess their expression levels. A transcript is defined as the predicted coding sequence (CDS) extended with 400 bp flanking regions at both sides.

Clustering analysis

Expression of genes in the 4-day-old culture of the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon was compared to the expression profiles of wild type cultures exhibiting 4 developmental stages of *S. commune* (monokaryon, stage I aggregates, stage II primordia and mushrooms; Chapter 5; Ohm *et al.*, 2010). For cluster analysis, only genes were used that had at least 4 transcripts per million in at least 1 of the 5 samples. Expression values were log2-transformed, after the values were increased with 1 in order to prevent negative log2 values. Hierarchical clustering of genes and samples and the subsequent analysis of the results were performed with the program MeV, which is part of the TM4 Software Suite (Saeed *et al.*, 2006). Euclidian distance was used as distance metric and average linkage clustering as linkage method.

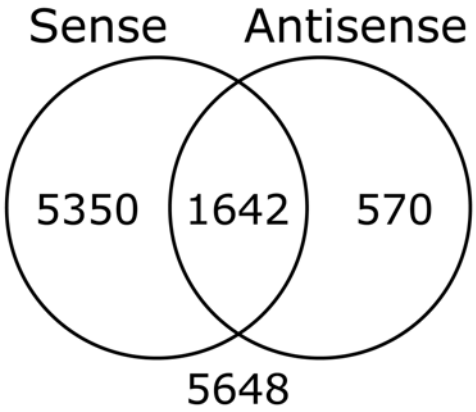

Figure 2. VENN diagram showing the number of genes expressed in sense and/or antisense direction in the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon. The majority of genes is either expressed only in sense direction (5350) or not expressed at all (5648).

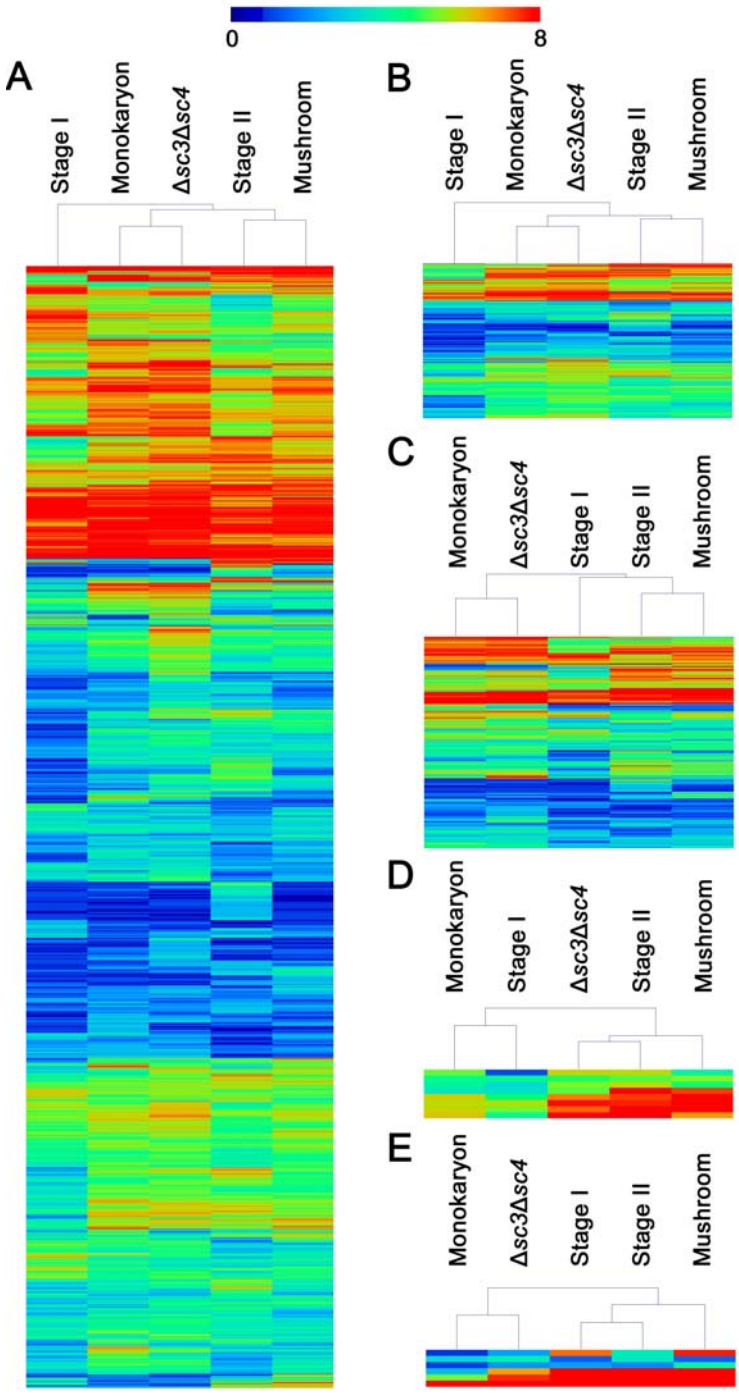

Figure 3. Heat maps depicting hierarchically clustered samples and expressed genes.
A. All genes. **B.** Genes encoding predicted transcription factors. **C.** Genes encoding secreted proteins. **D.** Genes encoding transcription factors known to be involved in the regulation of mushroom formation. **E.** Genes encoding known structural genes.

Representation analysis

Differentially regulated genes (at least two fold difference in expression) were identified using custom scripts. The genes *sc3* and *sc4* were removed from the dataset. FuncAssociate 2.0 (Berriz *et al.*, 2009) was used to study over- and under-representation of functional annotation terms in sets of differentially regulated genes. Default settings were used with a p-value of 0.05 as the cut off. Enrichment was studied of gene groups classified in Gene Ontology (GO; Ashburner *et al.*, 2000), eukaryotic orthologous groups (KOGs; Koonin *et al.*, 2004), KEGG metabolic pathways (Kanehisa *et al.*, 2004) and PFAM (Finn *et al.*, 2010) (See Chapter 5; Ohm *et al.*, 2010 for more details).

RESULTS

A wild type monokaryon that is grown from a mycelial homogenate for 4 days in the light or in the dark forms a vegetative substrate mycelium that is covered with a dense layer of aerial hyphae. A four day old culture of a fertile wild type dikaryon is covered with aggregates when it has been grown in the dark. These aggregates have developed into primordia in 4-day-old cultures that have been grown in the light. The primordia grow out into mature fruiting bodies from day 4 to day 8. In contrast to the wild type dikaryon, a four day old culture of the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon that has been grown in the light does not form fruiting body initials and only forms few aerial hyphae (Figure 1). Previously, the expression profiles have been described of the wild type developmental stages (Figure 1 of Chapter 5). Here, the expression profile was determined of the 4-day-old Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon. MPSS resulted in a total of 5.2 million tags. These tags represented 15824 unique sequences with an expression value of at least 4 TPM. Of these unique tags, 62.2% and 56.7% could be mapped to the genome sequence and the predicted transcripts, respectively, using a perfect match as the criterion. The mapped tags accounted for 71.7% and 71.2% of the total number of tags, for the genome sequence and predicted transcripts, respectively. These percentages are similar to numbers that were found for the different stages of wild type development (Chapter 5; Ohm *et al.*, 2010).

The genome-wide expression of the 4-day-old light-grown culture of the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon was compared to the expression profiles of the 4 developmental stages of the wild type (monokaryon, stage I aggregates, stage II primordia and mushrooms; Chapter 5; Ohm *et al.*, 2010). The ratio between the number of genes with sense and antisense gene expression in the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon (Figure 2) was similar to that in monokaryons, stage I aggregates and mushrooms (Figure 3C of Chapter 5).

However, many more genes were expressed in antisense direction in 4-day-old light-grown cultures of the wild type dikaryon that formed stage II primordia. 32.9% of the tags of this culture were in anti-sense direction, whereas this was only 14.2% in the case of the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon that had been grown for four days in the light.

Table 1. Differentially expressed genes and a summary of the functional annotation terms that are over-represented in these genes. For more information on over- and under-represented terms, see Supplementary Table 1. As an example, 1746 genes are at least 2 fold up-regulated in the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon compared to the wild type monokaryon, and the fungal specific transcription factor domain is over-represented in that gene set.

Comparison		Fold change ≥2	Differentially expressed genes (% of total)	Over-represented functional annotation terms (summary)
Wild type monokaryon (vegetative mycelium	Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon (vegetative mycelium)	Up	1746 (13.2%)	Fungal specific transcription factor domain
		Down	875 (6.6%)	Carbohydrate transport and metabolism, Phospholipase activity
Wild type dikaryon (stage II primordia)	Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon (vegetative mycelium)	Up	3063 (23.2%)	Protein metabolism
		Down	1457 (11.0%)	Hydrophobins, Fatty acid metabolism, Carbohydrate transport and metabolism, Secondary metabolites biosynthesis, transport and catabolism

Hierarchical clustering of the samples was performed to compare the expression profile of the 4-day-old culture of the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon with those of the wild type during the different stages of development. When all expressed genes were taken into account, Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* was most similar to the monokaryon (Figure 3A). A similar result was obtained when expressed genes encoding secreted proteins and putative transcription factors were analysed (Figure 3B and C). Interestingly, the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon and the dikaryon forming stage II primordia clustered when expression of a selected set of eight transcription factors was compared that are involved in mushroom formation (Hom1, Hom2, Wc2, Gat1, Fst3, Fst4, C2h2 and Bri1, see Chapter 5; Chapter 6; Chapter 7; Ohm *et al.*, 2010) (Figure 3D), In contrast, the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon clustered with the monokaryon when expression was compared of genes encoding structural proteins that are up-regulated during mushroom formation (*sc1*, *sc7*, *sc14*, *lccA* and *agg1* (Figure 3E)). Expression of the stress activated signalling pathways was not significantly affected in the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon

(data not shown), indicating that absence of hydrophobins does not pose stress to the fungus.

Differential gene expression was studied between the wild type monokaryon and the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon and between the wild type dikaryon and the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon. Fewer genes are differentially regulated in the first comparison than in the second comparison (Table 1). This difference is also reflected in the number of functional terms that were over-represented in the sets of differentially regulated genes. Interestingly, the fungal specific transcription factors and genes involved in carbohydrate metabolism are enriched in the upregulated genes of the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon when compared to the wild type monokaryon. Hydrophobin genes, and genes involved in carbohydrate and fatty acid metabolism are enriched in the genes that were down-regulated in the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon when compared to the wild type dikaryon.

DISCUSSION

Hydrophobins have been shown to fulfil a wide spectrum of functions in filamentous fungi in general and in *S. commune* in particular (Wösten & de Vocht, 2000). Here, it is described for the first time that the absence of hydrophobins severely affects global gene expression in the dikaryon.

4520 genes were differentially expressed (*i.e.* a change in expression of ≥2 fold) in a 4-day-old light grown culture of the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon when compared to the wild type dikaryon that had been grown under similar conditions. This difference in gene expression was accompanied with a severe phenotype. The wild type cultures had formed primordia, whereas the mutant strain only formed few aerial hyphae. This phenotype is more similar to that of the monokaryon. Intriguingly, only 2621 genes were differentially expressed (*i.e.* a change in expression of ≥2 fold) when expression of the 4-day-old light grown culture of the monokaryon and the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon was compared. The similarity in gene expression between the monokaryon and the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon is also reflected in the number of functional terms that were over-represented in the sets of differentially regulated genes (see Supplementary Table 1). The fungal specific transcription factors and genes involved in carbohydrate metabolism are enriched in the up-regulated genes of the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon when compared to the wild type monokaryon. These functional terms are also over-represented in the up-regulated genes during formation of aggregates and stage II primordia (Chapter 5; Ohm *et al.*, 2010). Hydrophobin genes and genes involved in carbohydrate and fatty acid metabolism were enriched in

the genes that are down-regulated in the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon when compared to the wild type dikaryon. These functional classes are up-regulated during formation of aggregates, primordia and mature fruiting bodies, respectively.

The similarity in gene expression between the monokaryon and the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon was also observed when the expression profile of the 4-day-old light-grown culture of the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon was clustered with that of 4-day-old mushrooms, with 4-day-old light and dark-grown cultures of the wild type dikaryon and 4-day-old light-grown cultures of the monokaryon. The monokaryon and the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon also clustered when expression of genes encoding predicted transcription factors, secreted proteins and putative structural mushroom proteins were compared. In contrast, the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon clustered with the light grown 4-day-old cultures of the dikaryon when expression was analysed of the 8 transcription factors that are involved in mushroom formation. These data indicate that the regulatory pathway that drives mushroom formation is in a stand-by mode in the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon. As a result, target genes of these transcription factors are not induced in the mutant strain. How can this be explained? It is proposed that an additional signal is required for further development of the mycelium into fruiting bodies. A signalling function for the SC3 and SC4 hydrophobins is unlikely. This is based on the observation that the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon does form fruiting bodies when the mycelium is grown under dryer conditions. This observation indicates that the inability to grow into the air prevents the generation of a signal that activates the regulatory machinery to induce the target genes of the 8 transcription factors. Possibly, the signal is a secreted molecule. In aerial hyphae this molecule would no longer be able to diffuse into the medium and would accumulate in the cell wall where it could bind to a receptor. Alternatively, the signal could be a relatively high oxygen concentration or a relatively low carbon dioxide concentration that may exist in the aerial hyphae, compared to the substrate hyphae.

Similar studies as reported here have recently been performed in the filamentous bacterium *Streptomyces coelicolor* (Claessen *et al.*, 2006; de Jong *et al.*, 2009) and in the smut fungus *Ustilago maydis* (Teertstra *et al.*, 2009). Whole genome expression analysis was performed in strains in which genes encoding hydrophobin-like molecules had been inactivated. Chaplins in *S. coelicolor* and repellents in *U. maydis* also self-assemble into amphipathic amyloid membranes and as such function in the formation of aerial hyphae. Expression of 244 genes was affected in a *S. coelicolor* strain that could no longer form aerial hyphae due to the absence of chaplins. Among these were

genes encoding structural and metabolic proteins, as well as transcription factors. In contrast, expression of only 31 genes was affected in a *U. maydis* strain in which the *rep1* gene was inactivated. In this case, expression of genes was affected that encode structural proteins. In fact, expression of transcription factor genes was not affected. The huge difference in differential expression between *U. maydis* on the one hand and *S. commune* and *S. coelicolor* on the other hand can be explained by the fact that the aerial hyphae of *U. maydis* do not develop in sporulating structures. In contrast, aerial hyphae of *S. commune* and *S. coelicolor* develop into fruiting bodies and chains of spores, respectively.

The data described in this Chapter in combination with what is found in *S. coelicolor* suggests that regulatory mechanisms involved in development of aerial structures (such as mushrooms) require signals that are present in the air or that are generated in aerial hyphae. In *S. coelicolo*r, this regulatory mechanism was dubbed the sky pathway (Claessen *et al.*, 2006).

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SUPPLEMENTARY DATA

All supplementary data can be found at http://tinyurl.com/robinohmthesis or obtained from the author.

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Chapter 9

Summary and general discussion

INTRODUCTION

Mushrooms are fungal adaptations for the aerial dispersal of sexual spores and they are predominantly produced in the phylum of the Basidiomycota. Approximately 14,000 mushroom-forming fungi have been identified in nature. Many of these fungi form a mutually beneficial symbiosis with roots of plants. Mushroom-forming fungi can also be pathogens of plants or animals and/or live as saprotrophs by degrading dead organic material.

The main economic value of mushrooms is their use as food (Kües, 2000; Kothe, 2001). The world-wide production of edible mushrooms amounts approximately 2.5 million tons annually. Apart from being a food source, fruiting bodies produce bioactive molecules (Lomascolo *et al.*, 1999; Kües, 2000; Kothe, 2001). Recently, mushrooms have been identified as promising cell factories for the production of pharmaceutical proteins (Berends *et al.*, 2009).

Despite their economical interest, relatively little is known about how mushrooms are formed. Many mushroom-forming fungi cannot be cultured in the lab nor genetically modified. This makes studying these organisms on the molecular level challenging. A notable exception is *Schizophyllum commune,* which is used as a model system to study the mechanisms underlying mushroom development (Kües & Liu, 2000; Wösten & Wessels, 2006). The life cycle of *S. commune* is completed in 10 days on a defined medium. Germination of basidiospores results in a sterile monokaryotic mycelium. A fertile dikaryon is established when two monokaryons fuse that have different alleles of the *matA* and *matB* mating type loci (for a review see Raudaskoski & Kothe, 2010). Proteins encoded in these loci activate signaling cascades and regulate genes encoding transcription factors. As a result, target genes are activated, which encode proteins that fulfill enzymatic or structural functions (*e.g.* hydrophobins) during mushroom development. Fruiting body formation also depends on environmental signals, such as light and temperature (Kües & Liu, 2000; Wösten & Wessels, 2006).

The first stage of mushroom development in *S. commune* is the aggregation of aerial hyphae into stage I aggregates. These aggregates develop into stage II primordia and further differentiate into mature fruiting bodies. Nuclear fusion (karyogamy) and meiosis take place in the basidia within the mature fruiting body. This results in basidiospores, which can give rise to new monokaryotic mycelia. In this Thesis, the regulation of mushroom formation was studied. For this, molecular tools had to be developed.

TOOLS FOR GENETIC ANALYSIS OF *S. COMMUNE*

S. commune is the only mushroom-forming fungus in which targeted gene deletions have been reported. However, targeted gene disruption in this basidiomycete is hampered by a low incidence of homologous recombination. Most genes were deleted with an average frequency of only 3% (Robertson *et al.*, 1996; van Wetter *et al.*, 1996; Horton *et al.*, 1999; Lengeler & Kothe, 1999; van Wetter *et al.*, 2000b; Lugones *et al.*, 2004; Schubert *et al.*, 2006; van Peer *et al.*, 2010). RNAi has been shown to be an alternative for gene inactivation by homologous recombination (de Jong *et al.*, 2006), but this does not seem to be the case for every gene (Lugones, unpublished results). Therefore, gene inactivation by homologous recombination remains the method of choice.

In order to facilitate the targeted deletion of genes by homologous recombination, a dedicated deletion vector was developed (**Chapter 2**; Ohm *et al.*, 2010a). This vector, called pDelcas, allows easy cloning of flanking regions of the gene that is to be deleted. Moreover, it enables efficient screening for transformants with a gene inactivation. Vector pDelcas consists of two antibiotic resistance cassettes. The flanks of the gene that is to be deleted are cloned at either side of the nourseothricin resistance cassette. The phleomycin resistance cassette is positioned elsewhere in the vector. As a consequence, phleomycin resistance is indicative of an integration of the construct by a single cross-over; *i.e.* an event not leading to a gene inactivation. By replica plating on a medium containing phleomycin, about 70% of the transformants could be eliminated in the screening process for a strain with a gene deletion. The gene deletion was confirmed in nourseothricin resistant and phleomycin sensitive transformants using a fast colony PCR protocol.

The *ku80* gene was inactivated in order to further reduce the number of transformants that have to be screened to obtain a strain with a gene deletion (**Chapter 3**; de Jong *et al.*, 2010). This gene is part of the non-homologous end joining (NHEJ) pathway, which is mainly responsible for ectopic integrations of transforming DNA by a single-crossover. Inactivation of components of NHEJ (*i.e. ku70* and *ku80* or both) increased the efficiency of targeted gene inactivation in a number of filamentous fungi (Ninomiya *et al.*, 2004; Krappmann *et al.*, 2006; Nayak *et al.*, 2006; Poggeler & Kuck, 2006; Takahashi *et al.*, 2006; Choquer *et al.*, 2008; Haarmann *et al.*, 2008). Up to a 100 fold decrease of transformation frequency was observed in the Δ*ku80* strain of *S. commune.* Notably, this was accompanied with an increase in the relative incidence of homologous recombination. Genes *sc15, jmj3* and *pri2*

were deleted in the Δ*ku80* strain. In total, 7 out of 10 transformants showed a gene deletion. The Δ*ku80* strain will be highly instrumental in the functional analysis of genes in *S. commune* and, in this way, contribute to our understanding of the biology of mushroom-forming basidiomycetes.

In **Chapter 4** the first inducible promoter from a mushroom-forming fungus is reported. The genome of *S. commune* contains 7 genes encoding putative heat shock proteins of the hsp26/42 family. Of these genes, 3 were not expressed at 25 °C (*hsp1-3*). Their promoters were used to induce expression of the gene encoding the red fluorescent protein dTomato. There was no fluorescence or mRNA accumulation at 25 °C, but after a heat shock of 1 hour at 42 °C strong fluorescence and mRNA accumulation were observed. This indicates that the promoter is not leaky under un-induced conditions, a phenomenon which is often observed with promoters from genes that are involved in carbohydrate degradation. Moreover, it was shown that the *hsp3* promoter showed a dose dependent induction. Weak and strong fluorescence of dTomato was observed after a heat shock at 37 and 42 °C, respectively. Notably, the *hsp3* promoter could be induced very locally in the mycelium simply by contact with a hot needle. Taken together, it is concluded that the *hsp3* promoter is an excellent inducible system for *S. commune.* It may also function in other basidiomycetes, given the fact that *S. commune* promoters are expressed in other members of this phylum (Alves *et al.*, 2004).

GENOME SEQUENCE OF *S. COMMUNE*

The genome sequence is an invaluable tool to study developmental processes in an organism. In **Chapter 5** the genome sequence of *S. commune* is reported (Ohm *et al.*, 2010b). The 38.5 MB genome contains 13,210 predicted genes. Comparison with other fungal genomes revealed that 53 protein families are over-represented in *S. commune* and the other two sequenced Agaricales (*i.e. Laccaria bicolor* (Martin *et al.*, 2008) and *Coprinopsis cinerea* (Stajich *et al.*, 2010)), whereas 4 protein families are under-represented. Over-represented PFAMs include the fungal hydrophobin domain, the transcription factor homeobox domain, the zf-MYND domain, and the fungal mating type pheromone.

The genome of *S. commune* contains 471 genes that are predicted to encode a putative transcription factor (**Chapter 5**; Ohm *et al.*, 2010b). Eight of these genes are contained in the *matA* mating type locus, which consists of the subloci *matAα* and *matAβ*. The organization of the *matAα* locus agreed with the published descriptions (Kothe, 2001). It contains two homeodomain

transcription factor genes. Interestingly, the *matAß* locus was shown to contain the highest homeodomain gene complexity of a fungal mating type locus described to date, with a total of 6 predicted homeodomain transcription factor genes. The genome sequence also revealed new insight into the *matB* locus. Five additional pheromone genes and 4 pheromone receptor-like genes were identified. Thus, the *matB* contains in total 16 pheromone genes and 5 pheromone receptor genes.

Whole genome gene expression was studied in 4 developmental stages (monokaryon, stage I aggregates, stage II primordia and mature mushrooms) using massively parallel signature sequencing (MPSS) (**Chapter 5**; Ohm *et al.*, 2010b). Approximately 60% of the 13,210 predicted genes were expressed in at least 1 developmental stage. Gene families that were differentially expressed during mushroom formation included hydrophobins, signal transduction and transciptional regulation. MPSS analysis also showed that anti-sense expression was found to occur widely in *S. commune* (**Chapter 5**; Ohm *et al.*, 2010b). About 20% of all sequenced mRNA tags originated from an anti-sense transcript and more than 5600 of the predicted genes showed anti-sense expression in one or more developmental stages. Anti-sense transcription was most pronounced in stage II primordia. In this stage, more than 4300 genes were expressed in both sense and anti-sense direction and more than 800 genes were expressed in the anti-sense direction only. Previously, MPSS showed anti-sense transcripts in *Magnaporthe grisea* (Gowda *et al.*, 2006). Little is known about the function of these transcripts in fungi. The circadian clock of *Neurospora crassa* is entrained in part by the action of an anti-sense transcript of a clock component locus (Kramer *et al.*, 2003), possible by RNA interference. It is tempting to speculate that anti-sense transcripts also regulate mRNA levels in *S. commune*. Natural anti-sense transcripts in eukaryotes have also been implicated in other processes such as translational regulation, alternative splicing and RNA editing (Lavorgna *et al.*, 2004). The anti-sense transcripts of *S. commune* may also have such functions. In all these cases, the anti-sense transcripts could especially be functional in the developmental switch that would occur when stage II primordia are formed.

REGULATION OF MUSHROOM FORMATION

Of the 471 predicted transcription factor genes of *S. commune*, 311 were expressed during mushroom development (**Chapter 5**; Ohm *et al.*, 2010b). Deletion strains were obtained for 10 of these genes. Mushroom formation in homozygous dikaryotic deletion strains of 2 of these genes (*pri2* and *jmj3*)

was not affected when compared to the wild type (**Chapter 3**; de Jong *et al.*, 2010). The other 8 transcription factors (*bri1*, *hom2*, *wc*-2, *fst4*, *c2h2*, *fst3*, *hom1* and *gat1*), on the other hand, do play a role in mushroom development (Figure 6 of **Chapter 7**; **Chapters 5, 6** and **7**; Ohm *et al.*, 2010b).

In the light, wild type dikaryons form asymmetrical colonies that have produced mushrooms after 10 days of growth. Homozygous dikaryons in which *bri1*, *hom2* or *wc-2* were deleted, formed symmetrical colonies and no mushrooms developed. Bri1 and Hom2 contain a DNA-binding BRIGHT domain and a homeodomain, respectively. WC-2 is homologous to the blue light receptor WC-2 of *N. crassa*. A similar role for WC-2 in *S. commune* and *N. crassa* is indicated by the fact that mushroom development in *S. commune* is also induced by blue light (Perkins & Gordon, 1969). Moreover, the phenotype of the Δ*wc-2*Δ*wc-2* strain of *S. commune* strongly resembles that of the wild type dikaryon grown in darkness. The symmetrical shape of colonies of the Δ*hom2*Δ*hom2*, Δ*bri1*Δ*bri1* and Δ*wc-2*Δ*wc-2* strains may be explained by the absence of an auto-inhibitory component, which is secreted in the wild type and inhibits hyphal growth (Klein *et al.*, 1997).

Genome-wide expression analysis of Δ*wc-2*Δ*wc-2* dikaryons showed that expression of dikaryon-specific hydrophobin genes is strongly reduced when compared to the wild type (**Chapter 7**). This may explain, at least in part, the absence of mushrooms in this mutant strain (see below). Mushroom formation in the Δ*wc-2*Δ*wc-2* strain could be induced by a heat shock by introducing a construct encompassing the *wc-2* gene under control of the inducible *hsp3* promoter. Genome-wide gene expression analysis of this strain before and after heat shock led to the identification of several downstream targets of WC-2, among which the previously described mushroom-specific gene *sc7* (Schuren *et al.*, 1993) and a gene encoding a putative signaling protein (**Chapter 7**).

Gene *fst4* encodes a transcription factor with a fungal specific Zn(II)2Cys6 zinc finger DNA binding domain. Homozygous dikaryons in which *fst4* was deleted had an irregular shape similar to the wild type (**Chapter 6**). This implies that in contrast to the Δ*hom2*Δ*hom2*, Δ*bri1*Δ*bri1* and Δ*wc-2*Δ*wc-2* strains, formation of the auto inhibitor was not affected. However, Δ*fst4*Δ*fst4* dikaryons did not develop stage I aggregates, and, as a result, mushrooms were not formed. Genome-wide expression analysis of the Δ*fst4*Δ*fst4* dikaryon showed that dikaryon-specific hydrophobin genes were lower expressed than in the wild type (**Chapter 6**). In this respect, deletion of *fst4* thus had a similar effect as deletion of *hom2* and *wc-2* (**Chapters 6** and **7**). Hydrophobins have been shown to play a role in mushroom development. For instance, Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryons grown from a homogenate do not form

mushrooms (**Chapter 8**; van Wetter *et al.*, 2000a). The role of the newly identified dikaryon-specific hydrophobins remains to be elucidated.

Gene *c2h2* encodes a transcription factor with a DNA binding zinc finger domain of the C2H2 type. Expression analysis showed that it is directly or indirectly regulated by the transcription factors Hom2, WC-2 and Fst4 (**Chapters 6** and **7**). Colonies of Δ*c2h2*Δ*c2h2* dikaryons were asymmetrical when grown in the light and they did form aggregates. These aggregates, however, did not develop into primordia. Different tissues are formed during primordia formation in the wild type and this is accompanied by an increase in antisense expression of genes (**Chapter 5**; Ohm *et al.*, 2010b). Whether C2h2 exerts its role by regulating antisense expression is not known, but it is an interesting topic for future research. It is clear that C2h2 functions downstream of Fst4.

Strains in which *fst3* (**Chapter 5**; Ohm *et al.*, 2010b), *hom1* or *gat1* (**Chapter 6**) were inactivated formed more but smaller mushrooms than the wild type. These genes encode transcription factors with a fungal specific Zn(II)2Cys6 zinc finger, homeodomain and GATA zinc finger DNA binding domains, respectively. Like *c2h2, hom1* is regulated directly or indirectly by Hom2, WC-2 and Fst4 (**Chapters 6** and **7**). The mushrooms of the Δ*fst3*Δ*fst3* dikaryon were smaller but otherwise similar to the wild type (**Chapter 5**; Ohm *et al.*, 2010b). From this it was proposed that Fst3 is involved in the local inhibition of primordia formation. This repressor may be important in the natural environment to ensure that mushrooms can fully develop with the limited resources that are available. Gene *fst3* may become activated by a signal from a developing mushroom. This would prevent outgrowth of neighbouring aggregates, thereby preventing competition between developing mushrooms for the limiting resources. The mushrooms produced by the Δ*hom1*Δ*hom1* and the Δ*gat1*Δ*gat1* dikaryon were not only smaller than those of the wild type but their morphology was also affected. The hymenium (i.e. the spore producing tissue) was enlarged in these mutant stains when compared to the wild type. From this we propose that *hom1* and *gat1* have a function in tissue development in *S. commune.* The smaller size of the mushrooms in the absence of *hom1* or *gat1* would result in a reduced flow of nutrients to the developing mushroom. This would enable more primordia to grow out, which would explain the higher number of mushrooms that develop in the Δ*hom1*Δ*hom1* and the Δ*gat1*Δ*gat1* dikaryon.

As mentioned above, the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon hardly forms aerial hyphae and mushrooms are not produced. This hydrophobin deletion strain provided the possibility to assess the importance of physical growth into the air for the expression of genes involved in fruiting body formation

(**Chapter 8**). It was shown that the expression profile of a 4-day-old culture of the Δsc3Δsc3Δsc4Δsc4 dikaryon was more similar to a 4-day-old culture of a wild type monokaryon that formed aerial hyphae than to full grown fruiting bodies or a 4-day-old culture of a wild type dikaryon that formed stage I or stage II primordia. More than 4500 genes were differentially expressed (*i.e.* a change in expression of ≥2 fold) in a 4-day-old light grown culture of the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon when compared to the wild type dikaryon that had been grown under similar conditions. Interestingly, the expression profile of the 8 transcription factor genes that are involved in mushroom formation (see above) was similar in the Δ*sc3*Δ*sc3*Δ*sc4*Δ*sc4* dikaryon and a wild type dikaryon forming primordia. This suggests that the regulatory machinery of mushroom formation is in a stand-by mode waiting for a signal of aerial growth to become activated.

Regulation of mushroom formation in *S. commune* may well apply to other mushroom-forming fungi. This is supported by the observation that the homologs of *fst3* and *fst4* are also up-regulated in young fruiting bodies of *Laccaria bicolor* compared to free-living mycelium. Furthermore, it was shown that the regulation of transcription factor genes positively correlates between *S. commune* and *L. bicolor* during mushroom formation (**Chapter 5**; Martin *et al.*, 2008; Ohm *et al.*, 2010b). The regulatory model of mushroom formation may be instrumental to explain why certain mushrooms can not be cultivated commercially and also represents an excellent lead to improve production of commercial mushrooms.

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Nederlandse samenvatting

INTRODUCTIE

Paddenstoelen worden gevormd door schimmels en zijn betrokken bij de vorming en de verspreiding van sporen van de geslachtelijke voortplanting. Paddenstoelen worden voornamelijk aangetroffen in het phylum van de Basidiomyceten. Er zijn ongeveer 14.000 paddenstoelvormende schimmels geïdentificeerd, waarvan vele een symbiose aangaan met de wortels van planten. Een bekend voorbeeld hiervan is de vliegenzwam. Paddenstoelvormende schimmels kunnen ook ziekteverwekkend zijn voor planten of dieren en/of dood organisch materiaal afbreken. Bekende voorbeelden van dergelijke paddenstoelvormende schimmels zijn de honingzwam (een ziekteverwekker van bomen) en het elfenbankje en de champignon (beide afbrekers van dood organisch materiaal).

De belangrijkste waarde van paddenstoelen vanuit economisch oogpunt is het feit dat ze als voedsel gebruikt kunnen worden. Wereldwijd wordt per jaar 2,5 miljoen ton eetbare paddenstoelen geproduceerd. Bovendien produceren paddenstoelen moleculen die interessant zijn voor de (medische) industrie en kunnen ze gebruikt worden voor de productie van dergelijke moleculen die van nature niet in de paddenstoel voorkomen.

Ondanks hun economisch belang is er relatief weinig bekend over hoe paddenstoelen worden gevormd. Veel paddenstoelvormende soorten kunnen niet in het lab gekweekt worden. Daarnaast is genetische modificatie vaak niet mogelijk, wat het doen van moleculair onderzoek belemmert. Een uitzondering hierop is *Schizophyllum commune*, in het Nederlands Waaiertje genoemd. Deze soort wordt daarom als modelsysteem gebruikt om paddenstoelvorming te onderzoeken. De levenscyclus van *S. commune* duurt ongeveer 10 dagen en kan volledig op gedefinieerd medium plaatsvinden. De ontkieming van de geslachtelijke basidiosporen leidt tot een steriel monokaryotisch mycelium. Een vruchtbaar dikaryon ontstaat indien twee monokaryons met verschillende mating type loci fuseren. De eiwitten die gecodeerd worden door deze loci activeren signaalroutes en reguleren genen die coderen voor transcriptiefactoren. Het resultaat hiervan is dat genen geactiveerd worden die coderen voor eiwitten die een structurele of enzymatische rol spelen bij de vorming van de paddenstoel. Daarnaast zijn externe factoren zoals licht en temperatuur belangrijk bij de beslissing om paddenstoelen te vormen. Het eerste stadium van de ontwikkeling van paddenstoelen in *S. commune* is de aggregatie van luchthyfen. Deze aggregaten ontwikkelen zich verder tot primordia en uiteindelijk tot volgroeide paddenstoelen. In gespecialiseerde structuren in de paddenstoel (de basidia)

vindt meiose plaats en worden de geslachtelijke sporen gevormd. Deze sporen worden verspreid, waarna de cyclus opnieuw kan beginnen.

In dit proefschrift wordt onderzoek naar de regulatie van paddenstoelvorming beschreven. Om dit onderzoek te kunnen doen werden ook een aantal moleculair genetische technieken voor *S. commune* ontwikkeld.

MOLECULAIR GENETISCHE TECHNIEKEN VOOR *S. COMMUNE*

S. commune is de enige paddenstoelvormende schimmel waarvoor technieken beschreven zijn om genen gericht uit te schakelen. Echter, de gerichte gendisruptie wordt belemmerd door een relatief lage frequentie van homologe recombinatie. Bij de meeste beschreven gendisrupties lag deze frequentie gemiddeld op slechts 3%. Om het proces van gerichte gendisruptie efficiënter te laten verlopen, werd een deletie-vector ontwikkeld (**Hoofdstuk 2**). De flanken van het gen dat gedisrupteerd wordt kunnen eenvoudig in deze vector, genaamd pDelcas, geplaatst worden en wel elk aan een kant van een nourseothricine resistentie cassette. De aanwezigheid van een phleomycine resistentie cassette elders in pDelcas maakt dat transformanten eenvoudig gescreend kunnen worden op de aanwezigheid van een gendisruptie. Bij een gendisruptie zal de transformant namelijk wel groeien op een medium met nourseothricine maar niet op een medium met phleomycine. Door deze screening kon een gendisruptie in ongeveer 70% van de transformanten eenvoudig worden uitgesloten. De overblijvende transformanten werden vervolgens met een snelle PCR screening geanalyseerd.

Om het aantal te screenen transformanten nog verder omlaag te brengen werd het gen *ku80* geïnactiveerd (**Hoofdstuk 3**). Dit gen speelt een essentiële rol bij de integratie van DNA op een willekeurige (ongewenste) plek in het genoom. Het disrupteren van dit gen leidde tot een sterke afname van de transformatie efficiëntie, maar dit ging gepaard met een sterke toename van de relatieve frequentie van homologe recombinatie. Deze Δ*ku80* stam vergemakkelijkt daardoor het uitschakelen van genen enorm en zal daardoor een belangrijke bijdrage leveren bij het ophelderen van mechanismen die ten grondslag liggen aan paddenstoelvorming in *S. commune*.

In **Hoofdstuk 4** wordt de eerste induceerbare promoter voor een paddenstoelvormende schimmel beschreven. Het genoom van *S. commune* bevat zeven genen die coderen voor een heat shock eiwit. Van drie van deze genen werd geen expressie gemeten bij 25 °C, terwijl bij 37 en 42 °C een sterke activatie plaatsvond. Voor een van deze promotoren (*hsp3*) werd aangetoond dat deze locaal geïnduceerd kon worden met een hete naald.

Deze promotor kan dus worden gebruikt om een gen op een bepaald moment op een bepaalde plaats tot expressie te laten komen.

GENOOM VAN *S. COMMUNE*

De genoomsequentie van een organisme bevat informatie over (onder andere) de ontwikkelingsprocessen in dat organisme. In **Hoofdstuk 5** wordt de genoomsequentie van *S. commune* beschreven. Het genoom is 38.5 miljoen basenparen groot en bevat 13.210 voorspelde genen. Een vergelijking met de genomen van andere organismen leidde tot de identificatie van eiwitfamilies die oververtegenwoordigd zijn in de paddenstoelvormende soorten, zoals hydrofobines en verscheidene klassen van transcriptiefactoren.

Het genoom van *S. commune* bevat 471 genen waarvan voorspeld wordt dat ze een transcriptiefactor coderen. Acht van deze genen bevinden zich op het *matA* mating type locus. Het *matAβ* sublocus bevat daarmee het voor schimmels tot nu toe hoogste aantal voorspelde (homeodomein) transcriptiefactoren. De genoomsequentie leverde ook nieuwe inzichten op voor het *matB* locus. Er zijn nu 16 feromoongenen en 5 feromoonreceptorgenen geïdentificeerd. Deze informatie is essentieel voor het begrip van (de evolutie van) de processen die plaatsvinden in de schimmel om een fertiel dikaryon te vormen.

Het genexpressieprofiel van *S. commune* werd onderzocht voor 4 ontwikkelingsstadia: monokaryon, aggregaten, primordia en volgroeide paddenstoelen (**Hoofdstuk 5**). Ongeveer 60% van de genen kwam tot expressie in minimaal 1 ontwikkelingsstadium. Genfamilies die differentieel tot expressie kwamen tijdens de groei van paddenstoelen waren onder andere hydrofobines, signaaltransductie-eiwitten en transcriptiefactoren. Ook werd aangetoond dat anti-sense genexpressie een wijdverbreid fenomeen is in het genoom van *S. commune*, en wel voornamelijk in het ontwikkelingsstadium met primordia.

REGULATIE VAN PADDENSTOELVORMING

Van de 471 voorspelde transcriptiefactor genen kwamen er 311 tot expressie tijdens paddenstoelvorming. Van tien van deze genen werd een deletiestam gemaakt. In twee gevallen (*pri2* en *jmj3*) leidde dit niet tot een afwijkend fenotype ten opzichte van het wildtype (**Hoofdstuk 3**). De andere acht transcriptiefactoren (*bri1*, *hom2*, *wc-2*, *fst4*, *c2h2*, *fst3*, *hom1* en *gat1*) blijken wel een rol te spelen bij paddenstoelvorming. Op grond van de fenotypes van de disruptiestammen en eerdere literatuur werd een model opgesteld voor paddenstoelvorming (**Hoofdstukken 5, 6** en **7**).

Wanneer wildtype dikaryons in het licht worden gegroeid, vormen ze een asymmetrische kolonie die na 10 dagen paddenstoelen heeft geproduceerd. Homozygote dikaryons waarin *bri1*, *hom2* of *wc-2* waren geïnactiveerd vormden symmetrische kolonies en geen paddenstoelen. Bri1 en Hom2 bevatten respectievelijk een DNA-bindend BRIGHT domein en een homeodomein. WC-2 is homoloog aan de blauwlichtreceptor WC-2 van *Neurospora crassa*. Een genexpressie analyse van Δ*wc-2*Δ*wc-2* en Δ*hom2*Δ*hom2* dikaryons toonde aan dat de expressie van dikaryon-specifieke hydrofobines verlaagd was ten opzichte van het wildtype. Dit verklaart, in ieder geval ten dele, de afwezigheid van paddenstoelen (zie **Hoofdstuk 8**). Bovendien kwamen in beide gevallen de genen *c2h2* en *hom1* (zie verderop in de tekst) lager tot expressie, wat erop wijst dat de regulatoren elkaar (in ieder geval indirect) reguleren. Paddenstoelvorming kon geïnduceerd worden in het Δ*wc-2*Δ*wc-2* dikaryon door het te transformeren met een construct waarin het *wc-2* gen onder controle is gebracht van de *hsp3* promoter. Indien een dagelijkse hitte schok gegeven werd, ontwikkelen zich paddenstoelen (**Hoofdstukken 6** en **7**).

Het gen *fst4* codeert voor een transcriptiefactor met een Zn(II)2Cys6 zink vinger DNA bindend domein dat specifiek is voor schimmels. Dikaryons waarin *fst4* is geïnactiveerd hadden net als het wildtype een onregelmatige koloniemorfologie, echter het Δ*fst4*Δ*fst4* dikaryon vormde geen paddenstoelen. Op grond hiervan werd geconcludeerd dat Fst4 op een later moment functioneert in de paddenstoelvorming dan Bri1, Hom2 en WC-2. Een genexpressieanalyse toonde aan dat ook in het Δ*fst4*Δ*fst4* dikaryon de expressie van hydrofobines en de regulatoren *c2h2* en *hom1* verlaagd waren (**Hoofdstukken 5** en **6**).

Het gen *c2h2* codeert voor een transcriptiefactor met een C2H2 zink vinger DNA bindend domein. De ontwikkeling van paddenstoelen in een dikaryon waarin dit gen is geïnactiveerd stopte in het stadium van aggregaten, waardoor volgroeide paddenstoelen niet werden gevormd. Hieruit kan geconcludeerd worden dat C2h2 een rol speelt op een later moment dan Fst4 (**Hoofdstuk 6**).

Dikaryons waarin de genen *fst3* (**Hoofdstuk 5**), *hom1* of *gat1* (**Hoofdstuk 6**) waren geïnactiveerd vormden meer, maar kleinere paddenstoelen dan het wildtype. Deze genen coderen voor transcriptiefactoren met respectievelijk een Zn(II)2Cys6 zink vinger DNA bindend domein dat specifiek is voor schimmels, een homeodomein en een GATA zink vinger DNA bindend domein. Mogelijk speelt Fst3 een rol bij de inhibitie van paddenstoelvorming door ervoor te zorgen dat naburige paddenstoelen niet door kunnen groeien. Een dergelijke repressor kan

belangrijk zijn in de natuurlijke situatie om ervoor te zorgen dat paddenstoelen kunnen groeien met de beperkte energiebronnen die beschikbaar zijn. In het geval van de dikaryons waarin *hom1* en *gat1* waren geïnactiveerd was ook de morfologie van de paddenstoelen aangetast, waardoor de paddenstoelen klein blijven. Doordat de paddenstoelen klein blijven kunnen er relatief meer paddenstoelen uitgroeien met het voedsel dat voorhanden is. Deze resultaten suggereren dat Fst3 eerder actief is in paddenstoelvorming dan Hom1 en Gat1.

Een genexpressieanalyse op dikaryons waarin de hydrofobinegenen *sc3* en *sc4* beiden zijn geïnactiveerd (en dus geen paddenstoelen vormden), toonde aan dat de genen die coderen voor structurele paddenstoeleiwitten over het algemeen lager tot expressie kwamen dan in het wildtype (**Hoofdstuk 8**). De transcriptiefactoren waarvan is aangetoond dat ze betrokken zijn bij paddenstoelvorming, daarentegen, komen juist wel tot expressie. Dit leidt tot de hypothese dat het regulatiemechanisme van paddenstoelvorming in een stand-by modus staat zolang er geen hyfen de lucht in groeien.

IMPACT VAN HET ONDERZOEK

Het is goed mogelijk dat de mechanismen van regulatie van paddenstoelvorming in *S. commune* ook van toepassing zijn op andere soorten. Dit wordt ondersteund door het feit dat de regulatoren homologen hebben in andere paddenstoelvormende schimmels en door het feit dat de homologen van *fst3* en *fst4* op een vergelijkbare manier tot expressie komen in *Laccaria bicolor*. Bovendien is aangetoond dat de regulatie van transcriptiefactorgenen positief correleert tussen *S. commune* en *L. bicolor* tijdens paddenstoelvorming (**Hoofdstuk 5**). Inzicht in de mechanismen van regulatie van paddenstoelvorming kan in de (nabije) toekomst worden gebruikt om de vraag te beantwoorden waarom sommige soorten (nog) niet commercieel gecultiveerd kunnen worden. Bovendien kan deze kennis worden toegepast om de commerciële productie van paddenstoelen te verbeteren.

Curriculum vitae

Robin Arthur Ohm was born on June 11th, 1981 in Hoorn, The Netherlands. He followed his secondary education at the Gemeentelijke Scholengemeenschap Doetinchem and the Comenius College in Hilversum, The Netherlands and graduated in 1999 with a Gymnasium-level diploma. In September of the same year he began his coursework in Biology at Utrecht University. As part of his studies he did an internship at the Hubrecht Laboratory of the Royal Netherlands Academy of Arts and Sciences in Utrecht under the supervision of Dr. F. van Eeden, followed by a second internship in the Molecular Microbiology group of the Department of Biology at Utrecht University under the supervision of Dr. A.M. Levin and Dr. R.P. de Vries. Robin obtained his MSc diploma in September 2005. In December of the same year he started his PhD with the Molecular Microbiology group of the Department of Biology at Utrecht University under the supervision of Dr. L.G. Lugones and Prof. Dr. H.A.B. Wösten. Research on the genetic regulation of mushroom formation in *Schizophyllum commune* was financially supported during this period by Technologiestichting STW and is described in this thesis. In August 2010 Robin started working at the Joint Genome Institute of the Lawrence Berkeley National Laboratory and the United States Department of Energy in Walnut Creek, California, United States, where he studies fungal genomics.

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