Environmental signaling and regulation of mushroom formation

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Environmental signaling and regulation of mushroom formation

Signalering van milieucondities en regulatie van paddenstoelvorming

(met een samenvatting in het Nederlands)

Proefschrift

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Voor Anastasia en Luna

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Fruiting body formation in basidiomycetes

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I. INTRODUCTION

Fruiting bodies have evolved in the fungal kingdom to produce and disperse sexual spores. These reproductive structures of the basidiomycetes generally contain specialised cells, basidia, in which the genetically different parental haploid nuclei fuse. The resulting diploid cells immediately undergo meiosis to form haploid basidiospores. In some cases diploid nuclei are already formed in the vegetative mycelium, as in *Armillaria* species (Ullrich and Anderson, 1978, Grillo *et al.,* 2000). After discharge, germination of the haploid basidiospores results in homokaryotic mycelia. These mycelia can fuse, depending on an often complex system of mating-type genes, to produce a heterokaryotic mycelium. This heterokaryotic mycelium is fertile and will form fruiting bodies under certain environmental conditions. Basidiomycetes behaving according to this scheme are in the majority and are called heterothallic (i.e. self-incompatible). From a teleological point of view this makes sense because it ensures that the diploid basidia produce recombinant meiotic progeny. A minority of basidiomycetes (about 10%; Whitehouse, 1949) is homothallic (i.e. self-compatible). Recently, it was shown that unisexual reproduction also creates diversity (Ni *et al.,* 2013), which would explain the existence of homothallic species from an evolutionary point of view.

This Chapter discusses the regulation of fruiting body formation in basidiomycetes and the role structural proteins and enzymes play in this process. The mushroom forming fungi *Schizophyllum commune* and *Agaricus bisporus* that were subject of this Thesis are introduced in Box 1 and Box 2.

II. DEVELOPMENT OF EMERGENT STRUCTURES

Formation of a feeding submerged mycelium

Fruiting bodies develop from a vegetative mycelium. Formation of this submerged mycelium starts with the germination of an asexual or sexual spore. Hyphae growing out of these spores grow at their tips, while branching subapically (Wessels, 1986; 1990). Hyphal fusion promotes the formation of an elaborate hyphal network.

The vegetative mycelium degrades and colonises organic substrates by enzymes that are secreted at tips of growing hyphae (Wösten *et al.,* 1991; Moukha *et al.,* 1993). The degradation products are taken up and serve as nutrients. They can also be transported to other parts of the mycelium, which would explain why some

Box 1 The life cycle of *Schizophyllum commune* and its role as a model organism

S. commune is a basidiomycete that produces fan-shaped fruiting bodies They are characterized by formation of split gills, hence the common name “split gill mushroom”. It grows on all continents and has been found to colonize over 150 different plant species (Cooke 1961). The life cycle of *S. commune* starts with the germination of a monokaryotic haploid spore that forms a sterile monokaryotic vegetative mycelium. Upon contact with a mycelium with a compatible mating type, dikaryotization takes place. Due to variations in allelic specificities for the mating type loci (Raper 1966, Koltin *et al.,* 1967), more than 23,000 compatible interactions can occur in nature. As a dikaryon, the colony will develop fruiting bodies under the right conditions. Blue light is required (Perkins 1969; Raudaskoski and Yli-Mattila 1985, Ohm *et al.,* 2012), while high CO_2 levels repress fruiting (Niederpruem, 1963; Raudakoski and Viitanen, 1982). Upon exposure to blue light and low CO_2 dikaryotic colonies start to grow irregular and to form aerial hyphae. These aerial hyphae aggregate and develop further into primordia. Primordia will eventually form fruiting bodies. These reproductive structures contain hymenium in which basidia are formed. In these basidia karyogamy and meiosis take place resulting in the formation of haploid basidiospores.

S. commune has a culinary value but is most known as a model organism for mushroom formation. It has a relatively short life cycle of 7 days and fruits on defined synthetic media. Its genome has been sequenced (Ohm *et al.,* 2011) and auxotrophic markers and antibiotic resistance cassettes are available for transformation (Schuren and Wessels, 1994; Mooibroek *et al.,* 1990; Scholtmeijer *et al.,* 2001; van Peer *et al.,* 2009a). Gene silencing has been described (de Jong *et al.,* 2006) as well as gene deletion by homologous recombination (Marion *et al.,* 1996; Robertson *et al.,* 1996; van Wetter *et al.,* 1996; Horton *et al.,* 1999; Lengerer and Kothe, 1999a; Lengerer and Kothe, 1999b; van Wetter *et al.,* 2000a; Lugones *et al.,* 2004; Schubert *et al.,* 2006; van Peer *et al.,* 2010b; Ohm *et al.,* 2010a; Ohm *et al.,* 2010b; Ohm *et al.,* 2011; Ohm *et al.,* 2012). Deletion of *ku80*, involved in DNA repair by non-homologous end joining, has strongly increased efficiency of gene deletion by homologous recombination (de Jong *et al.,* 2010).

fungi can grow for considerable distances over non-nutritive surfaces (Jennings, 1984). Mass transport of water and nutrients through the mycelium implies that the cytoplasm is continuous within the mycelium. This would be mediated by the large pores (diameters up to 500 nm) within the septa that compartmentalize hyphae. These pores, however, are not always open, at least in *Schizophyllum commune* (van Peer *et al.,* 2009b). As many as 90% of the subapical septa can be closed when *S. commune* is growing on glucose containing medium. Septal closure is strongly reduced when *S. commune* is grown in the absence of this carbon source, while high temperature, hypertonic conditions, or exposure to the antibiotic nourseothricin increase plugging incidence. So far, the plugging state of septa has not been assessed under natural conditions such as in wood and how this affects long and short distance transport of nutrients. Closure of the septal pore is mediated by the septal pore cap (SPC) (van Driel *et al.,* 2008; van Peer *et al.,* 2010). Inactivation of *spc33* that encodes a structural SPC protein results in reduced vegetative growth and retarded fruiting body formation (van Peer *et al.,* 2010b). It has been proposed that this is due to decreased turgor pressure as a result of the inability to close the septal pores. Septal closure may also allow for differentiation of hyphae within the vegetative mycelium, as was shown in *Aspergillus niger* (Wösten *et al.,* 2013).

Translocation of water and nutrients is essential for fruiting body development. Fruiting-body primordia of *S. commune* (Wessels, 1965) and *Coprinopsis cinerea* (*Coprinus cinereus*) (Moore, 1998) arise at the expense of constituents of the supporting mycelium, while expanding fruiting bodies feed on supporting mycelium and abortive fruiting-body primordia. At the moment, it is not clear how much of this is due to movement of cytoplasm or to degradation and resynthesis of cellular components. Woolston *et al.* (2011) showed that the heterologous protein β-glucuronidase is transported from the vegetative mycelium into the fruiting body but no evidence was obtained for translocation of its mRNA.

Formation of fruiting bodies from the submerged mycelium

Formation of fruiting bodies is a highly complex developmental process. A generalized scheme for formation of agaric fruiting bodies is as follows: (Moore, 1998; Kües, 2000) After a “critical mass” of submerged mycelium has been formed, hyphae escape the substrate to grow into the air. These aerial hyphae form aggregates, which are called

hyphal knots or nodules. These knots may result from a single hypha that branches intensely or arise from branches of neighbouring hyphae that grow towards and alongside each other. Within the knots, hyphae aggregate forming a fruiting body initial. These initials are the first fruiting body specific structures (Sánchez and Moore, 1999; Sánchez *et al.,* 2004; Kües *et al.,* 2004). Initials can further develop into primordia. Primordia are characterized by the presence of all tissues that occur in a mature fruiting body. Only part of the initials or primordia eventually will form fruiting bodies. Stochastic processes and competition for translocated materials may determine which initials grow out into mature fruiting bodies. Alternatively, and not mutually exclusive, developing fruiting bodies may repress outgrowth of neighboring initials. Such a mechanism was proposed based on the observation that inactivation of *fst3* in *S. commune* resulted in a high number of fruiting bodies (Ohm *et al.,* 2010b).

The lower part of primordia will develop into the stipe of a mature fruiting body, while the cap will be formed from the upper part. Within the cap different tissues develop, which are not formed from meristems, as in plants, but result from the interaction of individual hyphae. The outer part of the cap is called the veil, while in the inner part the pileus trama and gills (or pores) with a hymenium can be distinguished. In the hymenium different cell types are formed, among which the basidia. In the basidia, karyogamy and meioses take place, ultimately resulting in basidiospores. The complexity of fruiting body development is also exemplified by the fact that formation of the different tissues overlaps in time. Moreover, cells in the developing mushroom differ in diameter, length, the number of septa, nuclei and vacuoles, and molecular composition (e.g. the content of reserve carbohydrate; Moore, 1998). The different cell types are the result of localized growth as well as apoptosis (Umar and van Griensven, 1997a).

Not all basidiomycetes follow the general morphogenetic pathway of fruiting body formation (Reijnders and Stafleu, 1992; Watling, 1996; Clémonçon, 1997). For instance, the fruiting bodies of *S. commune* (Figure 1) result from indeterminate growth of fruiting body primordia. Expansion of these cup-shaped primordia is not the result of intercalary growth but is due to continued apical growth and differentiation of hyphae in the primordium (Wessels, 1993). Despite the variety in developmental programmes, their underlying regulation seems to be conserved, at least partly (see Section IIIB,C).

III. REGULATION OF FRUITING BODY FORMATION

A. Environmental signals

Emergence of fruiting bodies is accompanied by drastic changes in exposure to oxygen, carbon dioxide, and light. These environmental factors can exert a profound influence on fruiting body development. Temperature, humidity, volatiles, pH, salinity and availability of nutrients may also play a decisive role (Madelin, 1956, Manachère, 1980; Kües and Liu, 2000; Eastwood *et al.,* 2013). Development of fruiting bodies is often, if not always, the result of a combination of environmental conditions. For instance, it was proposed that the volatile 1-octen-3-ol controls the early differentiation of *Agaricus bisporus* vegetative hyphae to multicellular knots. A drop in temperature would control the differentiation of primordia in this basidiomycete, while the carbon dioxide level would determine the number of fruiting bodies that develop (Eastwood *et al.,* 2013). Similarly, fruiting in *Lentinula edodes* is induced by light, moisture, and low

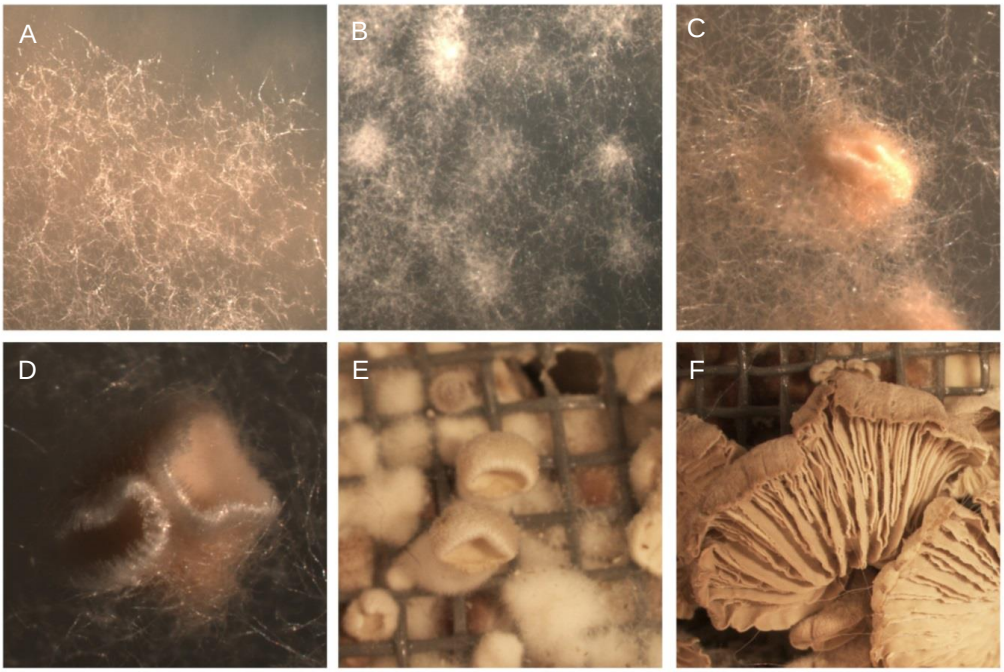

Figure 1. Fruit body development of *S. commune.* Aerial hyphae (A) aggregate into stage I aggregates (B), from which stage II primordia develop (C). These primordia form cup-shaped structures (D, E) that further differentiate into mature fruiting bodies (F).

temperature (Ishikawa, 1967; Matsumoto and Kitamoto, 1987; Nakazawa *et al.,* 2008). Molecular mechanisms underlying regulation of fruiting by CO_2 and light has been studied in some detail. This will be discussed in the following sections

Carbon dioxide as a signal for fruiting body formation

CO_2 represses fruiting body development in a variety of basidiomycetes including *A. bisporus* and *S. commune* (Niederpruem, 1963; Raudaskoski and Viitanen, 1982; van Griensven, 1988; Eastwood *et al.,* 2013). Carbonic anhydrase most likely functions in CO_2 sensing by converting this molecule in bicarbonate (Bahn and Muhlschlegel, 2006). This molecule stimulates adenylyl cyclase activity in *Candida albicans* and *Cryptococcus neoformans* (Klengel *et al.,* 2005 and Morgensen *et al.,* 2006) and this enzyme also plays a role in CO_2 sensing in fruiting body formation (Eastwood *et al.,* 2013; Yamagishi *et al.,* 2002; 2004; Chapter 3).

Light as a signal for fruiting body formation

Illumination for a few minutes is sufficient to induce primordia formation in *S. commune.* This stage was reported to be the only light sensitive step in mushroom development in this basidiomycete (Perkins, 1969; Raudaskoski and Yli-Mattila, 1985). However, we have found that a later stage(s) also requires light exposure. Light is also required for several stages of fruiting body development in, for example, *Coprinus congregatus* (Manachère, 1988) and *C. cinerea* (Tsusué, 1969; Lu, 1974; Kamada *et al.,* 1978). The latter has at least five light sensitive phases (Kües, 2000; Lu, 2000). Light is needed for the formation of initials, for maturation of primordia, and for karyogamy. On the other hand, it negatively impacts hyphal knot formation and completion of meioses. Thus, for fruiting bodies to develop, cycles of light and darkness are required. Light effects are local and are not spread systemically (Madelin, 1956; Kertesz-Chaloupková *et al.,* 1998).

Blue light initiates fruiting body formation in *C. cinerea* and *S. commune* (Perkins and Gordon, 1969; Durand, 1985). The molecular mechanisms underlying blue light regulation in these basidiomycetes have been partly elucidated. Yet, blue light regulation is much better understood in *Aspergillus nidulans* and *Neurospora crassa* (Purschwitz *et al.,* 2006; Bayram *et al.,* 2010). We here briefly discuss what is known about light signalling in *A. nidulans* in relation to reproduction to illustrate how

light signalling may operate in fruiting body formation in basidiomycetes. *A. nidulans* preferentially forms fruiting bodies in the dark. The phytochrome FphA is a red light receptor that represses sexual development (Blumenstein *et al.,* 2005), while the blue light LreA/LreB receptor complex stimulates this process. The latter is concluded from inactivation of *lreA* or *lreB* (Purschwitz *et al.,* 2008). Yet, light regulated development is more complex evidenced by the finding that fruiting body formation in a strain in which *lreA*, *lreB*, and *fphA* are inactivated is less affected than in the Δ*lreA* and the Δ*lreB* strains. LreA, LreB and PhyA form a light sensing complex that also includes an activator of sexual development called VeA (Kim *et al.,* 2002). VeA is also part of a complex with VelB, LaeA and VosA as core components (Bayram *et al.,* 2010). Like the Δ*veA* strain, the Δ*velB* strain does not form fruiting bodies (Bayram *et al.,* 2008b). LaeA is a negative regulator of sexual development (Sarikaya Bayram *et al.,* 2010) evidenced bt the finding that fruiting body development in the Δ*laeA* strain is markedly increased in the light. *A. nidulans* also has a second blue light sensor, CryA (Bayram *et al.,* 2008a). CryA functions as a negative regulator of sexual development probably by integrating near-UV and blue light, resulting in repression of VeA-mediated initiation of sexual development.

C. cinerea has homologues for LreA, LreB, VeA, VelB, LaeA, a putative phytochrome and a cryptochrome-like photolyase. The role of these proteins is not yet known except for the LreA and LreB homologues, called Dst1 and Wc-2, respectively (Terashima *et al.,* 2005; Nakazawa *et al.,* 2011). Gene *dst2* has also been proposed to encode a photomorphogenic protein (Kuratani *et al.,* 2010). Genes *dst1, wc-2,* and *dst2* were identified in a homokaryotic fruiting strain (i.e. with constitutively active A and B mating type pathways). This strain shows aberrant photomorphogenesis in that it forms dark stipes when grown in the dark (Kamada *et al.,* 2010). In other words, this strain does not need light for induction of initials but requires light for primordia maturation. Dark stipes in the wild-type are obtained when cultures containing primordia are incubated in the dark. The pileus and stipe tissues at the upper part of the primordium remain rudimentary in the case of dark stipes but the basal part of the primordium does elongate. Inactivation of *dst1, wc-2,* or *dst2* in the homokaryotic fruiter impaired fruiting body development and resulted in the dark stipe phenotype when subjected to light.

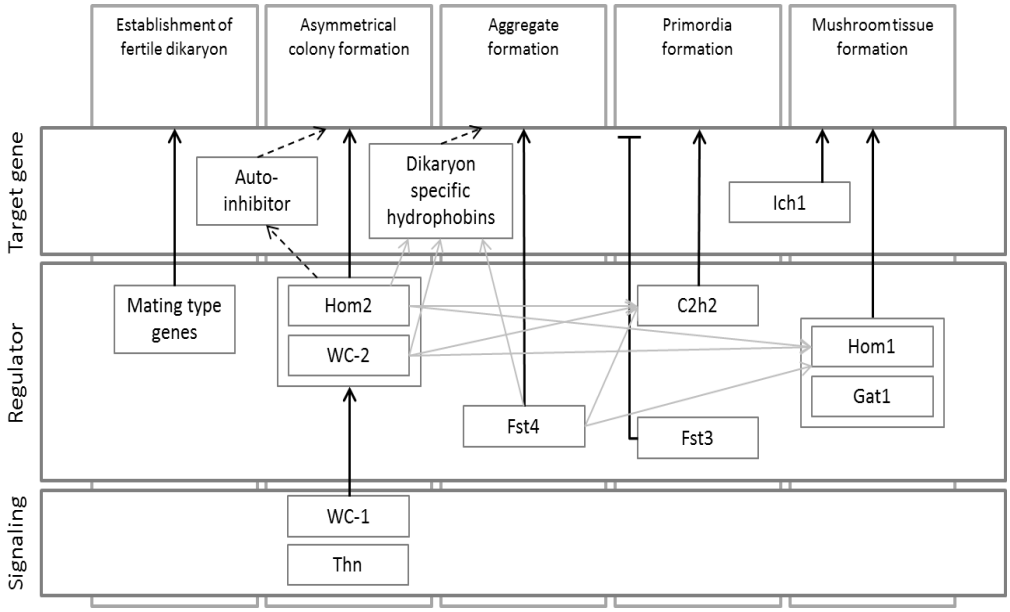

Figure 2. Model of regulation of fruiting body formation in *S. commune*. Depicted are transcription factors and proteins involved in signalling as have been investigated in *S. commune*. Black lines represent developmental stages in which these genes are involved. Grey lines represent interactions between transcription factors, as indicated by whole genome expression analysis (Ohm *et al.,* 2011). Dotted lines represent regulation of target genes and their role in specific stages of development.

The genome of *S. commune* contains orthologues of *lreA* and *lreB*, genes encoding a putative cryptochrome and phytochrome, and genes encoding homologues of VeA, VelB, LaeA, and Dst2 (Ohm *et al.,* 2010b). Genes *wc-1* and *wc-2* represent the *lreA* and *lreB* homologues of *S. commune.* Inactivation of these genes results in a blind phenotype. Dikaryons with both copies of *wc-1* or *wc-2* inactivated behave like monokaryons, unable to form mushrooms in white or blue light (Ohm *et al.,* 2013; Figure 2). The Wc-1 and Wc-2 proteins contain 2 and 1 protein binding Per-Arnt-Sim (PAS) domain, respectively. One of the PAS domains in Wc-1 is a modified version, called a Light-Oxygen-Voltage (LOV) domain. This domain is expected to bind flavin adenine dinucleotide (FAD), which is a chromophore that detects blue light. The Wc-2 protein has a predicted zinc-finger domain of the GATA-type. These data suggest that Wc-1 is the light receptor, whereas Wc-2 has a role as a transcriptional regulator. Compared to the wild type, 183 and 244 genes are more than 2 fold up- and

down-regulated in the Δwc-2Δwc-2 dikaryon, respectively (Ohm *et al.,* 2013). Hydrophobin genes are over-represented in the down-regulated genes (see Section IVA). Moreover, expression of the transcription factor genes *c2h2* and *hom1*, known to be involved in fruiting body development (Ohm *et al.,* 2011) (see section IIIC), is more than 2 fold decreased in the Δ*wc-2*Δ*wc-2* dikaryon. Genes *cry1* and *phy1* encoding the cryptochrome/photolyase and the phytochrome are also down-regulated in the Δ*wc-2*Δ*wc-2* dikaryon suggesting that they play a role in the photobiology of *S. commune* (Ohm *et al.,* 2013).

B. Mating-type Genes as Master Regulators

Heterokaryons are generally the life stage in heterothallic basidiomycetes capable of fruiting. Fertile heterokaryons are the result of a mating between two homokaryons that have different mating-type genes. Homokaryons of *S. commune* and *C. cinerea* contain one nucleus in each hyphal compartment, and therefore, are called monokaryons. On the other hand, the heterokaryons contain two nuclei in each hyphal compartment (one of each mating partner) and are called dikaryons. The dikaryons of *S. commune* and *C. cinerea* are characterized by a clamp connection at each septum, which is formed during synchronous mitotic division of the two nuclei. Nuclear distribution and presence of clamp connections is variable in the basidiomycetes. The homokaryon of *Agaricus bitorquis* is multikaryotic, while the fertile heterokaryon is dikaryotic but without clamp connections (Raper, 1976). The fertile heterokaryon of *A. bisporus* grows directly from a basidiospore that contains two nuclei with different mating types. The heterokaryon is multikaryotic and, like *A. bitorquis*, has no clamp connections (Raper *et al.,* 1972).

The mating-type genes are the master regulators of sexual development. When two homokaryons with different *A* and *B* mating type genes (in *S. commune* called *MATA* and *MATB*) fuse, a heterokaryon is formed with the propensity to form fruiting bodies. Nuclei that are exchanged migrate to the apical compartment of the recipient hypha, which is accompanied by septal dissolution. In the apical compartment the donated and recipient nuclei pair and hyphal dissolution is switched off. In fact, new septa are formed that are more resistant to dissolution and which

Box 2 The life cycle of *Agaricus bisporus* and its role as a food source

A. bisporus fruiting bodies nown as white button mushrooms are an important food source. A billion kilogram of this mushroom is produced in the European Union annually, of which 25 % in the Netherlands. The latter represents an annual sales of € 300 million, which makes this mushroom the third most important “vegetable” crop in the Netherlands after tomato and paprika. Mushrooms are a good source of digestible proteins with contents higher than most vegetables and only somewhat less than meat and milk. Moreover, they contain vitamins, minerals and bioactive compounds such as anti-cancer polysaccharides. The market for mushrooms is predicted to increase in the future due to their nutritional value and the increasing world population.

A. bisporus colonizes litter in nature and is considered a specialist degrader of humic-rich substances (Morin *et al.,* 2012). Under commercial conditions, *A. bisporus* is grown on compost originating from wheat straw, horse or chicken manure, and gypsum. Once the substrate is colonized, it is topped with a casing layer of peat. This layer supplies high water activity (Bels-Koning 1950; Flegg 1956; Kalberer 1987) as well as a microbial flora that is necessary for fruiting (Eger 1961). Other environmental signals for induction of fruiting are a decrease in temperature, CO_2, and 1-octen-3-ol (Eastwood *et al.,* 2013). Induction of fruiting starts with the formation of hyphal knots in the casing layer (Umar and van Griensven 1997b; Eastwood *et al.,* 2013). These structures develop into 1-2 mm initials (or undifferentiated primordia), that differentiate into primordia that have stipe and cap tissue (Umar and van Griensven, 1997b). Around 5-10% of the primordia will eventually become mushrooms (Noble *et al.,* 2003). Basidiospore-producing gill tissue is developed within the cap. This tissue will be exposed to air when the mushroom matures and the veil breaks.

Despite its commercial importance, molecular tools are hardly available for *A. bisporus*. Protoplast transformation has in only one case been reported (van de Rhee *et al.,* 1996). *Agrobacterium tumefaciens* mediated transformation is well established (Chen *et al.,* 2000; Burns *et al.,* 2005; Romaine and Schlagnhaufer 2007) but cannot be used for gene deletion. Gene silencing by RNAi has been successful in a few cases (Eastwood *et al.,* 2008; Costa *et al.,* 2009).

physically prevent nuclear migration (Wessels and Marchant, 1974). The nuclei in the dikaryotic hyphae divide synchronously. Nuclear division is accompanied by the formation of clamps. As a result, the apical and subapical compartments contain nuclei of both mating type.

The molecular structure of the mating-type loci has been uncovered in for instance *S. commune* and *C. cinerea*. The *A* genes of these fungi encode homeodomain proteins of the HD1 and HD2 type. These proteins form heterodimers in heterokaryons with nuclei containing different *A* genes. These dimers are active in clamp development and in initial pairing and migration of compatible nuclei. On the other hand, the *B* genes of *C. cinerea* and *S. commune* encode pheromones and G-coupled receptors for pheromones. Pheromones from one nucleus interact with receptors encoded by the other nucleus, and vice versa, in heterokaryons with nuclei containing different *B* genes. These interactions are involved in exchange and migration of the nuclei as well as fusion of the hook cell. Homokaryotic mutant strains have been isolated with a constitutive active *A* mating type gene (referred to as $MATA^{con}$ in *S. commune, Amut* in *C. cinerea*) and / or *B* mating type gene (referred to as $MATB^{con}$ in *S. commune*, *Bmut* in *C. cinerea*) (Raper *et al.,* 1965; Koltin, 1970; Swamy *et al.,* 1984). Activity of these loci is thus independent of a compatible locus donated by a sexual partner. The *S. commune* $MATA^{con}MATB^{con}$ homokaryon and the *Amut Bmut* homokaryons of *C. cinerea* form fruiting bodies like wild-type dikaryons (Swamy *et al.,* 1984; Boulianne *et al.,* 2000). Mutations in the *pcc1* gene of *C. cinerea* can also lead to fruiting in homokaryons (Uno and Ishikawa, 1971; Muraguchi *et al.,* 1999). The product of *pcc1* has been proposed to be a negative regulator of the *A* mating type pathway (Kamada, 2002; see below). The *frt1* gene of *S. commune* also initiates fruiting in a homokaryon. Certain homokaryons with introduced copies of this gene start to fruit independent of the mating type loci (see next section).

S. commune and *C. cinerea* are examples of tetrapolar species, where four compatible crosses can be made from the spores of a dikaryon. Bipolar species exist within the agaricomycetes as well, where the *B* locus has lost its ability to distinguish self from nonself (James *et al.,* 2006). This is in contrast to bipolar basidiomycetes that have linked all mating type genes on a single locus (Bakkeren and Kronstad, 1994, Lengerer *et al.,* 2002).

C. Other regulatory genes

Regulatory genes in establishment of the dikaryotic mycelium

Establishment of the dikaryotic mycelium strongly impacts gene expression. For instance, 26% of the genes of *S. commune* show a fold change ≥ 2 upon mating (Erdmann *et al.,* 2011). The *A* and *B* pathways regulate 27% and 42% of these genes, respectively, while the remaining 31% is controlled by both mating type loci. Among the *A* activated genes is *clp3*, a homolog of *clp1* in *C. cinerea* (Inada *et al.,* 2001). Gene *clp1* of *C. cinerea* was isolated by complementation of an *AmutBmut* mutant homokaryon that did not form clamps. Clamps are made independently from the homeodomain heterodimer when *clp1* is expressed from a constitutive promoter. The mode of action of Clp1 is not known and it does not contain any known structural motifs. Gene *pcc1* (see previous section) is another gene that seems to be part of the *A*-regulated pathway (Murata *et al.,* 1998). A homokaryotic strain with a mutated copy of this gene forms pseudoclamps and it has the capacity to form fully differentiated fruiting bodies. These data and its expression in a wild-type homokaryon indicate that *pcc1* is a repressor of the fruiting pathway in the absence of a functional *A* complex (i.e. in the homokaryon). Gene *pcc1* likely encodes a transcription factor because of its HMG box motif and nuclear localization signal. The presence of a pheromone responsive element in the *pcc1* promoter suggests that it is a pheromone responsive gene. Indeed, it is up-regulated by a compatible *B* mating interaction (Murata *et al.,* 1998). The fact that *pcc1* is also up-regulated by an activated *A* gene suggests that Pcc1 coordinates the activities of the *A* and *B* genes (Murata *et al.,* 1998). This hypothesis is supported by the finding that the *pcc1* mutant homokaryon has the potential to form fruiting bodies. It has been suggested that the repressor activity of *pcc1* is released by a compatible *A* gene interaction via *clp1* (Kamada, 2002).

FRT1 is a putative nucleotide-binding protein with a P-loop motif (Horton and Raper, 1995). Its gene was identified by its fruiting phenotype when transformed into certain homokaryons of *S. commune* (Horton and Raper, 1991). Experimental evidence indicates that homokaryotic strains that start to fruit have an endogenous *frt1* allele of a different kind (designated *frt1-2*; Horton *et al.,* 1999). In contrast, strains possessing *frt1-1* do not fruit when transformed with this allele (Horton and Raper, 1991). Homokaryons in which the *frt1* gene was disrupted were more fluffy compared to wild-type strains. The aerial hyphae of Δ*frt1* strains aggregate (Horton *et al.,* 1999),

like observed during the first stages of fruiting body development (Raudaskoski and Vauras, 1982; van der Valk and Marchant, 1978). This was accompanied by increased expression of the dikaryon-specific genes *sc1* and *sc4* (see section IV). From these data it was proposed that *FRT1* is part of a signal transduction pathway that represses expression of dikaryon-specific genes in the monokaryon (Horton *et al.,* 1995). However, absence of FRT1 is not sufficient to initiate full development of fruiting bodies. But how can haploid fruiting be explained when *frt1-1* is transformed into a *frt1-2* strain? It has been proposed that these proteins dimerise and by this relieve repression of the dikaryon-specific genes. The heterodimer would also activate genes, resulting in the formation of mature fruiting bodies (Horton *et al.,* 1999). Note that absence of *frt1* did not affect fruiting in the dikaryon (Horton *et al.,* 1999). This suggests that *frt1* is not component of the pathway in the dikaryon that leads to formation of fruiting bodies in the dikaryon.

Highly conserved small G proteins, called Ras, play an essential role in intracellular signaling (e.g. the MAPK, cAMP, and Cdc42 signaling pathways). Two *ras* genes have been identified in nearly all fungi. Ras exists in a GTP bound active form and a GDP bound inactive form. Guanine exchange factors and GTPase activating proteins (GAP) promote conversion of the active and inactive forms of Ras, respectively. Deletion of *gap1* of *S. commune* results in hampered clamp, gill, and spore formation (Schubert *et al.,* 2006). On the other hand, constitutively activated Ras1 results in dysfunctional dikaryotization, as non-self nuclei are not accepted. Furthermore, growth orientation, branching, fruiting body morphology and spore formation are affected (Knabe *et al.,* 2013). Since deletion of *gap1* increases Ras1 activity, an overlap of phenotypical traits with the *ras1* over-expression strain is expected. Indeed, PKA levels are increased in both strains, linking these proteins to the cAMP signaling pathway. Expression analysis of abovementioned transformant strains and a constitutively active Cdc42 mutant, involved in the MAPK pathway, revealed an overlap in target genes. This implies that Ras1 is also involved in MAPK signaling in *S. commune* (Weber *et al.,* 2005).

Regulatory genes involved in fruiting body formation in the heterokaryon

The *thn1* gene of *S. commune* acts early in the developmental pathway of fruiting body formation. A mutation in *thn1* leads to pleiotropic effects including absence of

aerial growth in the homokaryon and, when homozygous, in the dikaryon (Raper and Miles, 1958; Schwalb and Miles, 1967; Wessels *et al.,* 1991b). Thus, both aerial hyphae and fruiting body formation is affected. Thn1 is a putative RGS protein that converts an active GTP-bound Gα subunit into an inactive GDP-bound Gα. It was therefore proposed (Fowler and Mitton, 2000) that *thn1* regulates a heterotrimeric G protein signalling pathway that functions in the decision of the vegetative mycelium to start development of aerial hyphae and fruiting bodies. This function would be similar to that of FlbA in *A. nidulans* (Lee and Adams, 1994). Transcriptome analysis revealed 114 genes, mostly involved in cellular responses, that were affected in the *thn* mutant. This is in agreement with a role in G protein signalling (Erdmann *et al.,* 2012). Most of the pleiotropic effects of the *thn* mutation are overcome by growing the mutant near a wild-type colony (Schuren, 1999). A diffusible molecule smaller than 8 kDa would be responsible for this effect and may be part of the signalling cascade.

A set of transcription factor genes has been identified that is involved in different stages of fruiting body development in *S. commune* (Figure 2) (Ohm *et al*., 2010b; 2011; 2013). Inactivation of the homeodomain gene *hom2* results in symmetrical monokaryon-like colonies that form aerial hyphae but do not form aggregates, primordia and mature fruiting bodies. Fst4 is a zinc finger transcription factor positioned downstream of Hom2. Deletion of its encoding gene results in irregular dikaryon-like colonies that form aerial hyphae but do not form aggregates. Zinc finger protein C2h2 is involved in primordia formation. Inactivation of its encoding gene gives rise to irregular colonies that form aerial hyphae and aggregates. Deletion strains of *fst3, gat1* and *hom1* do form fruiting bodies. However, these fruiting bodies are smaller and are produced in a higher number. It was proposed that Fst3, a fungal specific transcription factor, is a local repressor of primordia formation (Ohm *et al.,* 2010b). This would imply the existence of communication between developing fruiting bodies. Such communication would ensure full outgrowth of mushrooms when resources are limited. Gat1, a GATA type zinc finger protein, and Hom1, a homeodomain protein, might play a role in expansion of the fruiting body. Expansion of fruiting bodies will go at the expense of outgrowth of neighbouring aggregates because of limited resources. Such a mechanism would not require communication between fruiting bodies. The *ich1* gene of *C. cinerea* also acts in later stages of fruiting body formation. Cap differentiation is blocked at an early stage of fruiting body

differentiation in the *ich1* mutant (Muraguchi and Kamada, 1998). In contrast to wild-type primordia, no rudimentary pileus is observed in the primordial shaft of the *ich1* mutant. The precise role of the gene is not yet known. It encodes a protein that contains a nuclear targeting signal and an S-adenosyl-L-methionine (SAM) binding motif (Kües, 2000), being characteristic for the enzyme family of methyltransferases (Faumann *et al.,* 1999).

The model presented in Figure 2 could also apply to other mushroom forming fungi. Genes *hom2*, *hom1*, *fst3*, *fst4*, *c2h2* and *gat1* are basidiomycete specific regulators (Todd *et al.,* 2014). These and the other transcription factors identified in *S. commune* that are involved in fruiting body development have homologues in *Laccaria bicolor* and *A. bisporus* (Ohm *et al.,* 2010b; Morin et al 2013). Similarly, homologues of *ich1* are present in *S. commune* and *A. bisporus*. Gene expression analysis of two *A. bisporus* varieties revealed 22 genes with homologues in *S. commune* that were significantly up-regulated in mature fruiting bodies. This group includes *fst3, fst4, c2h2* and *hom1.* Homologues of *hom2, fst4, fst3, gat1,* and *hom1* showed similar expression in *L. bicolor* (Morin *et al.,* 2013), while homoloques of *c2h2*, *fst3*, *hom1*, *gat1* showed similar expression in *C. cinerea* (Plaza *et al.,* 2014).

Whole genome expression analysis revealed that 284 genes are differentially expressed in the monokaryotic stage of *S. commune* (Ohm *et al.,* 2010b), whereas 128 genes are differentially expressed during the aggregate stage. Genes involved in hydrophobins, protein and energy production are up-regulated during aggregation, while genes involved in signal transduction, gene regulation, carbohydrate metabolism, and cell wall biogenesis are down-regulated. 467 genes are differentially expressed during primordia formation. Up-regulated genes are involved in signal transduction, gene regulation, carbohydrate metabolism, and cell wall biogenesis, while genes involved in protein and energy production are down-regulated. Genes involved in fatty acid metabolism are enriched in the 128 differentially expressed genes during fruiting body maturation, while gene regulation, glucose, alcohol and amino acid metabolism are over-represented in the down-regulated genes (Ohm *et al.,* 2010b). The high number of genes exclusively expressed during primordia development suggests this is a crucial developmental switch.

D. Nuclear positioning

The *sc1, sc4,* and *sc6* hydrophobin genes (see Section IVA) as well as *sc7* and *sc14* are expressed in dikaryons (*MATA*-on *MATB*-on) of *S. commune* but not in monokaryons (*MATA*-off *MATB*-off) and *MATA*-on *MATB*-off or *MATA*-off *MATB*-on mycelia (Mulder and Wessels, 1986; Wessels *et al.,* 1995). In contrast, the *sc3* hydrophobin gene is active in the monokaryon but is down-regulated in a *MATA*-off *MATB*-on mycelium (Ásgeirsdóttir *et al.,* 1995). From this it is expected that *sc3* would also be inactive in dikaryons (i.e. *MATA*-on *MATB*-on). Indeed, *sc3* mRNA levels are reduced in a fruiting dikaryon. However, under non-fruiting conditions (e.g. high CO_2 and darkness) high *sc3* expression is observed, while expression of *sc1, sc4, sc6, sc7,* and *sc14* is relatively low (Wessels *et al.,* 1987). Apparently, the *MATB* pathway, and possibly also the *MATA* pathway, is not active in at least part of the dikaryotic mycelium. This was explained by a regulatory mechanism that varies the distance between the nuclei in compartments of dikaryotic hyphae (Schuurs *et al.,* 1998). The distance of nuclei in dikaryotic aerial hyphae is relatively large (> 8 μm) and correlates with high *sc3* expression. In contrast, nuclear distance in fruiting bodies is small (< 2 μm), correlating with low *sc3* and high *sc4* expression. Increased distance of the nuclei (> 4 μm) would inactivate the *MATB*-on pathway, and possibly also the *MATA*-on pathway, resulting in a monokaryon-like gene expression (Wessels *et al.,* 1998).

IV. STRUCTURAL PROTEINS AND ENZYMES INVOLVED IN FRUITING

Hydrophobins

Hydrophobins are secreted proteins that fulfil a wide spectrum of functions in fungal growth and development (Wessels, 1997, Wösten and Wessels, 1997; Wösten, 2001). Class I and class II hydrophobins are distinguished based on hydropathy patterns and solubility characteristics, (Wessels, 1994). Basidiomycetes only have class I hydrophobins. Class I hydrophobins in a soluble state can affect hyphal wall composition (van Wetter *et al.,* 2000b). However, most functions of these hydrophobins are based on the property to self-assemble into an amphipathic membrane at hydrophilic / hydrophobic interfaces (Wösten *et al.,* 1993, 1994ab, 1995, 1999). This ~10 nm thick membrane is highly insoluble and consists of a mosaic of amyloid-like fibrils called rodlets (Wösten *et al.,* 1993; Wösten and de Vocht, 2000; de

Vocht *et al.,* 2002; Butko *et al.,* 2001; Scholtmeijer *et al.,* 2009). Upon self-assembly at the interface between the hydrophilic cell wall and a hydrophobic environment (the air or the hydrophobic surface of a host), the hydrophilic side of the amphipathic membrane will face the cell wall, while the hydrophobic side becomes exposed to the hydrophobic environment. Aerial hyphae and spores thus become hydrophobic, while hyphae that grow over a hydrophobic substrate become attached.

The role of hydrophobins in mushroom forming fungi has been best studied in *S. commune*. This fungus contains 13 hydrophobin genes (Ohm *et al.,* 2010b) including *sc1*, *sc3*, *sc4*, and *sc6* (Mulder and Wessels, 1986; Schuren and Wessels, 1990; Wessels *et al.,* 1995; de Vocht *et al.,* 1998). The *sc1*, *sc4*, and *sc6* hydrophobin genes are dikaryon-specific and are regulated by the mating type genes (Ruiters *et al.,* 1988), *fbf* (Springer and Wessels, 1989), *thn* (Wessels *et al.,* 1991b) and the transcriptional regulators involved in pre-aggregate development *wc-2*, *hom2,* and *fst4* (Ohm *et al.,* 2011, Ohm *et al.,* 2013). On the other hand, *sc3* is expressed both in the monokaryon and the dikaryon (see Section IIID). It is regulated by *thn*, *wc-2*, *hom2,* and *fst4* but not by *fbf*. Hydrophobins make up 6-8% of the proteins synthesized by *S. commune* at the time of emergent growth (Wessels *et al.,* 1991a,b). So far, SC3 and SC4 have been isolated and characterized and they will be discussed below.

Expression of *sc3* in monokaryons is induced after a feeding submerged mycelium has been established (Mulder and Wessels, 1986). SC3 secreted by submerged hyphae self-assembles at the medium-air interface, which is is accompanied by a huge drop in water surface tension. This enables hyphae to breach the interface of the aqueous environment and the air to form aerial hyphae (Wösten *et al.,* 1999). The water surface tension remains high in a Δ*sc3* strain and only few hyphae can escape the aqueous environment. In other words, in the absence of *sc3* expression hyphae are forced to remain growing in the aqueous substrate. Expression of *sc3* is thus a main event in onset of aerial growth. How the mycelium senses that the feeding mycelium is large enough to be able to support aerial growth by switching on SC3 production remains to be solved (Wösten and Willey, 2000). SC3 secreted by aerial hyphae cannot diffuse into the medium but will be confronted with the cell wall-air interface. As a result, SC3 assembles at the surface of these hyphae making them hydrophobic (Wösten *et al.,* 1994a).

SC3 also lowers the water surface tension in the dikaryon allowing aerial hyphae to grow into the air (van Wetter *et al.,* 2000a). The amount of SC4 in the medium is too low to complement for the absence of SC3 in a Δ*sc3*Δ*sc3* dikaryon. SC3 also coats aerial hyphae of the dikaryon and hyphae at the outer surface of fruiting bodies (Ásgeirsdóttir *et al.,* 1995). SC4 but not SC3 is located in the fruiting body context, in which it lines air channels that traverse the plectenchyma (Lugones *et al.,* 1999). The air channels of Δ*sc4*Δ*sc4* fruiting bodies readily fill with water in the absence of a hydrophobic coating (van Wetter *et al.,* 2000a). Thus, SC4 ensures gas exchange in the fruiting body tissue under moist conditions.

The ABH1 hydrophobin of *A. bisporus* (Lugones *et al.,* 1996; de Groot *et al.,* 1996) is an orthologue of SC4 of *S. commune*. Expression of *abh1* starts prior to primordia formation and remains high during primordia and fruiting bodies formation (Eastwood *et al.,* 2013). The protein not only lines air channels in the fruiting body tissue (Lugones *et al.,* 1999) but also coats the outer surface of the fruiting body (Lugones *et al.,* 1996). The HypB hydrophobin of *A. bisporus* is located at the border of the cap and the stipe tissue and has been proposed to protect the mushroom against bacterial infections (de Groot *et al.,* 1999).

The presence of multiple hydrophobin genes not only enables the fungus to produce hydrophobins at different stages of development (Kershaw *et al.,* 1998) but also to form hydrophobins that are tailored to fulfil specific functions (van Wetter *et al.,* 2000a). SC4 can substitute for SC3 in formation of hydrophobic aerial hyphae but hyphal attachment to hydrophobic surfaces is only partially restored. This is explained by a lower affinity of assembled SC4 for the cell wall of adhering hyphae when compared to SC3. Possibly, this is related to sugar binding specificities of these hydrophobins (van Wetter *et al.,* 2000a). The exposed carbohydrates of cell walls of aerial hyphae and hyphae in fruiting body tissue may be different, requiring different lectin specificities to ensure strong binding to the cell wall. Evolution of hydrophobins to fulfil specific functions is also indicated by sequence analysis. Hydrophobins involved in aerial hyphae formation (i.e. SC3 of *S. commune* (de Vocht *et al.,* 1998), ABH3 of *A. bisporus* (Lugones *et al.,* 1998), COH1 of *C. cinerea* (Ásgeirsdóttir *et al.,* 1997), and POH1 of *P. ostreatus* (Ásgeirsdóttir *et al.,* 1998)) are more related to each other than SC3 and ABH3 to the other hydrophobins of *S. commune* and *A. bisporus*, respectively. Similarly, the fruiting body specific hydrophobins of *S. commune* cluster

with *hypB* (de Groot *et al.,* 1999), *abh1,* and *abh2* (Lugones *et al.,* 1996; de Groot *et al.,* 1996) of *A. bisporus*. This suggests that functional similarity is reflected in the primary sequence of hydrophobins.

With the established roles of SC3, SC4, and ABH1 we are only at the beginning of our understanding of the functions of hydrophobins in fruiting. Dikaryons seem to express several hydrophobins that have specific properties or are expressed at a particular place. For instance, it has been suggested that hydrophobins are involved in aggregating aerial hyphae during fruiting body morphogenesis. The situation may even be more complex by the presence of proteins that can substitute for hydrophobins. For example, the SC15 protein can partly rescue the reduction of surface tension of the culture medium by SC3 (Lugones *et al.,* 2004).

Lectins

Lectins are carbohydrate-binding proteins that play diverse roles in fungi (Singh *et al.,* 2010). For instance, they function in pathogenesis in plants and insects, in predation of soil nematodes, and are involved in early stages of ectomycorrhizal symbiosis. Expression of galectin lectins is up-regulated in multiple stages of fruiting as in *C. cinerea* and *Agrocybe aegerita* (Boulianne *et al.,* 2000; Luan *et al.,* 2010). They play a role in defense but also seem to play a role in mushroom development. Addition of the galalectin AAL of *A. aegerita* to an established mycelium induces aggregation and primordia formation both in *A. aegerita* and *Auricularia polytricha* (Sun *et al.,* 2003; Luan *et al.,* 2010). In contrast, presence of the galalectin at the moment of inoculation results in growth inhibition and repression of fruiting (Luan *et al.,* 2010) showing that temporal expression of galactins is important for their role in mushroom development.

Haemolysins

Haemolysins are pore-forming proteins that are known for their ability to lyse red blood cells. Aggregation of haemolysins in the plasma membrane results in ~ 4 nm wide pores that permeabilise the membrane (Nayak *et al.,* 2013). Fungal homologs have been identified in the basidiomycetes *A. aegerita* (Fernandez Espinar and Labarère, 1997; Berne *et al.,* 2002), *P. ostreatus* (Berne *et al.,* 2002), and *S. commune* (Han *et al.,* 2010). There is strong evidence for the involvement of haemolysins in fruiting body formation. These proteins are specifically expressed in primordia and young fruiting

bodies of *A. aegerita* and *P. ostreatus* (Vidic *et al.,* 2005). In both species the concentration of protein was highest at the edge of lamellae, in basidia, and developing basidiospores. Notably, external addition of the hemolysin ostreolysin of *P. ostreatus* to mycelium boosted fruiting initiation in this fungus and increased the quantity and size of fruiting bodies (Berne *et al.,* 2007).

Oxidative enzymes

A role for laccases in oxidative cross-linking of hyphae in polypores has been proposed (Bu´Lock, 1967; Bu´Lock and Walker, 1967) but proof of a role in mushroom development is still lacking. Evidence for a role of cytochrome P450 enzymes in fruiting body formation is available. The *eln2-1* mutant of *C. cinerea* was isolated in a screen for developmental mutants (Muraguchi *et al.,* 1999). This mutant is characterized by dumpy fruiting body primordia. Cell morphogenesis and tissue organization in the primordial shaft is affected. As a result, the mature fruiting bodies have short stipes (Muraguchi and Kamada, 2000). The *eln2* gene is constitutively expressed and encodes a novel type of cytochrome P450 enzyme. These enzymes are involved in the oxidative, peroxidative, and reductive metabolism of numerous compounds. A deletion of 18 amino acids at the C-terminus is the cause of the mutant phenotype. Muraguchi and Kamada (2000) gave three explanations to explain the mutant phenotype. A changed catalytic activity may produce a toxic compound that affects development in the primordial shaft. Alternatively, activity of the truncated enzyme may not result in a product that is normally instrumental in development. Finally, the mutant enzyme may overproduce a normal metabolite or produce superoxide radicals. A disruption of the *eln2* gene should establish if this gene is indeed involved in fruiting body development. Expression of cytochrome P450 genes has also been shown in mushrooms of *A. bisporus* and *L. edodes* (de Groot *et al.,* 1997; Akiyama *et al.,* 2002; Hirano *et al.,* 2004) suggesting these enzymes play a general role in mushroom development.

Expansins

Expansins are non-enzymatic proteins that induce cell wall extension and cell wall modification (McQueen-Mason *et al.,* 1992; Cosgrove, 2005). These proteins are believed to act by disrupting non-covalent interactions between carbohydrate

polymers (McQueen-Mason and Cosgrove, 1995). Expansins were originally found in plants, but homologs have been identified in fungi. Basidiomycetes do not have strict homologues of expansins. Instead they have expansin-like proteins named loosenins with an N-terminal domain similar to that of plant expansins. LOOS1 was first isolated from the basidiomycete *Bjerkandera adusta* (Quiroz-Castañeda *et al.,* 2011) but they also occur in for instance *S. commune*, *A. bisporus*, *C. cinerea,* and *L. bicolor* (Suzuki *et al.,* 2014).

The main function of expansin(-like) proteins in fungi is believed to facilitate degradation of plant-derived substrates. However, evidence accumulates that these proteins also play a role in morphogenesis and cell wall modification. Deletion of the expansin-like protein EglD in *A. nidulans* results in increased resistance against lysing enzymes. This observation combined with the exclusive presence of EglD in conidial cell walls suggests that the expansin-like protein is involved in cell wall remodeling during germination (Bouzarelou *et al.,* 2008). A role of expansin(-like) proteins in extension of fruiting bodies of basidiomycetes is indicated by the fact that heat-induced repression of stipe wall extension in *F*. *velutipes* and *C*. *cinerea* can be rescued by a snail expansin-like protein (Fang *et al.,* 2014; Zhang *et al.,* 2014).

SCOPE OF THE THESIS

The fruiting bodies of basidiomycetes are the most complex fungal structures. The mechanisms underlying mushroom development at a molecular level is still a near black box. Recently, transcription factors have been identified in the model system *S. commune* that are involved in the process of mushroom formation. The aim of this thesis was to further characterize the regulatory network of fruiting body development in this basidiomycete and to link this network to environmental signalling. Furthermore, I started to modulate mushroom formation in *A. bisporus* using the knowledge obtained in *S. commune*. Inactivation of the transcription factor genes *wc-2*, *hom2* and *fst4* results in strains that are unable to fruit. **Chapter 2** describes that these deletion strains also form more biomass when compared to the wild-type. Increase in biomass is accompanied by increased expression of genes involved in carbohydrate metabolism. Expression studies also revealed that the *tea1* transcription factor gene is regulated by *wc-2*, *hom2* and *fst4.* Inactivation of this gene resulted in a strain that also produced higher biomass and that was impaired in fruiting. Together, this chapter

shows that regulators involved in initiation of mushroom formation also repress vegetative growth.

CO_2 represses fruiting body initiation in *S. commune.* **Chapter 3** describes that also later stages of fruiting body development are repressed by this compound. In addition, the involvement of the cAMP-Pka pathway in CO_2 sensing was shown. Addition of cAMP to the medium repressed fruiting body development, while overexpression of *pde2* encoding the protein degrading this compound resulted in mushroom development even at high CO_2 levels. Whole genome expression analysis revealed a set of transcription factors affected by CO_2, among which *wc-2*, *c2h2,* and *hom1*.

Chapter 4 presents evidence that Hom2 is a target of the CO_2-Pka signalling pathway. Biomass formation of a dikaryon in which *hom2* was inactivated was higher than the wild-type when grown in low CO_2. Notably, biomass formation of the wild-type was increased at high levels of this compound and similar to that of the *hom2* deletion strain grown in the presence of low or high CO_2. A constitutive active version of Hom2 was made by replacing serine residues for alanine. Introduction of the encoding gene resulted in mono- and dikaryotic strains that showed repressed vegetative growth and that immediately started to form fruiting bodies at low CO_2. Together, data indicate that Hom2 is inactivated post-translationally by the CO_2-cAMP-Pka signalling pathway. It is proposed that the active non-phosphorylated form of Hom2 represses vegetative growth, which is necessary for initiation of fruiting mediated by a yet unknown other CO_2 controlled transcriptional regulator .

In **Chapter 5** *c2h2* of *A. bisporus* was overexpressed in the commercial strain A15. This resulted in accelerated fruiting body formation and in a lower quality of the mushrooms. Data thus indicate that C2H2 has a role in early and late stages of fruiting development and that it is a target for strain breeding programs.

Results are summarized an discussed in **Chapter 6**.

Chapter 2

Transcription factors of *Schizophyllum commune* that link repression of vegetative growth and initiation of mushroom formation

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ABSTRACT

Transcription factors have been identified that are involved in mushroom formation in *Schizophyllum commune*. The DNA binding Bright domain protein Bril and the homeodomain protein Hom1 are involved in late stages of mushroom development, while the blue light receptor transcription factor Wc-2, the homeodomain protein Hom2, and the zinc-finger transcription factor Fst4 function in early stages of mushroom development. Here it is shown that Bril and Hom1 also stimulate vegetative growth, while biomass formation is repressed by Wc-2, Hom2, and Fst4. The Δ*bri1*Δ*bri1* and the Δ*hom1*Δ*hom1* strains formed up to 0.6 fold less biomass when compared to the wild-type. In contrast, Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2,* and Δ*fst4*Δ*fst4* strains formed up to 2.8 fold more biomass. RNA sequencing showed that repression of vegetative growth correlates with decreased expression of genes involved in carbohydrate metabolism. The TEA/ATTS domain transcription factor gene *tea1* was also down-regulated in the Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2* and Δ*fst4*Δ*fst4* strains. The Δ*tea1*Δ*tea1* strain produced 1.3 fold more biomass than the wild-type and was severely affected in fruiting body development. Together, these data show that transcription factors Wc-2, Hom2, Fst4, and Tea1 link mushroom initiation and repression of vegetative growth. This is the first time that homeodomain proteins have been show to modulate vegetative growth in eukaryotes.

INTRODUCTION

Mushrooms are the most conspicuous fungal structures. They are used as a food source or for their therapeutic compounds (Kües and Liu, 2000). The formation of mushrooms involves a complex developmental program. *Coprinopsis cinerea* and *Schizophyllum commune* are the model systems to study this program (Kües and Navarro-González, 2015). The life cycle of *S. commune* starts with a monokaryotic mycelium that results from the germination of a basidiospore. A fertile dikaryon is formed upon fusion of two monokaryons with compatible mating types. Blue light is required to initiate fruiting in the dikaryotic mycelium (Perkins and Gordon, 1969), whereas high CO_2 levels repress this developmental program (Niederpruem, 1963; Raudaskoski and Viitanen, 1982). Initiation of mushroom formation starts with asymmetrical colony growth, followed by aggregation of aerial hyphae, and

subsequent formation of primordia. These primordia can develop into fruiting bodies that form basidia within the hymenium. Karyogamy, meiosis, and one round of mitosis occur in the basidia, resulting in haploid, binucleate basidiospores.

Regulation of mushroom formation has been studied in *S. commune.* The blue light receptor complex consists of Wc-1 that has a blue light sensing domain and the transcription factor Wc-2. Inactivation of *wc-1* and / or *wc-2* results in a blind phenotype (Ohm *et al.,* 2013). Dikaryotic colonies of the homozygous deletion strains grow symmetrically in blue light (similar to dark-grown wild-type dikaryons) and do not produce aggregates, primordia, and fruiting bodies. Deletion of the homeodomain gene *hom2* and the DNA binding Bright domain protein gene *briI* shows a similar phenotype (Ohm *et al.,* 2011). In contrast, inactivation of the zinc finger transcription factor gene *fst4* results in dikaryons that still grow irregular in the light under low CO_2 conditions but aggregates, primordia, and fruiting bodies are not produced (Ohm *et al.,* 2011). Strains in which the Cys2His2 zinc finger protein gene *c2h2* has been inactivated are arrested at the aggregate stage (Ohm *et al.,* 2011). On the other hand, deletion strains of *fst3*, *gat1* or *hom1* form smaller fruiting bodies but in higher numbers (Ohm *et al.,* 2011). The zinc finger protein Fst3 was proposed to play a role in repression of outgrowth of fruiting bodies from primordia. On the other hand, Gat1, a GATA type zinc finger protein, and Hom1, a homeodomain protein, have been proposed to play a role in expansion of the fruiting body. Homologues of the *S. commune* transcription factors have been identified in *Agaricus bisporus*, *Laccaria bicolor* and *C. cinerea*. Expression studies suggest the existence of a core regulatory program for fruiting body development in basidiomycetes (Ohm *et al.,* 2010b; Morin *et al.,* 2012; Plaza *et al.,* 2014). Variations in expression of these genes would explain species-specific morphology and environmental sensing.

In this study, it is shown that Wc-1, Wc-2, Hom2, and Fst4 not only initiate mushroom formation but also repress vegetative growth. On the other hand, BriI and Hom1 were shown to stimulate biomass formation. Whole genome expression analysis indicates that repression of vegetative growth is the result of down-regulation of genes involved in carbohydrate metabolism. Expression analysis also revealed that the TEA/ATTS domain transcription factor gene *tea1* is down regulated in Δ*wc-1*Δ*wc-1,* Δ*wc-2*Δ*wc-2,* Δ*hom2*Δ*hom2,* and Δ*fst4*Δ*fst4*. Inactivation of *tea1* resulted in increased vegetative growth, and severely reduced formation of fruiting bodies. These

data indicate that Tea1 functions downstream of the blue light receptor complex, Hom2 and Fst4.

EXPERIMENTAL PROCEDURES

Culture conditions and strains

The compatible *S. commune* strains H4-8 (*mat*A43*mat*B41; FGSC 9210) (Ohm *et al.,* 2010b) and H4-8b (*mat*A41*mat*B43) (Ohm *et al.,* 2010a), their derived dikaryotic deletion strains Δ*wc-1*Δ*wc-1*, Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2*, Δ*fst4*Δ*fst4*, Δ*c2h2*Δ*c2h2*, Δ*fst3*Δ*fst3*, Δ*hom1*Δ*hom1,* Δ*bri1*Δ*bri1,* Δ*gat1*Δ*gat1* (Ohm *et al.,* 2010b; 2011, 2013), as well as Δ*hom2*Δ*hom2*Δ*fst4*Δ*fst4* and Δ*hom2*Δ*hom2*Δ*fst3*Δ*fst3* were used in this study. The Δ*ku80* H4-8 strain (de Jong *et al.,* 2010) was used for gene inactivation. Strains were grown in the dark or in the light (1200 lux white LED light; Conrad Electronic, Hirschau, Germany) at 25 °C on minimal medium (MM) containing 1% glucose and 1.5% agar, if applicable (van Peer *et al.,* 2009a). Liquid shaken cultures were inoculated with a mycelial homogenate (van Wetter *et al.,* 2000b) and grown at 250 rpm in 250 ml Erlenmeyers containing 100 ml MM. Agar cultures were inoculated with a point inoculum taken from the periphery of a 7-day-old colony. To assess growth on other carbon sources, glucose was replaced for 4% xylose, 3.4% sucrose, or 1% pectin.

Gene inactivation

Deletion vectors for *tea1* (protein ID 2519514; http://genome.jgi-psf.org/Schco3) and *c2h2d* (protein ID 2703923) were constructed using pDelcas that contains a nourseothricine and a phleomycin resistance cassette (Ohm *et al.,* 2010a). Upstream and downstream flanks of *tea1* and *c2h2d* were cloned at either site of the nourseothricine resistance cassette. To this end, the flanks were amplified by PCR using Taq polymerase and H4-8 chromosomal DNA as template. The 906 bp upstream flank and the 946 bp downstream flank of *tea1* were amplified using the primer combination Δ9519514ufw/Δ9519514urv and Δ9519514dfw/Δ9519514drv, respectively (Table 1). Primer pair combinations ∆2703923ufw/∆2703923urv and ∆2703923dfw/∆2703923drv were used to amplify the 897 bp upstream and 975 bp downstream flank of *c2h2d*, respectively (Table 1). The PCR products were cloned into pGEM-T Easy (Promega, Madison, USA). The upstream flanks were retrieved

from the resulting constructs using Van91I and introduced into the Van91I site of pDelcas, resulting in pDel-2519514-UF and pDel-2703923-UF. The downstream flanks were retrieved from the pGEM-T

easy derived constructs using SfiI and introduced into the SfiI site of pDel_2519514-UF and pDel_2703923-UF. This resulted in the knock-out constructs pDelcas-2519514 and pDelcas-2703923.

Table 1. Primers used in this study.

∆2519514ufw	GGCCTAATAGGCCTAGAATGCGCTCTCCGTC
∆2519514urv	GGCCTCGCAGGCCAGGGAGGATGACGCAAAG
∆2519514dfw	GGCCTGCGAGGCCGTCCGTGTTCTTGGATAC
∆2519514drv	GGCCTATTAGGCCGTGCGTTGTTTCGTTTCC
2519514 ufcfw	TCCACGCTGGCTGAATAG
2519514 dfcrv	TCGATGTGAGGTACTGTC
Nourdelrev	TAAGCCGTGTCGTCA
sc3tersqf	CCGGGAATTCCAGAT
2519514 ifw	CCGACTTCGATATCACTC
2519514 irv	TCGGCGATGCAAGAAGTC
tea1 comp fw	CATGGCTAGCAGGTGATGCAGCGCGACGATAG
tea1 comp rv	GGATCCTTAGATCATGAAAGCGCCGCC
∆2703923ufw	GGCCTAATAGGCCCTGTCACGCACCAGTACG
∆2703923urv	GGCCTCGCAGGCCGGGCGAACGTGAGATAAG
∆2703923dfw	GGCCTGCGAGGCCGCTGTGGACGGTCTTAAC
∆2703923drv	GGCCTATTAGGCCCATTGCACGAGTCCATTC

Transformation

Deletion constructs were introduced in H4-8Δ*ku80*. Transformation was done as described (van Peer *et al.,* 2009). $1 \cdot 10^7$ protoplasts were incubated with 20 µg vector DNA and regenerated overnight without antibiotic. Selection took place for 4 days at 30 °C on MM plates containing 8 µg ml^{-1} nourseothricin. Transformants were transferred to a second selection plate containing 20 µg ml^{-1} phleomycin to distinguish between homologous and ectopic integrations. Gene deletion was confirmed by PCR using primers outside the flanks and inside the nourseothricin cassette. Primer pairs Δ2519514ufcfw/nourdelrev and Δ2519514dfcrv/sc3tersqf were used to screen for *tea1*

deletion (Table 1), while primer pairs c2h2D UFCFW/nourdelrev and c2h2D DFCRV/sc3tersqf were used to confirm *c2h2d* deletion (Table 1).

Biomass of colonies

Colonies were grown as liquid shaken cultures or on agar medium on a PC-membrane (diameter 76 mm, pore size 0.1 µm; Osmonics, GE Water Technologies). Mycelium of liquid cultures was separated from the medium using Miracloth filter (Merck Millipore, Billerica, USA). Mycelium was freeze-dried and weighed. Statistical analyses was done with an independent sample t-test (p-value ≤ 0.05) using IBM SPSS 20.

Whole genome expression analysis

The wild type dikaryon and strains Δ*wc-1*Δ*wc-1*, Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2*, Δ*fst4*Δ*fst4*, Δ*c2h2*Δ*c2h2*, Δ*fst3*Δ*fst3*, Δ*hom1*Δ*hom1*, Δ*bri1*Δ*bri1,* and Δ*gat1*Δ*gat1* were grown for 5 days in the dark at 22˚C, after which they were transferred to the light. Colonies (biological duplicates) were harvested at the moment the wild-type dikaryon formed aggregates (day 8) or fruiting bodies (day 12). H4-8 colonies were also harvested at the moment they were transferred to the light (day 5) and when they had formed primordia (day 10). Mycelium was frozen in liquid nitrogen and homogenized using the TissueLyser II (Qiagen, Düsseldorf, Germany). RNA was extracted using TriZol (Life technologies, Carlsbad, USA) and purified using the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany). Quality of RNA was checked using the BioAnalyzer and sent to ServiceXS (Leiden, the Netherlands) for Illumina Next Generation Sequencing.

RNA-Seq Analysis Pipeline

The RNA-Seq pipeline used the STAR aligner (Dobin *et al.,* 2013) to align the 100 bp paired end reads to the *S. commune* v3.0 genome (http://genome.jgi-psf.org/Schco3/Schco3.home.html). The size of introns was limited to a maximum of 1500 bp based on the largest intron sizes in the genome annotation.

Abundance estimation and differential expression were performed by Cufflinks version 2.1.1 (Trapnell *et al.,* 2012a), and Cuffdiff (Trapnell *et al.,* 2012b). Enrichments of GO terms were analysed within sets of differentially expressed genes. Proteins

annotated to contain a DNA-binding or regulatory protein domain were defined as transcription factors (Ohm *et al.,* 2010b).

RESULTS

Genes involved in mushroom development also control vegetative growth

Dikaryotic colonies of wild-type H4-8, Δ*wc-1*Δ*wc-1*, Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2*, Δ*bri1*Δ*bri1,* Δ*fst4*Δ*fst4*, Δ*c2h2*Δ*c2h2*, Δ*fst3*Δ*fst3*, Δ*hom1*Δ*hom1*, and Δ*gat1*Δ*gat1* were grown for 6 days on PC membranes on glucose MM in the dark. The Δ*hom2*Δ*hom2* strain formed 1.4 fold more biomass when compared to H4-8, while Δ*bri1*Δ*bri1* and Δ*hom1*Δ*hom1* formed 0.4 and 0.6 fold less biomass, respectively (Figure 1). Biomass formation of the other strains was not significantly different from H4-8. Strains Δ*hom2*Δ*hom2*Δ*fst3*Δ*fst3* and Δ*hom2*Δ*hom2*Δ*fst4*Δ*fst4* also formed more biomass than the wild-type. Biomass of both strains was similar to that of Δ*hom2*Δ*hom2* in the case of the agar cultures (Figure 2A). Notably, the Δ*hom2*Δ*hom2*Δ*fst4*Δ*fst4* strain formed 1.7 fold more biomass in liquid shaken cultures when compared to Δ*hom2*Δ*hom2* (Figure 2B). These data show that *bri1* and *hom1* stimulate vegetative growth, while *hom2* and *fst4* repress biomass formation of dikaryotic strains when glucose is used as a carbon source.

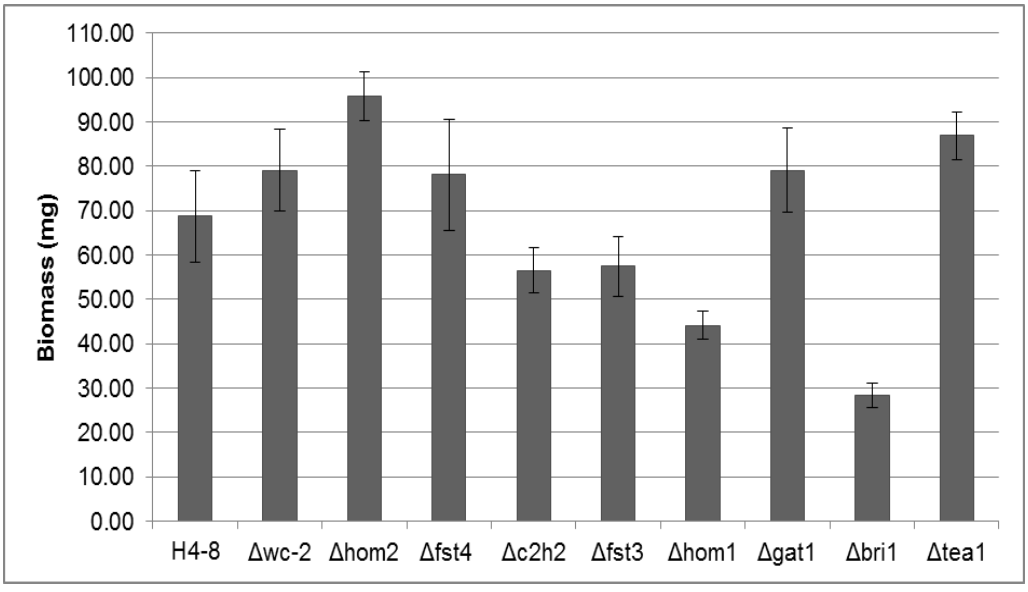

Figure 1 Biomass of 6-day-old dark-grown agar cultures of the wild-type dikaryon and transcription factor deletion strains using glucose as carbon source.

Biomass formation of Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2* and Δ*fst4*Δ*fst4* was assessed on xylose, pectin, and sucrose (Figure 3). The Δ*fst4*Δ*fst4* strain formed 2.8 fold more biomass on xylose when compared to H4-8 (Figure 3A). Moreover, Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2,* and Δ*fst4*Δ*fst4* formed more biomass on sucrose (1.2, 1.4, and 1.6 fold, respectively) and pectin (1.3, 1.4, and 1.6 fold, respectively) when compared to H4-8 (Figure 3B, C). Together, these data show that Wc-2, Hom2 and Fst4 repress vegetative growth on different carbon sources.

Reduced vegetative growth of the Δ*bri1*Δ*bri1* and Δ*hom1*Δ*hom1* strains may slow down fruiting body development. Therefore, fruiting was monitored after 7 days (Ohm *et al.,* 2011) and 15 days of culturing. The Δ*hom1*Δ*hom1* strain formed more but smaller mushrooms both after 7 days and 15 days (Figure 4). In contrast, Δ*bri1*Δ*bri1* had not formed fruiting bodies after 7 days but did so after 15 days showing that fruiting in this strain is delayed and not abolished as reported previously (Ohm *et al.,* 2011).

Genome-wide expression analysis

RNA composition of wild-type H4-8 was determined during vegetative growth in the dark, after transfer to the light, and during aggregate, primordium, and fruiting body formation. Expression of *wc-1*, *wc-2*, *hom2*, *fst4*, *fst3*, *gat1,* and *bri1* changed less than 2 fold during development when compared to the vegetative mycelium grown in

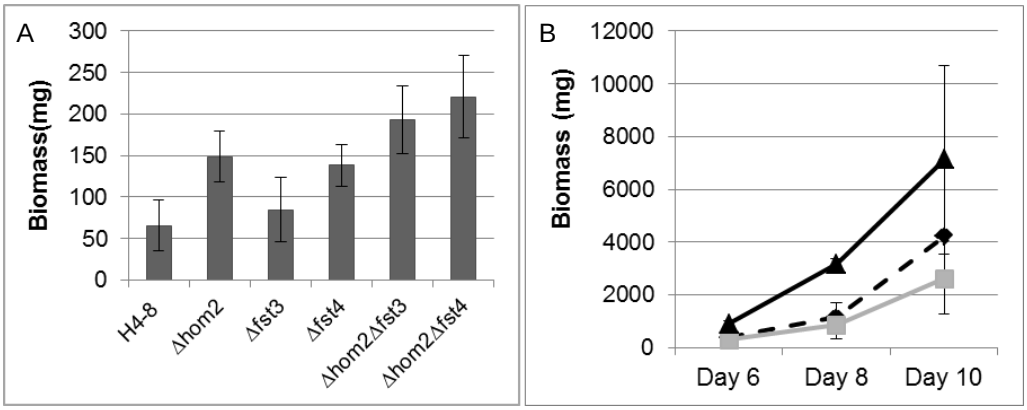

Figure 2 Biomass of 6-day-old dark-grown cultures of the wild-type dikaryon and transcription factor deletion strains that had been grown on agar medium (A) or as liquid shaken cultures (B) using glucose as a carbon source. Δ*hom2*Δ*fst4* (solid black), Δ*hom2* (dotted black), Δ*hom2*Δ*fst3* (solid light grey).

the dark (Table 2). In contrast, *c2h2* and *hom1* expression increased gradually with a maximum fold change of 4.7 and 2, respectively, during the fruiting body stage.

Expression profiles of wild type, Δ*wc-1*Δ*wc-1*, Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2*, Δ*fst4*Δ*fst4*, Δ*c2h2*Δ*c2h2*, Δ*fst3*Δ*fst3*, Δ*gat1*Δ*gat1*, Δ*hom1*Δ*hom1,* and Δ*bri1*Δ*bri1* strains were compared at the moment the wild-type formed aggregates (day 8) and fruiting bodies (day 12). Principal component analysis of the RNA profiles of 8-day-old colonies revealed a first and second component explaining 38 and 29% of the variation, respectively. Two distinct clusters were observed (Figure 5A). The first cluster consisted of Δ*wc-1*Δ*wc-1*, Δ*wc-2*Δ*wc-2,* and Δ*hom2*Δ*hom2* that are all affected in early stages of fruiting body development. The second cluster consisted of Δ*gat1*Δ*gat1* and Δ*fst3*Δ*fst3* that are affected in late stages of development. The other deletion strains did not cluster but rather showed a gradual change in expression. Principal component analysis of the RNA profiles of 12-day-old colonies revealed a first and second component explaining 72 and 7% of the variation, respectively. In this case, Δ*wc-1*Δ*wc-1*, Δ*wc-2*Δ*wc-2,* Δ*hom2*Δ*hom2,* and Δ*fst4*Δ*fst4* clustered, whereas the other strains clustered with the wild-type (Figure 5B).

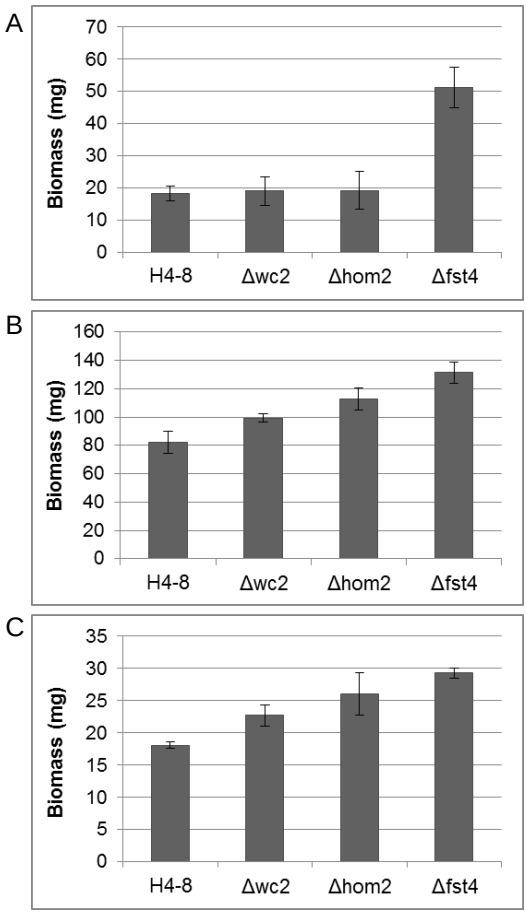

Figure 3 Biomass of 6-day-old dark-grown agar cultures of the wild-type dikaryon and transcription factor deletion strains using 4% xylose (A), 3.4% sucrose (B), and 1% pectin (C) as carbon source.

Table 2. Temporal expression of the blue light sensor gene *wc-1* and transcription factor genes involved in fruiting body development in the wild-type dikaryon. Values are expressed in FKPM. Green shaded boxes indicate a significant ≥2 fold up-regulation when compared to dark grown vegetative mycelium.

	Veg Myc	Veg Ind	Aggregate	Primordia	Fruiting body
wc-1	37.30	40.06	54.83	51.97	62.23
wc-2	38.29	46.19	39.88	61.66	47.47
hom2	107.64	135.84	115.99	104.15	58.63
tea1	9.39	14.73	18.88	62.40	10.48
fst4	112.88	159.43	140.70	178.65	119.08
c2h2	22.06	29.61	47.48	87.84	105.36
fst3	103.23	97.82	102.70	119.53	115.64
gat1	75.01	65.00	72.79	61.87	126.09
hom1	114.38	108.69	149.72	200.48	234.15
bri1	30.03	34.22	30.56	34.99	29.95
c2h2d	3.72	8.76	16.57	39.17	54.94

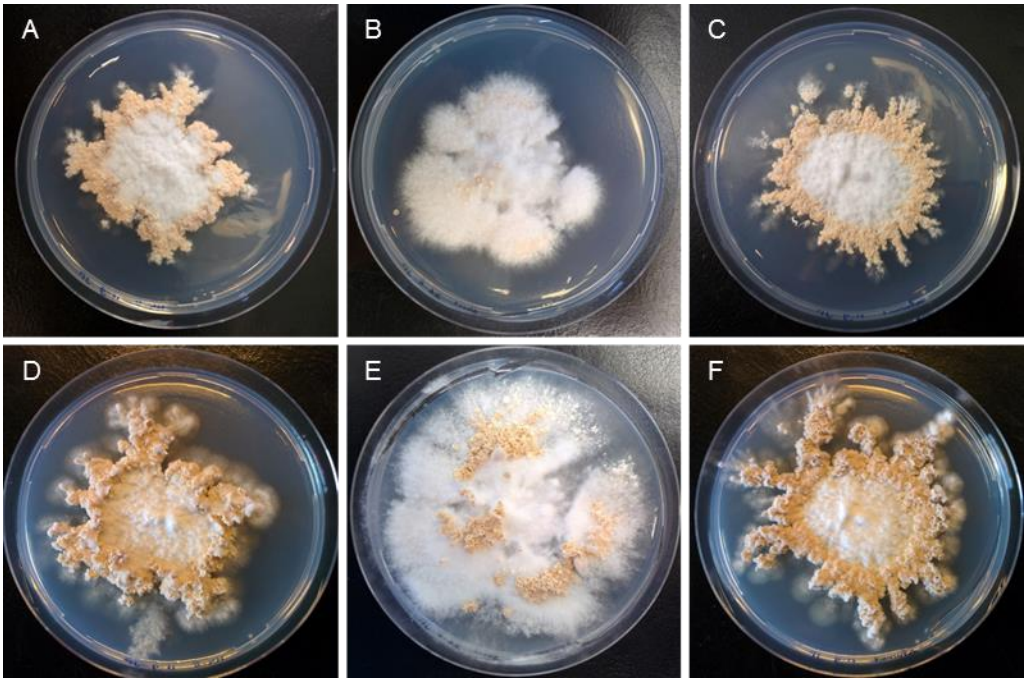

Figure 4. Fruiting body development of the wild-type dikaryon (A,D), Δ*bri1*Δ*bri1* (B, E), and Δ*hom1*Δ*hom1* (C, F) after 7 (A-C) and 15 (D-F) days of growth.

The number of up- and down-regulated genes were between 86 and 1392 and 131 and 1463, respectively, when expression of the 8- and 12-day-old cultures of the deletion strains was compared with the wild type (Table 3). Enriched functional categories in the up-regulated genes of 8-day-old colonies of Δ*wc-1*Δ*wc-1*, Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2,* and Δ*fst4*Δ*fst4* were mainly linked to carbohydrate metabolism, when compared to the 8-day-old wild-type colonies (Table 4). Down-regulated functional groups were linked to energy transfer in these strains. Functional groups involved in energy transfer and carbohydrate metabolism were up-regulated in 12-day-old colonies of Δ*wc-1*Δ*wc-1* and Δ*wc-2*Δ*wc-2* (Table 5). Down-regulated genes were enriched for nucleosome, catalytic and oxidoreductase activity for Δ*wc-1*Δ*wc-1* and Δ*wc-2*Δ*wc-2.* The functional group ATPase activity was also overrepresented in the down-regulated genes of Δ*wc-2*Δ*wc-2*. In 12-day-old colonies of Δ*hom2*Δ*hom2* functional groups involved in translation, energy transfer, and carbohydrate metabolism were overrepresented in the up-regulated genes, while functional groups involved in transport and energy transfer were overrepresented in the down-regulated genes. In 12-day-old colonies of Δ*fst4*Δ*fst4* functional groups involved in carbohydrate processes and energy transfer were enriched in the up-regulated genes. Groups involved in oxidoreductase activity, metabolic process and nucleosome, amongst others, were enriched in the down-regulated genes. Functional categories involved in energy transfer and carbohydrate metabolism were up-regulated in 8-day-old colonies of Δ*c2h2*Δ*c2h2*, while processes involved in cytoplasm, ATP binding, nucleosome and peptidase activity were enriched in the down-regulated genes. In 12-day-old colonies of Δ*c2h2*Δ*c2h2* up-regulated genes were enriched in functional groups related to energy transfer, carbohydrate metabolic process, and chitin catabolic process. Down-regulated genes were enriched in groups involved in cell wall, catalytic activity, tryptophan and fatty acid synthesis. No functional groups were overrepresented in the up-regulated genes in 8-day-old and 12-day-old colonies of Δ*hom1*Δ*hom1* but functional groups related to chitinase activity, carbohydrate metabolism and energy transfer were enriched in the down-regulated genes. 12-day-old Δ*hom1*Δ*hom1* colonies showed overrepresentation of functional groups related to amino acid synthesis, oxidoreductase, and carbohydrate metabolism activity in the down regulated genes. The functional categories that were enriched in the up-regulated genes of 8-day-old colonies of Δ*fst3*Δ*fst3* and Δ*gat1*Δ*gat1* were mainly

Table 3. Number of genes significantly up- and down-regulated compared to wildtype at the moment the wild-type had formed aggregates (AG) and fruiting bodies (FB).

	Up-regulated		Down-regulated	
	AG	FB	AG	FB
Δ*wc1*Δ*wc1*	415	1195	431	1298
Δ*wc2*Δ*wc2*	375	1392	500	1421
Δ*hom2*Δ*hom2*	494	1267	652	1462
Δ*fst4*Δ*fst4*	1267	1150	1226	1330
Δ*c2h2*Δ*c2h2*	668	306	644	327
Δ*fst3*Δ*fst3*	400	86	482	131
Δ*hom1*Δ*hom1*	194	317	247	400
Δ*gat1*Δ*gat1*	480	662	556	991

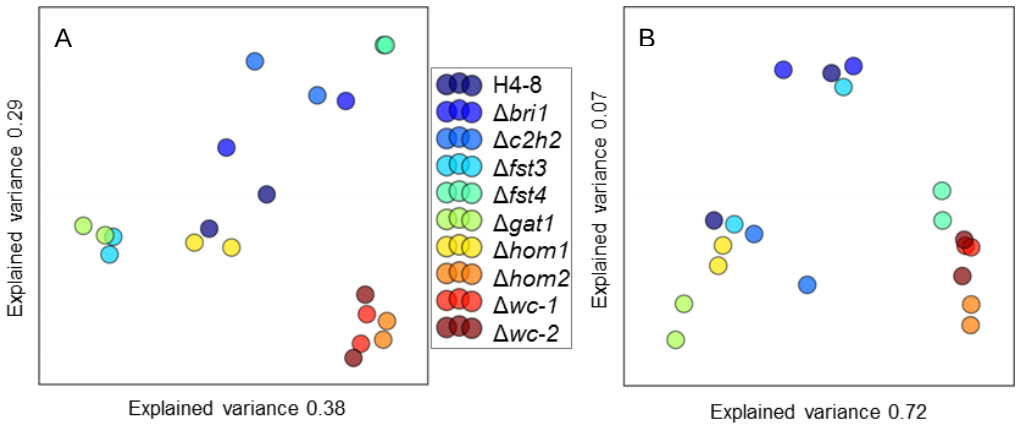

Figure 5 Principal component analysis of expression profiles of the deletion strains during aggregation (A) and fruiting body formation (B).

involved in energy transfer, tryptophan metabolism, and cell wall processes. Genes involved in energy transfer and carbohydrate metabolism were enriched in the down-regulated genes. Groups involved in carbohydrate metabolism, methyltransferase, and leucine synthesis were enriched in the up-regulated genes in 12-day-old colonies of

Δ*fst3*Δ*fst3*. Down-regulated genes were mainly enriched in cell wall processes and chitinase activity. Functional groups were up-regulated for sphingolipid metabolism in 12-day-old colonies of Δ*gat1*Δ*gat1*. Down-regulated genes were mainly enriched for translation and cell wall. No functional groups were overrepresented in the up-regulated genes of 8-day-old and 12-day-old colonies of the Δ*bri1*Δ*bri1* strain. In contrast, functional groups related to metabolic process, carbohydrate metabolism, transcription and cell wall were overrepresented in the down-regulated genes of 8-day-old colonies. Down-regulated genes in 12-day-old Δ*bri1*Δ*bri1* colonies were mainly enriched for carbohydrate metabolism, hydrolase activity and transcription repressor activity.

Between 1 and 42 transcription factor genes were exclusively ≥ 2 fold differentially expressed in one of the deletion strains when compared to the wild-type (Figure 6). Expression of *hom1* and *c2h2* had decreased in 12-day-old Δ*wc-1*Δ*wc-1* colonies. In 12-day-old colonies of Δ*wc-2*Δ*wc-2 hom2* expression was increased, while *c2h2* was down-regulated. Expression of *c2h2*, *gat1,* and *hom1* was down-regulated in 12-day-old Δ*hom2*Δ*hom2* colonies. In 8-day-old Δ*fst4*Δ*fst4* colonies expression of *hom1* was down-regulated and in 12-day-old colonies *hom2* expression increased, while *c2h2* and *hom1* expression decreased. In contrast, *hom1* levels were up-regulated in 8-day-old Δ*fst3*Δ*fst3* colonies. Similarly, expression of *hom1* and additionally *c2h2* was increased in 8-day-old colonies of Δ*gat1*Δ*gat1*. In 8-day-old Δ*bri1*Δ*bri1* colonies *gat1* expression was increased, while *hom2* was increased in 12-day-old colonies (Figure 7).

Gene tea1 is involved in fruiting body development and represses vegetative growth

Transcription factor gene *tea1* (protein ID 2519514) showed ≥ 2 fold decreased expression in Δ*wc-1*Δ*wc-1*, Δ*wc-2*Δ*wc-2,* Δ*hom2*Δ*hom2,* Δ*briI*Δ*briI,* and Δ*fst4*Δ*fst4* when compared to the wild-type during aggregation (8-day-old colonies), while it was up-regulated ≥ 2 fold in Δ*fst3*Δ*fst3*, Δ*hom1*Δ*hom1,* and Δ*gat1*Δ*gat1.* A ≥ 2 fold differential expression of *tea1* was not observed when the wild-type had formed fruiting bodies (12-day-old colonies), explained by reduced expression of *tea1* in 12-day-old wild-type colonies. Gene *c2h2d* (proteinID 2703923) was also down-regulated in Δ*wc-1*Δ*wc-1*, Δ*wc-2*Δ*wc-2,* Δ*hom2*Δ*hom2,* and Δ*fst4*Δ*fst4*, while it was up-regulated

≥2 fold in Δ*gat1*Δ*gat1* during aggregation (Figure 7). Furthermore, expression of *c2h2d* was decreased >2 fold in 12-day-old colonies of Δ*wc-1*Δ*wc-1*, Δ*wc-2*Δ*wc-2,* Δ*hom2*Δ*hom2,* Δ*fst4*Δ*fst4*, Δ*fst3*Δ*fst3,* and Δ*gat1*Δ*gat1*. Gene *c2h2d* has a predicted C2H2 DNA binding domain and *tea1* is a predicted TEA/ATTS transcription factor. Expression of *tea1* and *c2h2* in the wild-type strain peaked during primordia and fruiting body formation, respectively (Table 2).

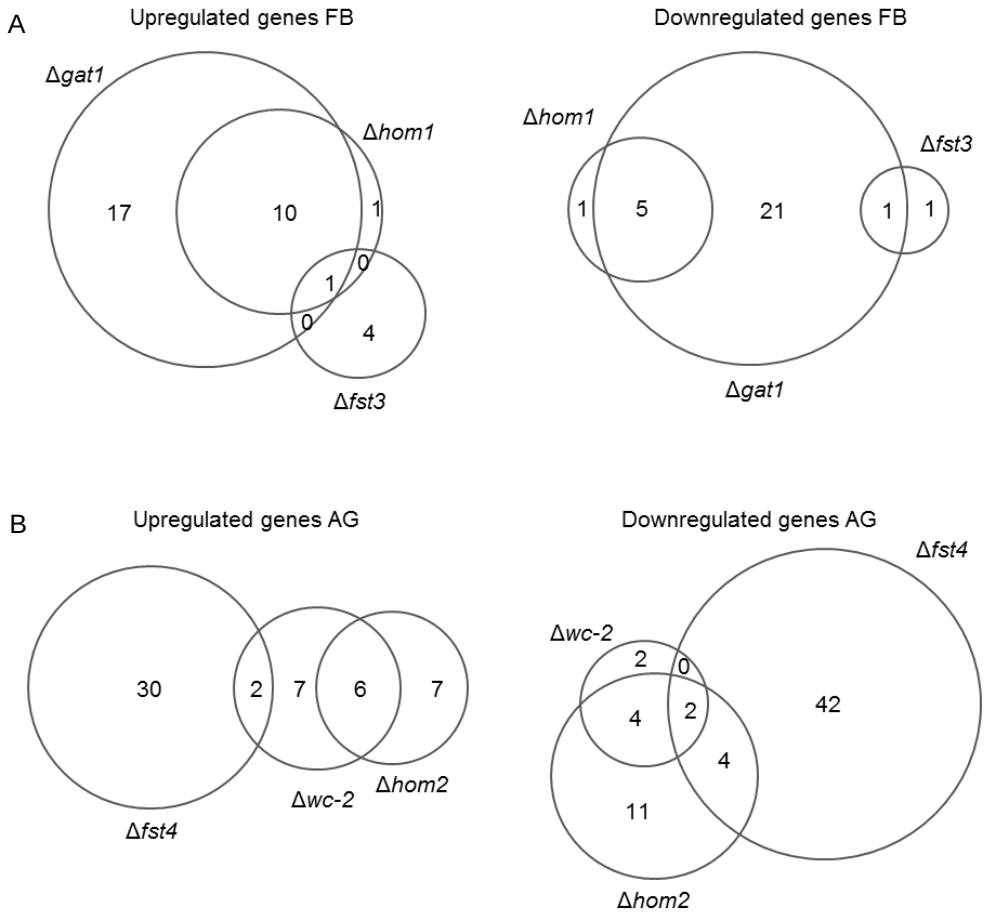

Figure 6 Venn diagrams showing overlapping differentially expressed transcription factor genes in Δ*fst3*Δ*fst3,* Δ*hom1*Δ*hom1,* and Δ*gat1*Δ*gat1* (A) and Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2,* and Δ*fst4*Δ*fst4* (B) when compared to the wild-type aggregate and fruiting body stage, respectively.

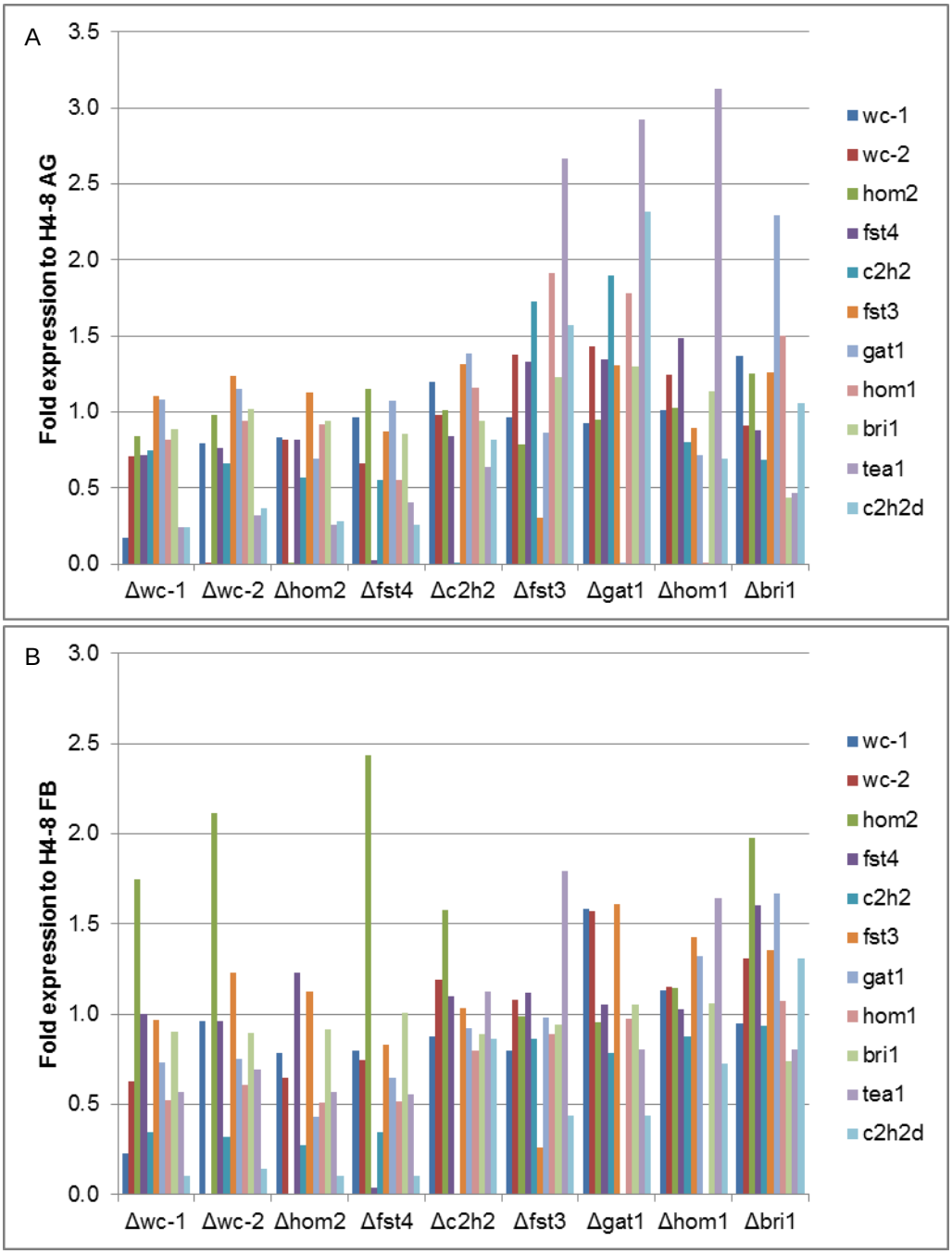

Figure 7 Expression of *wc-1*, *wc-2*, *hom2*, *fst4*, *c2h2*, *fst3*, *gat1*, *hom1*, *bri1*, *tea1,* and *c2h2d* in dikaryotic transcription factor deletion strains when compared to the wild-type during aggregation (A) and fruiting body formation (B).

Deletion constructs were made for *c2h2d* and *tea1.* No deletion strains were obtained for *c2h2d* after screening 100 transformants. In contrast, deletion of *tea1* in the H4-*8*Δ*ku80 was* successful. PCR analysis confirmed inactivation of *tea1* in one of the transformants. The transformant was crossed with the compatible wild-type H4-8b and siblings were selected with a deleted *tea1* gene and an intact *ku80* gene. One of these siblings was crossed with a H4-8 strain to obtain a Δ*tea1* strain with compatible mating types. These monokaryons were then crossed to obtain the Δ*tea1*Δ*tea1* strain.

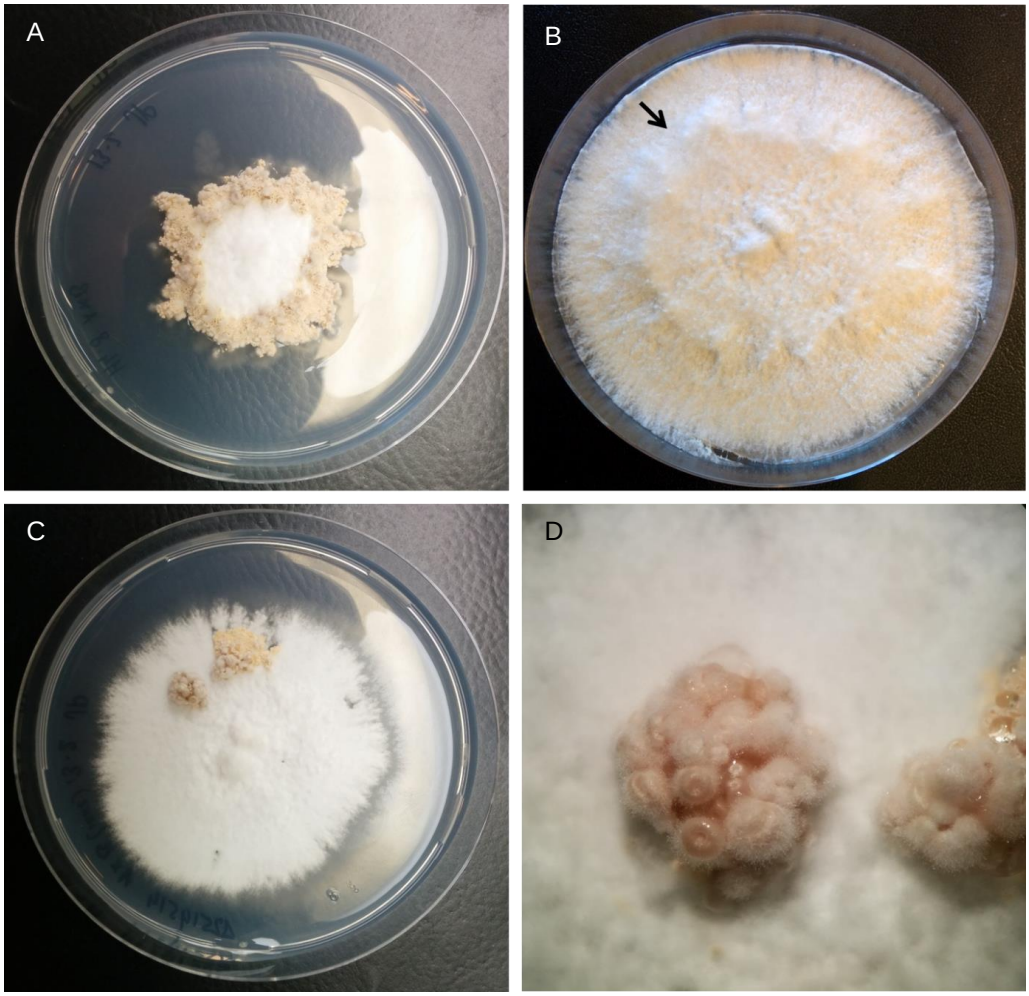

Figure 8 Aerial growth of dikaryotic colonies of H4-8 (A) and Δ*tea1* (B, C, D). Δ*tea1* produced less dense and high aerial hyphae when transferred to light (B) and only forms small clusters of fruiting bodies (C and D). Arrow indicates the transition from dense aerial hyphae production to thinner aerial hyphae production upon light induction.

The Δ*tea1*Δ*tea1* strain showed a 1.3 fold increase in biomass when compared to the wild-type. This increase was similar to that of Δ*hom2*Δ*hom2* (Figure 1). Transfer to the light did not induce irregular vegetative growth as observed in the wild-type. Interestingly, newly formed light-exposed mycelium did not produce aerial hyphae (Figure 8B). This resulted in a distinct border between the dark-grown mycelium and mycelium grown in the light. Fruiting body formation was almost completely abolished. Instead of the typical ring of fruiting bodies, only local clusters of fully-developed mushrooms were formed (Figure 8C, D). These clusters were made at random positions in the colony; they were not restricted to the center, or the periphery. Together, these data show that *tea1* represses vegetative growth and promotes fruiting body formation in the wild-type dikaryon.

DISCUSSION

The transcription factor genes *wc-2*, *hom2*, *fst4*, *briI, c2h2*, *fst3*, *gat1*, and *hom1* have been reported to be involved in fruiting body formation in *S. commune* (Ohm *et al.,* 2010b; 2011; 2013). We here showed that the homeodomain domain protein Hom2, the zinc finger transcription factor Fst4 and the blue light complex transcription factor Wc-2 are not only involved in early stages of fruiting body development but also repress vegetative growth. In contrast, the DNA binding BRIGHT domain protein BriI and the homeodomain protein Hom1 stimulated vegetative growth. The latter protein is involved in late stages of mushroom development, while BriI was shown not to be essential for fruiting. Based on phenotypic analysis, whole genome expression analysis of the deletion strains, and inactivation of gene *tea1* we propose a modified model for mushroom development (Figure 9).

The Δ*bri1*Δ*bri1* strain does not fruit after the standard growth period of 10 days (Ohm *et al.,* 2011). However, we here showed that fully developed mushrooms had formed after 4 weeks. This shows that BriI is not required for fruiting. Delayed mushroom development may be the result of reduced growth speed. Lower biomass formation may well be explained by the fact that functional categories metabolic process, carbohydrate metabolism, catalytic activity, transcription, and cell wall are down-regulated in the deletion strain in 8-day-old colonies. 12-day-old colonies showed down-regulation of functional categories carbohydrate metabolism, hydrolase activity, and transcription repressor activity. As a consequence of reduced biomass

formation, a quorum sensing pathway may become activated at a later moment delaying the switch to fruiting body formation (Wösten and Willey, 2000). Notably, BriI deletion has an effect on expression of *tea1* and of *gat1.* The repression of *tea1* may be a direct or an indirect effect due to its stimulatory effect on *gat1* expression. Together, BriI stimulates vegetative growth but has no role in mushroom formation except for its effect on *tea1* and *gat1* expression (Figure 9).

Genes *hom2* and *wc-2* are involved in the switch from vegetative growth to fruiting. Inactivation of these genes abolishes early stages of fruiting body formation (Ohm *et al.,* 2011; 2013) but also increased the vegetative growth rate. Strain Δ*wc-2*Δ*wc-2* formed more biomass on sucrose and pectin when compared to the wild-type, while Δ*hom2*Δ*hom2* formed more biomass on glucose, sucrose, and pectin. Expression of *hom2* and *wc-2* is rather constant during development, suggesting post-transcriptional regulation of these genes. In the case of Wc-2, this may be accomplished by the interaction with the blue light sensor Wc-1 (Ohm *et al.,* 2013). Preliminary results indicate that Hom2 activity is regulated by phosphorylation of its Pka sites (Chapter 4). Increased growth rate was associated with enrichment of up-regulated genes associated to carbohydrate metabolism in 8-day-old colonies, and of functional groups involved in carbohydrate metabolism and energy transfer in 12-day old colonies. Deletion of *wc-2* and *hom2* resulted in a ≥ 2 fold down-regulation of *c2h2* in 12-day-old colonies. Down-regulation was also observed in 8-day-old colonies, although the effect was less pronounced. Together, this confirms that Wc-2 and Hom2 stimulate *c2h2* expression (Ohm *et al.,* 2011; 2013; Figure 9).

Gene *fst4* is constitutively expressed during the *S. commune* life cycle. Like Hom2 and Wc-2 it is involved in the switch from vegetative growth to fruiting. Strain Δ*fst4*Δ*fst4* grows irregular in the light like the wild-type but does not aggregate. It formed more biomass than the wild-type on xylose, sucrose, and pectin but not on glucose. This indicates that Fst4 and Hom2 represent different parts of the repression pathway of vegetative growth. In liquid shaken cultures with glucose as carbon source these pathways may merge explaining why Δ*hom2*Δ*hom2*Δ*fst4*Δ*fst4* formed more biomass than Δ*hom2*Δ*hom2*. Strain Δ*fst4*Δ*fst4* showed enrichment of carbohydrate metabolism in the up-regulated genes of 8-day-old and 12-day-old-colonies similar to that observed in Δ*wc-2*Δ*wc-2* and Δ*hom2*Δ*hom2*. The fact that *fst4* expression is not affected in Δ*wc-2*Δ*wc-2* and Δ*hom2*Δ*hom2* strengthens the hypothesis that Fst4 and

Hom2 represent different pathways. Gene *fst4* stimulates *c2h2* like *hom2* and *wc-2* do. This indicates that Fst4, Hom2, and Wc-2 input are channeled into the fruiting pathway via *c2h2* (Figure 9B).

Transcription factor gene *tea1* was down-regulated in Δ*wc-1*Δ*wc-1*, Δ*wc-2*Δ*wc-2,* Δ*hom2*Δ*hom2,* Δ*brlI*Δ*brlI,* and Δ*fst4*Δ*fst4* when compared to the aggregating wild-type (8-day-old colonies), while it was up-regulated in Δ*fst3*Δ*fst3*, Δ*hom1*Δ*hom1,* and Δ*gat1*Δ*gat1.* This indicates it is up-regulated during early stages of development while it is repressed during late stages of mushroom formation. This agrees with the expression profile in the wild-type. The Δ*tea1*Δ*tea1* strain formed more biomass on glucose when compared to the wild-type. Moreover, it was severely affected in mushroom formation. Only local clusters of fully developed mushrooms were formed in the deletion strain. This phenotype may be explained by a reduced sensitivity of a signaling pathway leading to a developmental switch from “off” to “on”.

Expression of *c2h2* increased >2 fold when RNA profiles of 5- and 8-day-old colonies were compared. The increased expression during the aggregation stage and further increased expression in primordia and fruiting bodies agrees with the phenotype of Δ*c2h2*Δ*c2h2* forming aggregates but not primordia and fruiting bodies (Ohm *et al.,* 2011; Figure 9). C2H2 did not affect biomass formation implying that it is downstream of the switch between vegetative growth and mushroom development.

Deletion of *fst3*, *gat1* or *hom1* results in more, but smaller mushrooms (Ohm *et al.,* 2011). In addition, Hom1 and Gat1 are involved in mushroom tissue formation (Ohm *et al.,* 2011). Gene *fst3* is constitutively expressed and its expression is not affected by any of the other transcriptional regulators. This suggests that Fst3 is subject to post-transcriptional regulation. The fact that a higher number of genes are differentially expressed in 8-day-old colonies (wild-type forming aggregates) when compared to 12-day-old colonies (wild-type forming fruiting bodies) (i.e. 882 and 217 genes, respectively) suggest that Fst3 exerts its effect already early in mushroom development. Up-regulated functional groups were involved in energy transfer and cell wall processes in 8-day-old colonies, while down-regulated functional groups were involved in carbohydrate metabolism. In 12-day-old colonies these groups were regulated in the opposite direction. Expression of *gat1* was highest during fruiting body formation. It is repressed by Bri1 in 8-day-old colonies, while it is activated by Hom2 during fruiting body formation. Colonies of 8 days old were enriched for groups

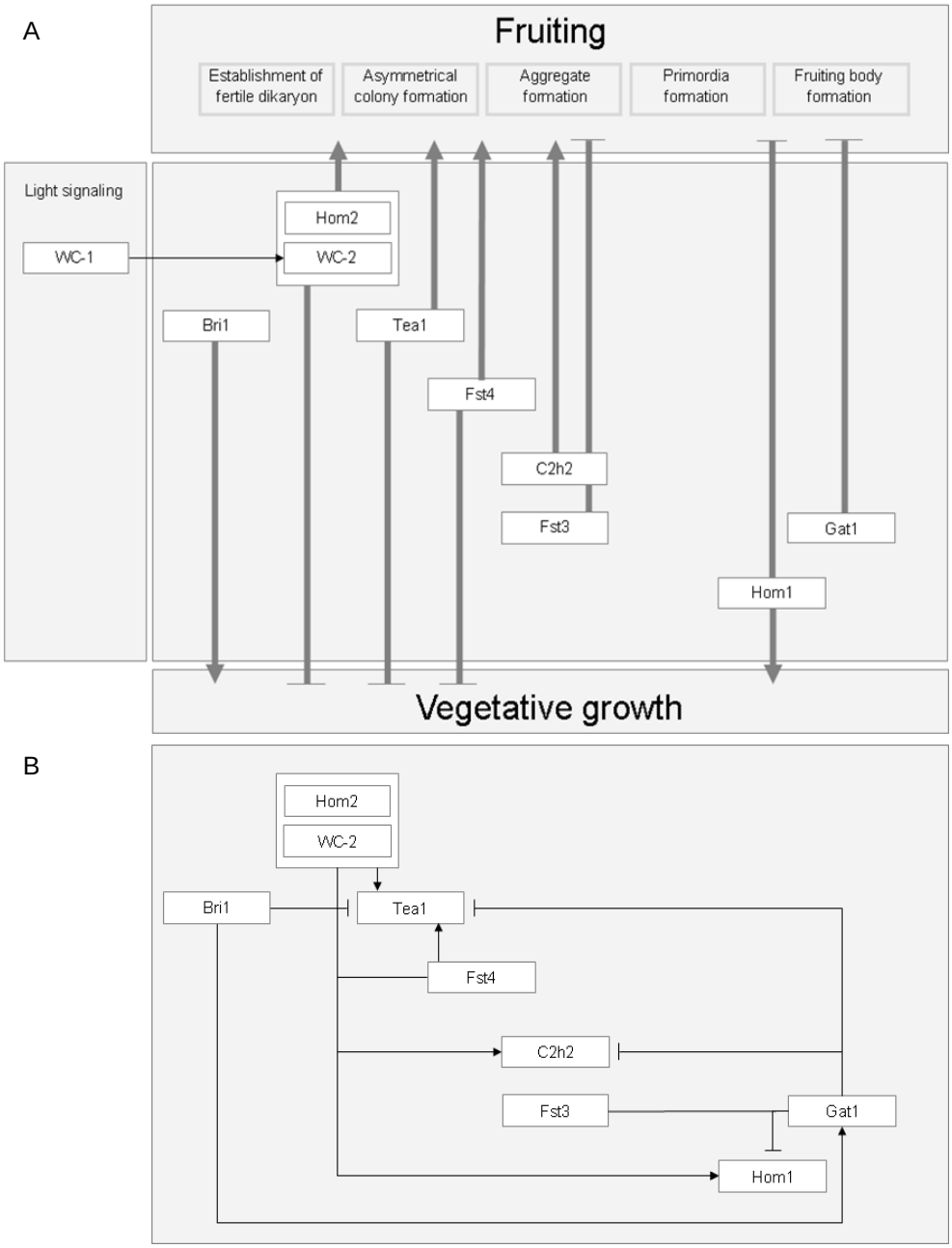

Figure 9 Regulatory model of activation and repression of vegetative growth and initiation and maturation of fruiting body formation in *S. commune*. Transcription factor genes control both vegetative growth and fruiting body development (A) and influence each other's expression levels (B).

involved in energy transfer and cell wall processes in up-regulated genes, while groups involved in carbohydrate metabolism were down-regulated. Groups involved in translation and cell wall were enriched in down-regulated genes in 12-day-old colonies. Expression of *hom1* gradually increases upon progression of fruiting. Expression analysis showed that Wc-1, Hom2 and Fst4, and probably Wc-2, stimulate expression of Hom1. Notably, Hom1 stimulates formation of biomass of the vegetative mycelium. This suggests that Hom1 operates at two distinct stages of development (Figure 9). It may well be that Hom1 functions in mushroom formation by stimulation of biomass formation. This would explain the reduced size of the fruiting bodies in Δ*hom1*Δ*hom1*. Notably, both Fst3 and Gat1 repress *hom1* expression. Functional groups involved in chitinase activity, carbohydrate metabolism, and energy transfer were enriched for down-regulated genes in 8-day-old colonies. Functional groups involved in amino acid synthesis were enriched for down-regulated genes in 12-day-old colonies.

The model of development of *S. commune* may apply to mushroom forming basidiomycetes as well. Hom2, Hom1, Fst3, Fst4, C2H2, and Gat1 are basidiomycete-specific regulatory proteins (Todd *et al.,* 2014). Homologues of these genes were identified in *L. bicolor* and *A. bisporus* (Ohm *et al.,* 2010b; Morin *et al.,* 2013). The homologues for *fst4*, *fst3*, *c2h2* and *hom1* were up-regulated during sexual development in two *A. bisporus* varieties (Morin *et al.,* 2013), while homologues of *hom2*, *fst4*, *c2h2*, *fst3*, *gat1* and *hom1* showed similar expression profiles in *L. bicolor* when compared to *S. commune* (Morin *et al.,* 2013). Expression patterns of *c2h2*, *fst3*, *hom1* and *gat1* were also found to be similar in *C. cinerea* (Plaza *et al.,* 2014).

This is the first time a direct link has been shown between repression of vegetative growth and induction of sexual reproduction. Previously, a link has been shown between vegetative growth and asexual development in *Aspergillus* (Krijgsheld *et al.,* 2013). This link involves trimeric G-protein signaling. The activity of the Gα-subunit of *Aspergillus* is regulated by the FlbA protein (Yu *et al.,* 1996). Inactivation of this gene results in a strain that cannot initiate asexual development. Notably, *S. commune* has a homologue of *flbA* called *thn.* Inactivation of this gene results in a strain unable to form fruiting bodies (Fowler and Mitton, 2000). This suggests that similar signaling pathways are involved in the decision to stop vegetative growth and to invest in reproduction in ascomycetes and basidiomycetes.

ACKNOWLEDGEMENTS

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Table 4. Enrichment of GO terms in up- and down-regulated genes of 8-day-old colonies of Δ*wc-1*Δ*wc-1* and transcription factor deletion strains Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2*, Δ*fst4*Δ*fst4*, Δ*c2h2*Δ*c2h2,* Δ*fst3*Δ*fst3,* Δ*hom1*Δ*hom1*, Δ*gat1*Δ*gat1,* and Δ*brlI*Δ*brlI* when compared to the aggregating wild-type strain.

Δwc-1Δwc-1

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0005975	carbohydrate metabolic process	0004497	oxidoreductase activity
0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	0015171	monooxygenase activity
0008643	carbohydrate transport	0006865	amino acid transmembrane transporter activity
0008643	L-arabinose isomerase activity	0016491	electron transport
0005351	sugar:hydrogen symporter activity	0005618	amino acid transport
0016810	hydrolase activity, acting on carbon-nitrogen (but not peptide)	0006118	metabolic process
0005506	iron ion binding	0005199	glutathione transferase activity
0016614	oxidoreductase activity, acting on CH-OH group of donors	0004194	structural constituent of cell wall
0009082	branched chain family amino acid biosynthetic process	0006810	cell wall
0008812	choline dehydrogenase activity		

Δwc-2Δwc-2

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0005975	carbohydrate metabolic process	0016491	oxidoreductase activity
0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	0004497	monooxygenase activity
0005506	iron ion binding	0043039	amino acid transmembrane transporter activity
0004497	monooxygenase activity	0006118	electron transport
0050381	unspecific monooxygenase activity	0006865	amino acid transport

0020037	heme binding	0008152	metabolic process
0016614	oxidoreductase activity, acting on CH-OH group of donors	0004364	glutathione transferase activity
0008812	choline dehydrogenase activity	0005199	structural constituent of cell wall
0009082	branched chain family amino acid biosynthetic process	0005618	cell wall
0006066	alcohol metabolic process	0050162	oxalate oxidase activity

Δhom2Δhom2

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0005975	carbohydrate metabolic process	0004497	monooxygenase activity
0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	0006810	transport
0030246	carbohydrate binding	0020037	heme binding
0016491	oxidoreductase activity	0006118	electron transport
0003824	catalytic activity	0005215	transporter activity
0005215	transporter activity	0050381	unspecific monooxygenase activity
0008152	metabolic process	0005506	iron ion binding
0016021	integral to membrane	0015171	amino acid transmembrane transporter activity
0008422	beta-glucosidase activity	0006865	amino acid transport
0006118	electron transport	0016614	oxidoreductase activity, acting on CH-OH group of donors

Δfst4Δfst4

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0005975	carbohydrate metabolic process	0005524	ATP binding
0008733	L-arabinose isomerase activity	0003677	DNA binding

0005351	sugar:hydrogen symporter activity	0000166	nucleotide binding
0008643	carbohydrate transport	0008152	metabolic process
0005215	transporter activity	0005737	cytoplasm
0006810	transport	0017111	nucleoside-triphosphatase activity
0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	0016491	oxidoreductase activity
0016021	integral to membrane	0003824	catalytic activity
0030246	carbohydrate binding	0016887	ATPase activity
0015171	amino acid transmembrane transporter activity	0005643	nuclear pore

Δc2h2Δc2h2

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0005506	iron ion binding	0005737	cytoplasm
0004497	monooxygenase activity	0005524	ATP binding
0006118	electron transport	0008152	metabolic process
0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	0003824	catalytic activity
0020037	heme binding	0016491	oxidoreductase activity
0008733	L-arabinose isomerase activity	0044267	cellular protein metabolic process
0050381	unspecific monooxygenase activity	0000786	nucleosome
0005351	sugar:hydrogen symporter activity	0006334	nucleosome assembly
0050660	FAD binding	0004298	threonine endopeptidase activity
0008643	carbohydrate transport	0004299	proteasome endopeptidase activity

Δfst3Δfst3

Up-regulated genes in mutant strain	Down-regulated genes in mutant strain

GO term	Functional annotation	GO term	Functional annotation
0005199	structural constituent of cell wall	0005506	iron ion binding
0005618	cell wall	0004497	monooxygenase activity
0016491	oxidoreductase activity	0020037	heme binding
0008152	metabolic process	0005975	carbohydrate metabolic process
0050660	FAD binding	0006118	electron transport
0016614	oxidoreductase activity, acting on CH-OH group of donors	0004553	hydrolase activity, hydrolyzing O-glycosyl compounds
0033754	indoleamine 2,3-dioxygenase activity	0050381	unspecific monooxygenase activity
0016717	oxidoreductase activity, acting on paired donors, with oxidation	0004568	chitinase activity
0020037	heme binding	0008843	endochitinase activity
0005215	transporter activity	0006032	chitin catabolic process

Δhom1Δhom1

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
	NONE	0004568	chitinase activity
		0008843	endochitinase activity
		0004497	monooxygenase activity
		0016491	oxidoreductase activity
		0005975	carbohydrate metabolic process
		0004553	hydrolase activity, hydrolyzing O-glycosyl compounds
		0008115	sarcosine oxidase activity
		0006118	electron transport
		0006032	chitin catabolic process
		0006725	aromatic compound metabolic process

Δgat1Δgat1

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0005199	structural constituent of cell wall	0005975	carbohydrate metabolic process
0005618	cell wall	0005506	iron ion binding
0016491	oxidoreductase activity	0020037	heme binding
0008152	metabolic process	0006118	electron transport
0004497	monooxygenase activity	0004497	monooxygenase activity
0050660	FAD binding	0005524	ATP binding
0020037	heme binding	0004316	3-oxoacyl-[acyl-carrier-protein] reductase activity
0033754	indoleamine 2,3-dioxygenase activity	0004553	hydrolase activity, hydrolyzing O-glycosyl compounds
0016614	oxidoreductase activity, acting on CH-OH group of donors	0050381	unspecific monooxygenase activity
0006118	electron transport	0005215	transporter activity

Δbri1Δbri1

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
	NONE	0008152	metabolic process
		0005975	carbohydrate metabolic process
		0003824	catalytic activity
		0000786	nucleosome
		0005215	transporter activity
		0005199	structural constituent of cell wall
		0006334	nucleosome assembly

0005618	cell wall
0006810	transport
0016491	oxidoreductase activity

Table 5. Enrichment of GO terms in up- and down-regulated genes of 12-day-old colonies of Δ*wc-1*Δ*wc-1* and transcription factor deletion strains Δ*wc-2*Δ*wc-2*, Δ*hom2*Δ*hom2*, Δ*fst4*Δ*fst4*, Δ*c2h2*Δ*c2h2,* Δ*fst3*Δ*fst3,* Δ*hom1*Δ*hom1*, Δ*gat1*Δ*gat1,* and Δ*brlI*Δ*brlI* when compared to the fruiting wild-type strain.

Δwc-1Δwc-1

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0005506	iron ion binding	0008152	metabolic process
0020037	heme binding	0016491	oxidoreductase activity
0004497	monooxygenase activity	0000786	nucleosome
0006118	electron transport	0006334	nucleosome assembly
0050381	unspecific monooxygenase activity	0003824	catalytic activity
0005975	carbohydrate metabolic process	0006810	transport
0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	0016020	membrane
0005524	ATP binding	0004497	monooxygenase activity
0005215	transporter activity	0016021	integral to membrane
0016810	hydrolase activity, acting on carbon-nitrogen (but not peptide)	0006118	electron transport

Δwc-2Δwc-2

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation

0020037	heme binding	0000786	nucleosome
0005506	iron ion binding	0006334	nucleosome assembly
0004497	monooxygenase activity	0003677	DNA binding
0050381	unspecific monooxygenase activity	0003824	catalytic activity
0005975	carbohydrate metabolic process	0008152	metabolic process
0006118	electron transport	0006260	DNA replication
0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	0042624	ATPase activity, uncoupled
0005215	transporter activity	0016491	oxidoreductase activity
0005524	ATP binding	0042623	ATPase activity, coupled
0006032	chitin catabolic process	0008186	RNA-dependent ATPase activity

Δhom2Δhom2

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0003735	structural constituent of ribosome	0006810	transport
0005840	ribosome	0005215	transporter activity
0006412	translation	0008152	metabolic process
0005975	carbohydrate metabolic process	0016020	membrane
0005506	iron ion binding	0016021	integral to membrane
0020037	heme binding	0016491	oxidoreductase activity
0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	0003824	catalytic activity
0005622	intracellular	0006865	amino acid transport
0004497	monooxygenase activity	0015171	amino acid transmembrane transporter activity
0006118	electron transport	0004497	monooxygenase activity

Δfst4Δfst4

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0005975	carbohydrate metabolic process	0016491	oxidoreductase activity
0005215	transporter activity	0008152	metabolic process
0006508	proteolysis	0003824	catalytic activity
0005506	iron ion binding	0000786	nucleosome
0020037	heme binding	0030170	pyridoxal phosphate binding
0050381	unspecific monooxygenase activity	0006334	nucleosome assembly
0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	0006118	electron transport
0004497	monooxygenase activity	0006810	transport
0006118	electron transport	0008483	transaminase activity
0008236	serine-type peptidase activity	0005199	structural constituent of cell wall

Δc2h2Δc2h2

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0005506	iron ion binding	0003824	catalytic activity
0020037	heme binding	0008152	metabolic process
0004497	monooxygenase activity	0030170	pyridoxal phosphate binding
0006118	electron transport	0005199	structural constituent of cell wall
0050381	unspecic monooxygenase activity	0005618	cell wall
0016711	flavonoid 3'-monooxygenase activity	0005992	trehalose biosynthetic process
0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	0005488	binding
0005975	hydrolase activity, hydrolyzing O-glycosyl compounds	0004316	3-oxoacyl-[acyl-carrier-protein] reductase activity

0006032	carbohydrate metabolic process	0033754	indoleamine 2,3-dioxygenase activity
0005618	chitin catabolic process	0006164	purine nucleotide biosynthetic process

Δfst3Δfst3

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0004565	beta-galactosidase activity	0004568	chitinase activity
0009341	beta-galactosidase complex	0008843	endochitinase activity
0008168	methyltransferase activity	0005576	extracellular region
0005975	carbohydrate metabolic process	0004553	hydrolase activity, hydrolyzing O-glycosyl compounds
0009316	3-isopropylmalate dehydratase complex	0006032	chitin catabolic process
0003861	3-isopropylmalate dehydratase activity	0005199	structural constituent of cell wall
0004035	alkaline phosphatase activity	0005618	cell wall
0005385	zinc ion transmembrane transporter activity	0030246	carbohydrate binding
0006829	zinc ion transport	0050381	unspecific monooxygenase activity
0005315	inorganic phosphate transmembrane transporter activity	0005975	carbohydrate metabolic process

Δhom1Δhom1

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
	NONE	0003824	catalytic activity
		0016491	oxidoreductase activity
		0004316	3-oxoacyl-[acyl-carrier-protein] reductase activity
		0008152	metabolic process
		0006526	arginine biosynthetic process

		0016021	integral to membrane
		0003939	L-iditol 2-dehydrogenase activity
		0004932	mating-type factor pheromone receptor activity
		0003991	acetylglutamate kinase activity
		0004358	glutamate N-acetyltransferase activity

Δgat1Δgat1

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
0017059	serine C-palmitoyltransferase complex	0005840	ribosome
0004758	serine C-palmitoyltransferase activity	0003735	structural constituent of ribosome
		0006412	translation
		0005622	intracellular
		0005839	proteasome core complex
		0005199	structural constituent of cell wall
		0005618	cell wall
		0008152	metabolic process
		0006118	electron transport
		0006526	arginine biosynthetic process

Δbri1Δbri1

Up-regulated genes in mutant strain		Down-regulated genes in mutant strain	
GO term	Functional annotation	GO term	Functional annotation
	NONE	0005975	carbohydrate metabolic process
		0004553	hydrolase activity, hydrolyzing O-glycosyl compounds

0016491	oxidoreductase activity
0004364	glutathione transferase activity
0005618	cell wall
0003824	catalytic activity
0004568	chitinase activity
0005199	structural constituent of cell wall
0008843	endochitinase activity
0016564	transcription repressor activity

Chapter 3

CO_2 represses fruiting body formation in *Schizophyllum commune* through the cAMP pathway

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ABSTRACT

CO_2 has profound effects on functioning of organisms. Therefore, it is sensed throughout the tree of life, in most cases via the cAMP signaling pathway. Here it is shown that CO_2 represses all developmental stages of fruiting body formation in *S. commune*. Addition of cAMP in the medium mimicked high CO_2 conditions by repressing fruiting body development. On the other hand, overexpression of *pde2*, a gene encoding phosphodiesterase that degrades cAMP, resulted in fruiting body development even at elevated CO_2 levels. This effect was overcome by adding the Pde2 inhibitor IBMX to the medium. Whole genome expression analysis revealed that genes involved in energy production are overrepresented in the up-regulated genes under high CO_2 levels, while genes involved in carbohydrate metabolism were overrepresented in the down-regulated genes. Moreover, a set of transcription factor genes have been identified that are affected by CO_2 levels, among which *wc2*, *c2h2,* and *hom1* that were previously shown to be involved in fruiting body development.

INTRODUCTION

Mushrooms are the most conspicuous structures formed by fungi. Their formation is affected by environmental factors such as O_2 and CO_2 levels, light, temperature, humidity, volatiles, pH, salinity, and availability of nutrients (Madelin, 1956, Manachère, 1988; Kües and Liu, 2000; Eastwood *et al.,* 2013; Pelkmans *et al.,* 2016). Fruiting body development is often, if not always, the result of a combination of such environmental conditions (Pelkmans *et al.,* 2016). Formation of white button mushrooms by *Agaricus bisporus* is repressed by 1-octen-3-ol and high CO_2 levels, while low temperature stimulates mushroom formation. On the other hand, fruiting body development is responsive to blue light in *Schizophyllum commune*, while also in this case high CO_2 levels repress fruiting (Perkins, 1969; Raudaskoski and Yli-Mattila, 1985, Ohm *et al.,* 2013; Eastwood *et al.,* 2013). CO_2 signaling pathways have been unraveled, at least partly, in different life forms including fungi (Bahn and Mühlschlegel, 2006). CO_2 can freely diffuse across the membrane into cells. Carbonic anhydrase (CA) converts this molecule into the biologically active compound bicarbonate. CA increases the turnover of CO_2 10 million-fold (Mitchell, 2005). Bicarbonate induces active site closure of soluble adenylyl cyclase (AC) (Steegborn *et*

al., 2005). As a result, cyclic adenosine 3',5'-monophosphate (cAMP) is synthesized. This second messenger binds to the regulatory subunit of cAMP-dependent protein kinases (PKAs). Consequently, the two regulatory subunits are released from the catalytic subunits of the PKA complex enabling phosphorylation of target proteins. Phosphodiesterase (PDE) degrades cAMP by breaking its phosphodiester bond. As a result, PKA activity will be reduced. Low and high affinity PDEs have been identified. The high affinity PDEs generally maintain a basal level of cAMP, while low affinity PDEs regulate fluctuating levels of cAMP (Agarwal *et al.,* 2013).

S. commune produces monokaryotic basidiospores that, after germination, form a vegetative mycelium. This mycelium becomes fertile upon fusion with a partner with a compatible mating type. The resulting dikaryon initiates fruiting at low CO_2 conditions and exposure to blue light. Formation of fruiting bodies starts with a shift from regular concentric growth to irregular growth. In the next step, aerial hyphae form aggregates that differentiate into primordia. These primordia further develop into fruiting bodies with a hymenium in which basidia are formed. In the latter structure, karyogamy and meiosis occurs resulting in haploid basidiospores that can give rise to a new haploid mycelium. Here it is shown that high CO_2 levels repress all the developmental stages of fruiting body formation in *S. commune.* Moreover, evidence is presented that CO_2 is sensed via cAMP.

EXPERIMENTAL PROCEDURES

Culture conditions and strains

The compatible *S. commune* strains H4-8 (*mat*A43*mat*B41; FGSC 9210) (Ohm *et al.,* 2010b) and H4-8b (*mat*A41*mat*B43) (Ohm *et al.,* 2010a) were used. Colonies were grown at 25 °C on minimal medium (MM) containing 1% glucose and 1.5% agar (van Peer *et al.,* 2009a) either or not containing 2.5 mM cAMP (Sigma-Aldrich, St. Louis, USA) or 2.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich, St. Louis, USA). Agar cultures were inoculated with a point inoculum taken from the periphery of a 7 day-old colony. Strains were grown for 3 days in the dark and were either or not transferred to 3000 lux white light using CE Conrad LEDs (Conrad Electronic, Hirschau, Germany). High CO_2 levels were obtained by sealing the plates twice with Parafilm (Sigma-Aldrich, St. Louis, USA) and once with duct tape. Defined CO_2 conditions (30% CO_2) were created by mixing pressurized CO_2 with ambient air

through water. In this case colonies were grown on 20 ml agar medium in closed 100 ml bottles.

Overexpression construct of phosphodiesterase pde2

A 3.4 kb fragment containing the orthologue of high affinity *pde2* gene of *Saccharomyces cerevisiae* (ProteinID 2636760; http://genome.jgi-psf.org/Schco3) was amplified by PCR using Phusion Hot Start II High-Fidelity DNA polymerase (Thermo Fisher Scientific, Waltham, USA), primers CGTGCCAGATATGAAGG and GCAAGAAAGCCGAACAG, and H4-8 chromosomal DNA as template. The fragment contained 1.2 kb 5' flanking region, the coding region, and 150 bp 3' flanking sequence. The amplified product was inserted into pGEM-phleoB (Ohm *et al.,* 2011) that was cut with SwaI. The resulting construct was transformed to protoplasts of H4-8 spores using PEG transformation (van Peer *et al.,* 2009). Transformants were selected on MM plates containing 25 µg ml^{-1} phleomycin and transferred to non-selective plates for phenotypic analysis.

Whole genome expression analysis

RNA was isolated from colonies of the dikaryotic 4-8 strain that had been grown at 22 ˚C in open or sealed plates in the dark. Colonies were snap-frozen in liquid nitrogen and homogenized using the TissueLyser II (Qiagen, Düsseldorf, Germany). RNA was isolated using TriZol (Life technologies, Carlsbad, USA) and purified with the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany). RNA quality was checked by gel electrophoresis and sent to ServiceXS (Leiden, the Netherlands) for Illumina Next Generation Sequencing.

The RNA-Seq pipeline used STAR aligner (Dobin *et al.,* 2013) to align the 100 bp paired end reads to the *S. commune* v3.0 genome (http://genome.jgi-psf.org/Schco3). The size of introns was limited to a maximum length of 1500 bp, which is the largest intron size according to the genome annotation. Abundance estimation and differential expression were performed by Cufflinks (Trapnell *et al.,* 2012a) and Cuffdiff (Trapnell *et al.,* 2012b). GO term enrichments were analyzed using sets of differentially expressed genes. Proteins annotated with a DNA-binding or regulatory protein domain were defined as transcription factors (Ohm *et al.,* 2010b).

RESULTS

CO_2 represses all stages of fruiting body development

Colonies were exposed to 30% CO_2 at different stages of fruiting body development to assess at which moment CO_2 impacts development. To this end, the H4-8 dikaryon was grown in bottles at ambient CO_2 until aggregates, primordia or fruiting bodies had been formed. At these developmental stages the air in the bottle was flushed with an air mixture containing 30% CO_2. In all cases development was halted and the developmental structures were overgrown by aerial hyphae (data not shown).

cAMP represses fruiting body formation

Colonies were grown for 3 days in the dark and 4 days in the light in ambient air or high CO_2 on medium either or not containing 2.5 mM cAMP. Colonies grown in open plates failed to initiate fruiting in the presence of exogenous cAMP (Figure 1C). Growth of these colonies was similar to colonies grown in sealed plates in the

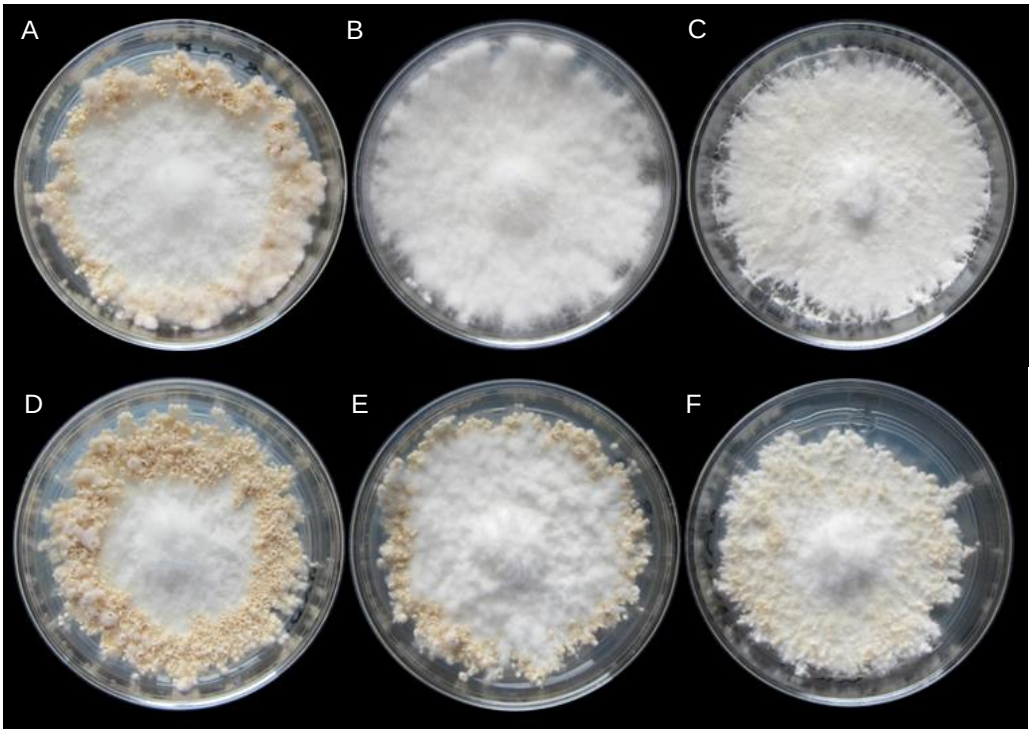

Figure 1 Dikaryon of H4-8 (A-C) and the *pde2pde2* overexpressor (D-F) grown for 7 days in ambient air (A, D), sealed plates (B, E), and in ambient air with 2.5 mM cAMP added to the medium (C, F).

absence of cAMP (Figure 1B). These results suggest that cAMP functions in CO_2 sensing.

S. commune contains all genes of the cAMP signaling pathway

Components of the cAMP signaling pathway are highly conserved in the eukaryotes. CA converts CO_2 to bicarbonate, AC and PDE regulate cAMP levels, while PKA functions as the effector protein of this second messenger. The *S. commune* genome contains two predicted CA genes, *sca1* (protein ID 2605577) and *sca2* (protein ID 2611292). The former gene is the orthologue of the single *S. cerevisae* CA gene *nce103* as indicated by bidirectional BLAST (Table 1). Gene *sca2* does not have an orthologue in *S. cerevisiae*, but it does have one in for instance *Coprinopsis cinerea* (65.9% identity to protein ID 1462 [http://genome.jgi.doe.gov/Copci1]). WoLF PSORT (Horton *et al.,* 2007) predicted that Sca1 is transported to mitochondria, while Sca2 would remain in the cytosol. The *S. commune* genome contains a single AC gene, *sac1* (protein ID 2510338), that has a bidirectional hit with the *S. cerevisae* AC gene *cyr1* with predicted nuclear localization (Table 1). Furthermore, it contains two predicted PDE genes that have bidirectional hits with the two *pde* genes of *S. cerevisae*. Genes *pde1* (protein ID 2607053) and *pde2* (protein ID 2636760) are homologous to the low-affinity and the high-affinity yeast genes *pde1* and *pde2,* respectively. Pde1 of *S. commune* has a predicted nuclear localization, while Pde2 would reside in mitochondria. A single regulatory PKA unit, *pkr1* (protein ID 2687768), was found in *S. commune*. This gene is the predicted ortologue of *bcy1 of S. cerevisae* and its protein has a predicted nuclear localization. The catalytic PKA subunit encoding genes *pkac1, pkac2* and *pkac3* (protein ID 2484274, 2630814 (Yamagishi *et al.,* 2004), and 2201031, respectively) were identified in the *S. commune* genome. Their encoded proteins have a predicted mitochondrial, nuclear, and cytosolic localization, respectively. Gene *pkac1* shows 49.5%, 46.9% and 49.1% identity to yeast *tpk1*, *tpk2* and *tpk3,* respectively, while *pkac2* shows 63.3%, 63.2% and 63.3% identity to respectively *tpk1*, *tpk2* and *tpk3*. Gene *pkac3* shows 36.2% identity to both *tpk1* and *tpk3*. Thus, *tpk1*, *tpk2,* and *tpk3* show highest identity with *pkac2* and the second highest hit for these genes is *pkac1*.

Tabel 1. Components of the cAMP-PKA pathway in *S. commune*, their yeast orthologues, and the highest probable localization as predicted by WoLF PSORT (Horton *et al.,* 2007).

Gene	Function	*S. cerevisiae* Homologue (bidirectional hit)	Identity (%)	Predicted localization	Number of nearest neighbors
sca1	Carbonic anhydrase	*nce103*	46.4	Mitochondria Cytoplasm	24 1.5
sca2	Carbonic anhydrase	none		Cytoplasm Cyto/Nucleus Nucleus	17 13 5
sac1	Adenylyl cyclase	*cyr1*	40.7	Nucleus Cyto/Nucleus Cytoplasm	10.5 9.3 7
pde1	3'5'-cyclic nucleotide phosphodiesterase	*pde1*	36.5	Cyto/Nucleus Nucleus Cytoplasm	14 12.5 12.5
pde2	3'5'-cyclic nucleotide phosphodiesterase	*pde2*	39.7	Mitochondria Nucleus Cytoplasm	13 6 2
pkr1	Protein kinase A regulatory subunit	*bcy1*	43.6	Nucleus Cyto/Nucleus Cytoplasm	15.5 12.5 8.5
pkac1	Protein kinase A catalytic subunit	*tpk1* *tpk2* *tpk3*	49.5 46.9 49.1	Mitochondria Nucleus Cytoplasm	13 6 6
pkac2	Protein kinase A catalytic subunit	*tpk1* *tpk2* *tpk3*	63.3 63.2 63.3	Nucleus Cytoplasm Cyto/Perox	10 6.5 6.5
pkac3	Protein kinase A catalytic subunit	*tpk1* *tpk3*	36.2 36.2	Cytoskeleton Cytoplasm	19 4

Overexpression of pde2 overrules the effects of cAMP and CO_2

Gene *pde2* of *S. commune* was overexpressed to assess its impact on CO_2 sensing. Compatible transformants were crossed, after which the dikaryons were screened for reduced sensitivity to exogenous cAMP. Two dikaryons (18x4 and 22x4) were chosen from the strains that formed fruiting bodies in open plates in the presence of 2.5 mM cAMP in the medium. QPCR showed 5 and 10.7 fold higher levels of *pde2* mRNA, respectively, in these dikaryons when compared to wild-type.

Colonies of wild-type and 18x4 dikaryons were grown in sealed plates to assess whether *pde2* overexpression results in mushroom formation. Both strains

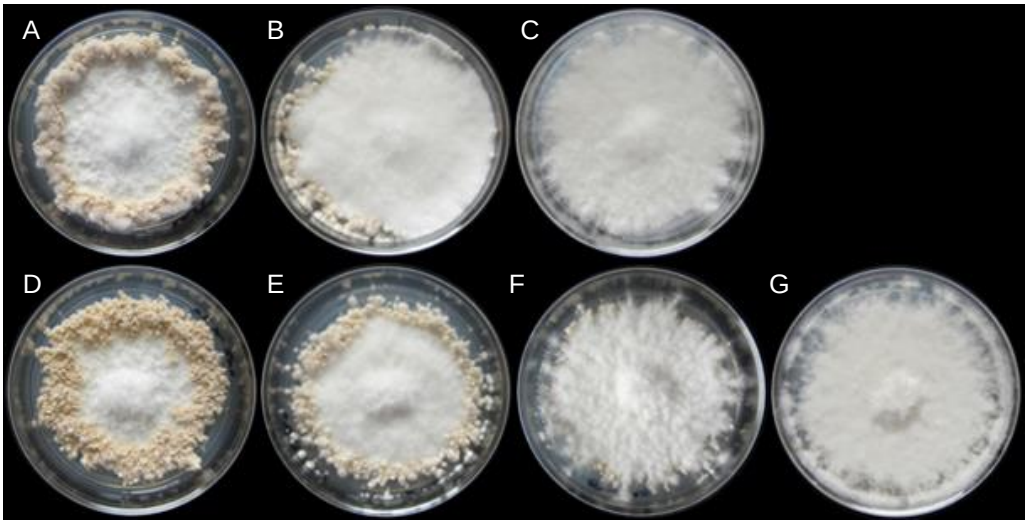

Figure 2 Dikaryons of H4-8 (A-C) and a *pde2pde2* overexpressor (D-G) grown for 7 days in ambient air (A, D), ambient air with 2.5 mM IBMX (B, E), sealed plates (C, F) and sealed plate with 2.5 mM IBMX added to the medium (G).

failed to produce fruiting bodies in a sealed 9 cm plate. To lower CO_2 levels in the sealed plates, both strains were grown in an open 4 cm plate positioned in a sealed 9 cm plate. Neither strain started fruiting body formation in this setting. The *pde2* overexpressing strain did fructify when grown in a 4 cm open plate within a sealed 10 cm sealed square plate, whereas the wild-type did not (Figure 1B,E). However, the ring of fruiting bodies was reduced when compared to that of wild-type or over-expression colonies grown in open plates (Figure 1A,D). This was also the case when the overexpression strain was grown in open plates in the presence of 2.5 mM cAMP (Figure 1F). The Pde2 inhibitor IBMX was added to the medium to confirm that increased fruiting body formation under sealed conditions was caused by over-expression of *pde2*. Transformant 18x4 was not able to initiate fruiting in sealed plates upon addition of IBMX to the medium (Figure 2G). Addition of IBMX also partly repressed fruiting in open plates (Figure 2B, E), confirming that Pde2 plays a role in fruiting under ambient CO_2 conditions. Together, the results support a link between high CO_2 levels, high cAMP levels, and repression of fruiting body formation.

Table 2. Expression levels (FPKM) of genes of the cAMP pathway, the blue sensor *wc-1,* and the regulatory genes involved in fruiting body development in colonies grown in the dark in ambient air or at high CO_2.

	Expression level (FPKM)	
Genes	Ambient Air	High CO_2
cAMP signaling pathway		
sca1	124.20	95.19
sca2	925.37	243.44
sac1	39.44	33.12
pde1	34.59	31.09
pde2	38.19	28.95
pkr1	159.07	146.22
scpkac1	38.95	32.44
scpkac2	81.95	71.90
scpkac3	1.68	1.83
Fruiting involved regulatory genes		
wc-1	50.81	47.39
wc-2	36.80	22.27
hom2	131.47	95.18
fst4	128.59	148.98
c2h2	46.92	19.04
fst3	117.77	83.61
hom1	182.02	88.44
gat1	47.07	41.74

Whole genome expression analysis

Whole genome expression analysis was performed on colonies grown in the dark in sealed or open plates. Genes *sca1*, *ac1*, *pde1, pde2*, *pkr1*, *scpkac1*, *scpkac2,* and *scpkac3* that encode components of the cAMP pathway did not respond to changes in CO_2 levels (Table 2). In contrast, transcript levels of *sca2* were 3.8 fold increased under high CO_2 conditions when compared to ambient air. This indicates that *sca2* is regulated at the transcriptional level.

A total of 548 and 735 genes were up- and down-regulated under low CO_2 conditions, respectively. Genes up-regulated at high CO_2 levels were enriched in electron transport and energy production (Table 3), while down-regulated genes were

enriched in genes involved in carbohydrate metabolism (Table 3). 18 transcription factors were up- and down-regulated when high and low CO_2 conditions were compared (Table 4), which included *wc-2, c2h2,* and *hom1*.

Table 3. Enrichment of GO terms in the up- and down-regulated genes when comparing wild-type dikaryotic colonies grown in the dark in ambient air to colonies grown in high CO_2.

Up-regulated genes in high CO_2		Down-regulated in high CO_2	
GO term	Functional annotation	GO term	Functional annotation
0016491	oxidoreductase activity	0005975	carbohydrate metabolic process
0003824	catalytic activity	0008733	L-arabinose isomerase activity
0009481	aa3-type cytochrome c oxidase	0005215	transporter activity
0009483	caa3-type cytochrome c oxidase	0008643	carbohydrate transport
0009482	ba3-type cytochrome c oxidase	0005351	sugar:hydrogen symporter
0009485	cbb3-type cytochrome c oxidase	0004553	hydrolase activity, hydrolyzing hydrolyzing O-glycosyl compounds
0008152	metabolic process	0006810	transport
0004129	cytochrome-c oxidase activity	0016021	integral to membrane
0006118	electron transport	0016810	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds
0005215	transporter activity	0005991	trehalose metabolic process

Table 4. Transcription factors up- or down-regulated in wild-type dikaryotic colonies grown in the dark at high or ambient CO_2 levels.

Up-regulated in high CO_2			Down-regulated in high CO_2		
Protein ID	Annotated domain	Fold change	Protein ID	Annotated domain	Fold change
2616011	SANT, DNA binding	1.7	2635354	TEA/ATTS	0.4
1183987	Forkhead-associated	2.3	2618564	Zn2-Cys6	0.6
2633009	CCHC type	2.4	1194000 (*c2h2*)	C2h2 type	0.4
2645489	CCHC type	2.5	2532312	C2h2 type	0.5
1163017	C2h2 type, ABC transporter	4.7	2488590	C2h2 type	0.6
2629570	C2h2 type, ABC transporter	1.7	2620880	Fst factor	0.3
2445444	CCHC type	10.0	2131645	C2h2 type	0.3
2625693	MADS-box	1.6	1191738	Zn2-Cys6	0.5
2628320	C2h2 type, ABC transporter	2.5	2577038	Forkhead-associated	0.6
2354220	C2h2 type, ABC transporter	1.7	2703601	Helix-loop-helix DNA binding	0.4
2558936	Lambda-repressor like	1.7	2533051	Zn2-Cys6	0.5
2629777	C2h2 type, ABC transporter	2.4	2604883	C2h2 type	0.5
2731703	Jumonji	1.8	2601101	C2h2 type	0.5
2640943	SANT, DNA binding	1.6	2703742	Zn2-Cys6	0.6
2642179	Helix-loop-helix DNA binding	2.2	2632356 (*hom1*)	Homeodomain box	0.5
2615671	Zn2-Cys6	1.6	2608172 (*wc-2*)	Gat type	0.6
2638002	bZIP	1.7	1159645	C2h2 type	0.5

DISCUSSION

Diffusion of CO_2 that is produced by a vegetative mycelium is reduced in a compact substrate when compared to open air. Consequently, hyphae within such a substrate are exposed to increased levels of CO_2, while hyphae growing at the surface of the substrate will experience ambient CO_2 levels. As such, the concentration of CO_2 is a signal for hyphae whether or not to initiate fruiting body formation. So far, it was not known whether CO_2 represses only mushroom initiation or that it impacts more stages of development. By transferring colonies from low to high CO_2 at different time points it was shown that CO_2 represses all stages of development. This implies that local accumulation of CO_2 between developing mushrooms will repress fruiting body outgrowth. Windy conditions may stimulate or even be a requisite for full development of *S. commune* mushrooms. This may also be the case for other mushroom forming fungi.

Not only high CO_2 levels but also absence of light represses full development of *S. commune* fruiting bodies once their formation has been induced. Light is at least required for aggregate formation and for maturation of *S. commune* fruiting bodies (unpublished data). Fruiting body formation in *C. cinerea* has even five or more light sensitive phases (Kües, 2000; Lu, 2000). Light is needed for the formation of *C. cinerea* initials, for maturation of primordia, and for karyogamy. On the other hand, it negatively impacts hyphal knot formation and completion of meiosis. Thus, environmental control on fruiting body formation functions throughout the developmental program.

The cAMP-PKA signaling pathway is the most used mechanism in prokaryotes and eukaryotes to sense CO_2. All genes of a cAMP-PKA pathway are present in *S. commune*. The first step in CO_2 sensing is the conversion of this compound in bicarbonate. This step is accelerated by the enzyme CA. *S. commune* is predicted to have a mitochondrial (Sca1) and a cytosolic (Sca2) version of this enzyme. The former would play a role in gluconeogenesis (Henry, 1996), while the latter would be involved in conversion of exogenous CO_2. Gene *sca2* was the only gene of the CO_2-cAMP-PKA signaling pathway that was differentially expressed when low and high CO_2 conditions were compared. This indicates that regulation of the CO_2 signaling pathway at the transcriptional level only occurs at the beginning of this pathway. Transcriptional regulation of CA genes is also observed in *C. albicans*, *S.*

cerevisiae, and *C. glabrata* and is independent from the cAMP-PKA pathway (see Cottier *et al.,* 2013). Such an independent regulation makes sense considering the fact that the cAMP-PKA pathway is also involved in signaling of other environmental conditions. For instance, Ras increases cAMP levels in *S. cerevisae* as a response to carbon levels (Tamanoi, 2011).

Addition of cAMP to the medium resulted in symmetrical colonies that do not form aggregates, primordia or fruiting bodies. Thus, high cAMP levels repress fruiting body formation. Our findings are in line with Yamagishi *et al.* (2002; 2004) but contradict those of Schwalb (1974; 1978), Yli-Mattila (1987), and Uno and Ishikawa (1971). The different outcomes may be due to the concentrations of cAMP that were applied, by using monokaryotic instead of dikaryotic strains, or by using other growth conditions. Our findings that high cAMP levels repress fruiting in *S. commune* are supported by Pde overexpression and inhibition studies. The high affinity *pde2* of *S. cerevisiae* regulates basal cAMP levels by degrading this compound. Overexpression of its *S. commune* orthologue resulted in fruiting under repressing levels of exogenous cAMP or CO_2. On the other hand, the Pde2 inhibitor IBMX repressed fruiting under low CO_2 conditions (i.e. in open plates), supporting a role of Pde2 in fruiting under ambient CO_2 conditions. Together, these data show a clear link between high CO_2 and cAMP levels and repression of fruiting.

High cAMP levels activate Pka activity by dissociation of the two regulatory subunits from the catalytic subunits. We were unable to show differences in cAMP concentration in hyphal extracts from colonies grown at high or low CO_2 (data not shown). Similar results were reported by Yamagishi *et al.* (2004) and Schubert *et al.* (2006). In addition, we were unable to show PKA activity in colonies grown at high or low CO_2. Absence of Pka activity in the wild-type was also reported by Knabe *et al.* (2013) but they did show Pka activity in Δ*gap1* and constitutive *ras1* transformants. Clearly, absence of Pka activity and differences in cAMP levels is not expected when dikaryotic wild-type colonies grown at high and low CO_2 conditions are compared. These results can be explained when we assume that cAMP production and Pka activity relevant for repression of fruiting take place in the nucleus. Protocols used by Knabe *et al.* (2013) and by ourselves only measure cytosolic cAMP and Pka activity. The existence of nuclear cAMP microdomains are known to be regulated by soluble AC (sAC) (Zippin *et al.,* 2004). This enzyme is bicarbonate dependent, in contrast to

transmembrane AC (tAC), which is responsive to G-protein signaling. A single AC was identified in the genome of *S. commune*, which is of the sAC type. *C. albicans* also possesses a single AC of the sAC type. This protein called Cyr1 functions as an integrator of multiple signals (temperature, bacteria, CO_2 and Ras) mediated by LRR, PP2C, CYCc, and putative Ras interaction domains (Wang, 2013). *S. commune* sAC contains similar domains, and is thus expected to transmit different environmental signals. This protein as well as Pde1, Pkr1, and Pkac2 are predicted to have a nuclear localization. So, at least a minimal set of proteins involved in cAMP synthesis and Pka activity is predicted to reside in the nucleus. Other components may be imported in the nucleus by interacting with Sac1, Pde1, Pkr1, or Pkac2.

Previously, transcription factors have been described that are involved in mushroom development. The blue light receptor complex contains the transcription factor Wc-2. Inactivation of its gene results in colonies that grow symmetrical in blue light (similar to dark-grown wild-type dikaryons) and that do not produce aggregates, primordia, and fruiting bodies (Ohm *et al.,* 2013). Deletion of the homeodomain gene *hom2* shows a similar phenotype (Ohm *et al.,* 2011), while inactivation of the zinc

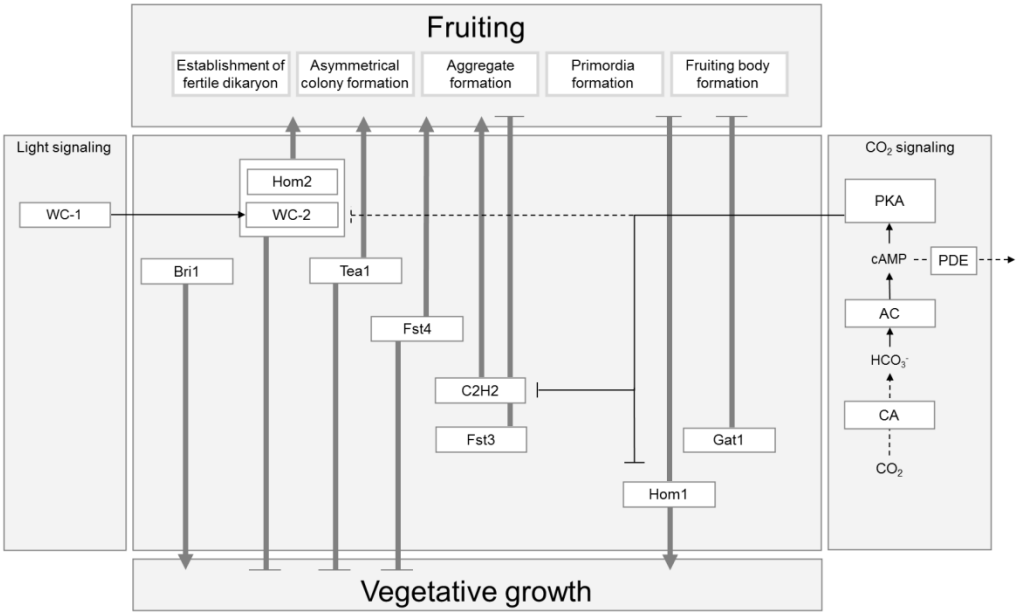

Figure 3 Model showing the link between CO_2 signaling and transcription factors involved in fruiting and vegetative growth in *S. commune*. Dotted line from Pka to Wc-2 represents a significant but <2-fold effect of CO_2 on Wc-2.

finger transcription factor gene *fst4* results in dikaryons that still grow irregular in fruiting inductive conditions but aggregates, primordia, and fruiting bodies are not produced (Ohm *et al.,* 2011). Strains in which the Cys2His2 zinc finger protein gene *c2h2* has been inactivated are arrested in the aggregate stage (Ohm *et al.,* 2011), while deletion strains of *fst3*, *gat1* or *hom1* form smaller fruiting bodies but in higher numbers (Ohm *et al.,* 2011). The zinc finger protein Fst3 was proposed to play a role in repression of outgrowth of fruiting bodies from primordia, while Gat1, a GATA type zinc finger protein, and Hom1, a homeodomain protein, would play a role in expansion of the fruiting body. Genes *wc-2, c2h2,* and *hom1* were shown to be significantly down-regulated under high CO_2 conditions. This shows a link between CO_2 signalling, light perception, and regulators of mushroom development (Figure 3). Additional transcription factor genes have been identified that are either up- or down-regulated under high CO_2 conditions. Part of these genes may also encode regulators of mushroom formation.

ACKNOWLEDGEMENTS

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Chapter 4

Post-translational modification of the homeodomain protein Hom2 keeps the mushroom forming fungus *Schizophyllum commune* in the vegetative stage

Jordi F. Pelkmans, Robin A. Ohm, Han A.B. Wösten, and Luis G. Lugones

ABSTRACT

High CO_2 levels repress mushroom formation in *S. commune* via the cAMP-protein kinase A (Pka) pathway. Here, it was addressed whether the homeodomain protein Hom2 is a target for Pka. Biomass of wild-type and Δ*hom2*Δ*hom2* colonies were compared after growth at low (0.04%) and high (5%) CO_2 levels. The Δ*hom2*Δ*hom2* dikaryon formed 2 fold more biomass than the wild-type at ambient CO_2 levels, while biomass of both strains was similar at 5% CO_2. These data supported a link between CO_2 sensing and Hom2. Bioinformatic analysis revealed 4 predicted Pka RRXS phosphorylation motifs in Hom2. The serine codons of these 4 motifs were replaced by alanine. Introduction of this *hom2*^{con} derivative in the ∆*hom2*∆*hom2* dikaryon resulted in strains that showed radial growth inhibition and prompt fructification at low CO_2. This was even the case in sterile monokaryons. High CO_2 levels or the presence of 2.5 mM cAMP in the medium still inhibited fruiting in *hom2*^{con} dikaryotic strains. This implies the existence of another regulator of fruiting that is subject to Pka phosphorylation. Together, it is proposed that Pka phosphorylates Hom2, thereby maintaining the mycelium in the vegetative phase. Dephosphorylation of Hom2 and an unknown other protein at low CO_2 switches the dikaryon from the vegetative into the generative phase. The fact that all *hom2* orthologues of Agaricomycetes contain 2-5 RRXS motifs indicates that the role of Hom2 in fruiting is conserved.

INTRODUCTION

Mushroom formation is a complex process governed by responses of the fungus to environmental cues. Such cues are levels of O_2 or CO_2, light intensity, temperature, humidity, volatiles, pH, salinity and availability of nutrients (Madelin, 1956, Manachère, 1988; Kües and Liu, 2000; Eastwood *et al.,* 2013; Pelkmans *et al.,* 2016). Formation of white button mushrooms by *Agaricus bisporus* is repressed by 1-octen-3-ol and high CO_2 levels, while low temperature stimulates mushroom formation. On the other hand, fruiting body development is responsive to blue light in *Schizophyllum commune,* while also in this case high CO_2 levels repress fruiting (Perkins, 1969; Raudaskoski and Yli-Mattila, 1985, Ohm *et al.,* 2013; Eastwood *et al.,* 2013).

Signalling of blue light in *S. commune* is mediated by the blue light sensor Wc-1 and the transcription factor Wc-2. Inactivation of one or both of the encoding genes results in a blind phenotype (Ohm *et al.,* 2013). The deletion strains are completely blocked in fruiting body development. Colonies grow regular in the light like wild-type dikaryons in the dark and the progressive stages of mushroom development (aggregates, primordia, and fruiting bodies) are not observed. Recently, evidence was obtained that CO_2 is sensed via the cAMP protein kinase A (Pka) pathway. Addition of cAMP to the medium blocked fruiting in the light under low CO_2 conditions (Chapter 3). On the other hand, overexpression of *pde2* that encodes phosphodiesterase with cAMP degrading activity, resulted in fruiting body development even at elevated CO_2 levels. This effect was overcome by adding the Pde2 inhibitor IBMX to the medium. High cAMP levels would release the regulatory subunits of the Pka complex. As a result, the Pka catalytic subunits will phosphorylate serine or threonine residues in target proteins containing RRXS/T motifs (Taylor *et al.,* 2005). Phosphorylation of transcription factors can influence their stability, their ability to interact with other proteins, their cellular localization, and their ability to bind DNA (Whitmarsh and Davis, 2000).

Various transcription factors involved in fruiting have been identified in *S. commune*. Deletion mutants of the transcription factor Wc-2 of the blue light sensing complex and the homeodomain protein Hom2 show phenotypes similar to *S. commune* wild-type grown at high CO_2 or high cAMP levels (Ohm *et al.,* 2011). In these mutants no overall and local growth arrest is seen (the latter indicated by asymetrical growth of colonies) and no aggregates, primorida, and fruiting bodies are formed. Here, it is shown that activity of Hom2 is modulated by CO_2 and that this homeoprotein is a target for phosphorylation by PKA. Introduction of a constitutive active Hom2 lacking PkA phosphorylation sites resulted in dramatic growth inhibition and instantaneous fruiting initiation. Together, these results link Hom2 activity to CO_2 sensing.

MATERIALS AND METHODS

Culture conditions and strains

The monokaryotic strain *S. commune* H4-8 (*mat*A43 *mat*B41; FGSC 9210; Ohm *et al.,* 2010b), the dikaryon resulting from the cross between H4-8 and the compatible

isogenic strain H4-8b (Ohm *et al.,* 2010a), and their derived Δ*hom2* strains were used in this study. Cultures were grown on minimal medium (MM) with 2% glucose and 1.5% agar (van Peer *et al.,* 2009) starting from a point inoculum taken from the periphery of a 7-day-old colony. Colonies were routinely grown at 30 °C in the dark in sealed plates. After 4 days, the 2 layers of parafilm and the covering layer of duct tape were removed and growth of the colonies was prolonged for 3 days at 25 °C with 1200 lux white LED light (Conrad Electronic, Hirschau, Germany). In the case of experiments with controlled 0.04% CO_2 conditions, colonies were grown in a 13 liter box with an air flow of water saturated air of 15 l h^{-1}, while colonies were grown in a CO_2 incubator (Binder, Tattlingen, Germany) in the case of experiments with controlled 5% CO_2 conditions.

Quantification of biomass

Colonies were grown as described above with the modification that the inoculum was placed on a cellophane membrane (Versteden Papier, Tilburg, The Netherlands) overlaying the agar medium. The membranes had been weighed, wetted, and sterilized before they had been placed on the medium. Plates were incubated using different aeration regimes. After 5 days of growth the membranes with the colonies were dried for 24 h at 65 °C and weighed. Weight of the membrane before growth was subtracted to obtain the mycelial biomass.

Expression vector with mutant Hom2

Plasmid pHom2 is a pGMTphleoB derivative containing the *S. commune* gene *hom2* (Prot ID 2634429; genome.jgi.doe.gov/Schco3/Schco3.home.html) (Ohm *et al.,* 2011). The 1832 bp HindIII/SbfI fragment of pHom2 containing *hom2* coding and flanking sequences was cloned in pSP72 using the same sites, resulting in pHom2HF. In the next step, a 692 *hom2* coding fragment was synthesized (Genscript, Piscataway, NJ, USA), in which the serine codons in the 4 RRXS motifs were replaced for alanine. A unique PvuI site was removed as a consequence of the substitution in the second motif. A fusion PCR was performed using T7 and SP6 primers annealing at both sides of the multiple cloning site of pHom2HF and as templates the 692 bp fragment and pHom2HF. The fusion fragment resulted in a mixture of a 1922 bp wild-type *hom2* fragment and a 1922 bp fragment with the 4 serine codons replaced. The wild-type

fragment was digested with PvuI. The remaining 1922 bp band was cut with HindIII/SbfI and used to replace the corresponding fragment in pHom2. This resulted in plasmid pHom2* that contains the *hom2*^{con} gene.

Restriction PCR

RNA was extracted from 10-day-old colonies grown on MM plates on top of a polycarbonate membrane using Trizol (Thermo Scientific, Waltham, MA, USA). cDNA was made using the QuantiTect Reverse Transcription Kit (Qiagen, Düsseldorf, Germany). In the next step, cDNA was amplified by PCR with the intron spanning primers Hom2RTFw (GAGAGGTGTACAG**GTCTG**) and Hom2RTRv (ACTGGTTCGAGGTC**ATAG**). Sequences that anneal at the beginning and end of exon 2, respectively, are indicated in bold. The primer sequences in normal font anneal at the end of exon 1 (Hom2RTFw) and at the beginning of exon 3 (Hom2RTRv). The amplified wild-type 1117 bp fragment was cut with PvuI resulting in fragments of 609 and 508 bp. In contrast, the *hom2*^{con} fragment could not be cut by this enzyme, leaving the 1117 bp band intact.

Bioinformatics

RRXS domains were identified in the protein sequences of *S. commune* using a custom Python script. Enrichment analyses were performed with Python and R to analyze over- and under-representation of functional annotation terms in sets of genes using the Fisher Exact test. The Benjamini-Hochberg correction was used to correct for multiple testing using a *p*-value ≤ 0.05. Orthologues of *S. commune hom2* in other basidiomycetes were identified by the presence of a homeobox PFAM domain (PF00046; Finn *et al.,* 2014) and a reciprocal best blastp hit to *hom2*. These hits were manually curated using MycoCosm (Grigoriev *et al.,* 2014) based on the available expression and homology data for that genome. The protein sequences were aligned using MAFFT version 7.123 (Katoh and Standley, 2013) with the E-INS-i method. The alignment was visualized and manually curated using Jalview 2.8.2 (Waterhouse *et al.,* 2009). Conserved RRXS sites were identified in the alignment. A phylogenetic tree was reconstructed using RaxML version 8.1.16 (Stamatakis, 2006) with the PROTGAMMAWAG model with 100 rapid bootstrap partitions. The tree and domain structures were visualized using the ETE Toolkit version 2.2.

RESULTS

Hom2 activity is related to CO_2 levels surrounding the colony

The Δ*hom2*Δ*hom2* dikaryon produces more biomass than the wild type when grown in open Petri dishes (Chapter 2). Here, biomass of 5-day-old wild-type and Δ*hom2*Δ*hom2* colonies was compared after growth under controlled CO_2 conditions; i.e. at low (0.04% [400 ppm]) and high (5% [50000 ppm]) CO_2 levels. The Δ*hom2*Δ*hom2* dikaryon formed 2 fold more biomass than the wild-type at ambient CO_2 levels (Figure 1). In contrast, biomass of both strains was similar at 5% CO_2 being 10% higher when compared to the Δ*hom2*Δ*hom2* dikaryon grown at 0.04% CO_2. The differences in biomass correlated with the radial growth rate. The Δ*hom2*Δ*hom2* dikaryon formed at both CO_2 concentrations large colonies, while this was only observed at high CO_2 for the wild-type (Figure 1). These data and the fact that Δ*hom2*Δ*hom2* dikaryons form colonies at low CO_2 that resemble the phenotype of the non-fruiting wild-type colonies grown at high CO_2 suggest that Hom2 is a target of the CO_2-cAMP-Pka signaling pathway.

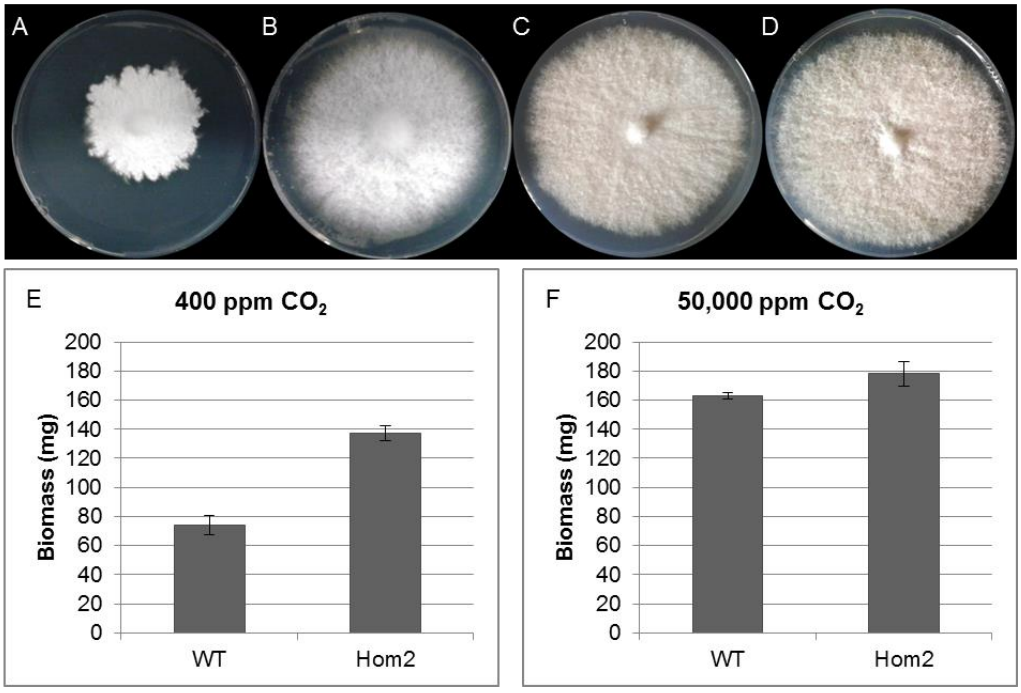

Figure 1. Dikaryotic wild-type (A, C) and Δ*hom2*Δ*hom2* (B, D) colonies grown at 400 (A, B) and 50000 ppm CO_2 (C, D)) and their corresponding biomass (E, F).

Table 1 The number of genes that contain 1 to 11 conserved RRXS motifs in their sequence as found in the genome of *S. commune*.

Number of RRXS motifs	1	2	3	4	5	6	7	8	9	10	11
	2320	535	141	46	28	9	4	5	2	2	2

Hom2 as a target for protein kinase A

High CO_2 leads to high cAMP levels that stimulate Pka activity (Chapter 3). Pka phosphorylates serine or threonines in RRXS/T motifs, the former being 37 fold more efficient than the latter (Kemp *et al.,* 1977). Therefore, a Pka phosphorylation motif search in the *S. commune* genome was restricted to the RRXS motif. Table 1 shows the frequency of RRXS motifs in genes of *S. commune*. The group with ≥ 3 boxes is enriched for genes involved in signal transduction (Supplementary Table 1).

Hom2 is one of the 46 proteins predicted to contain 4 RRXS motifs. The ortologues of Hom2 were identified in the genomes of Agaricomycetes. Homology of reciprocal best blastp hits was limited to the homeobox domain. However, all predicted orthologues had 3-5 RRXS boxes, of which 3 were conserved (Figure 2).

Modification of the RRXS motifs of Hom2 has a profound effect on colony phenotype

The serine codons within the RRXS motifs of *hom2* were replaced for alanine codons to remove the predicted phosphorylation sites. This *hom2*^{con} derivative was introduced in *S. commune* spore protoplasts. Two types of transformants were observed. 90% of the colonies had a growth speed similar to regenerating wild-type protoplasts, while 10% grew very slowly. Four slow growing transformants represented monokaryons, while 3 of these strains represented fertile dikaryons or showed a common matA interaction. Such strains can be formed in the regeneration medium between non-transformed and / or transformed cells since the mixture of protoplasts contains all possible mating type combinations. The dikaryons and common matA strains were re-protoplasted to obtain monokaryons with a transformed nucleus. Restriction PCR was

done using RNA from colonies of the wild-type and from transformant T4. Cutting with PvuI visualizes the relative expression of the endogenous and introduced *hom2* alleles. This analysis revealed that *hom2*^{con} was much higher expressed than the endogenous copy (Figure 3F).

All 7 monokaryotic transformants grew slower than the wild type at 30 ˚C in the dark at high CO_2, but to a different extent (Figure 3B-D). They formed irregular colonies, whereas the wild-type grew symmetrical. Notably, strain T4 that formed the smallest colonies produced aggregate-like structures. When colonies were transferred to fruiting conditions (low CO_2, light, 25 ˚C), strain T4 started immediately to form non-sporulating fruiting bodies, while the other transformants produced such mushrooms

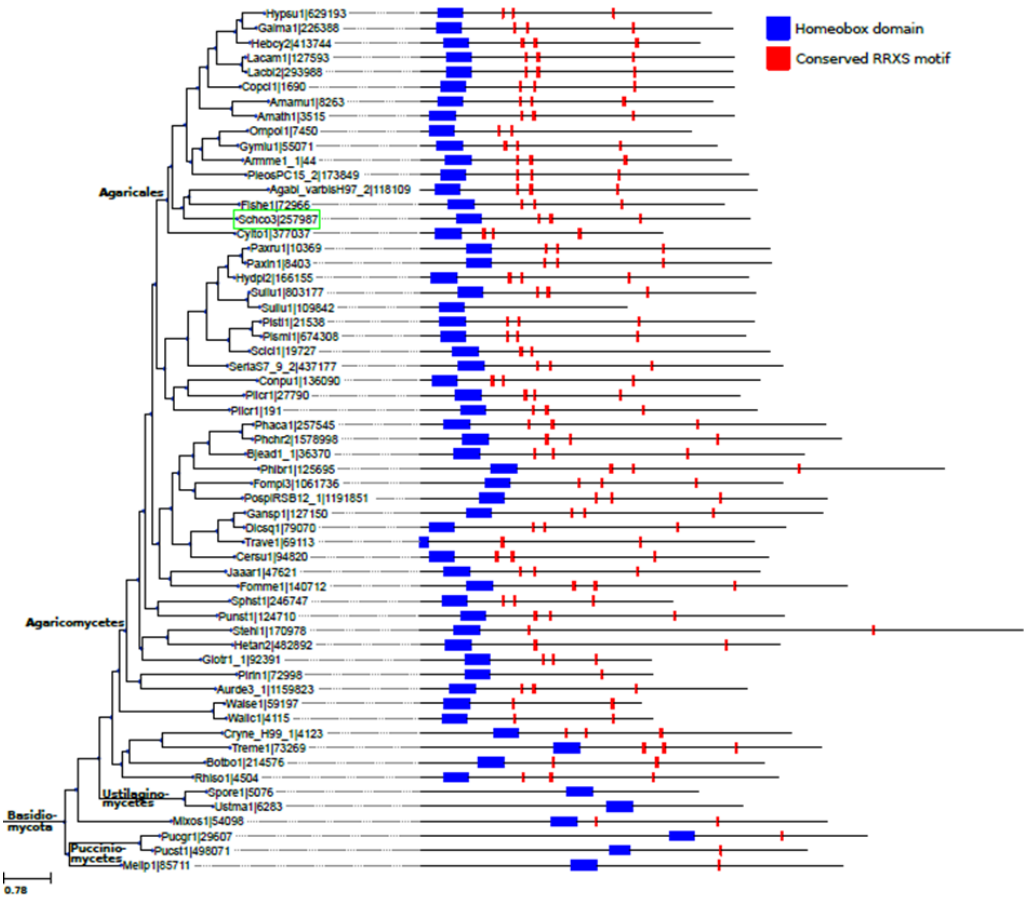

Figure 2 Phylogenetic tree and domain structure of putative Hom2 orthologs. Hom2 of *S. commune* is indicated with a green box.

after 4 to 5 days. Strains growing slower generally formed fruiting bodies earlier than the ones that grew quicker. Strain T4 formed non-sporulating ear like structures in the dark at high CO_2 at 30 ˚C when grown on a polycarbonate membrane (Figure 3E). These colonies did not form an extended feeding mycelium and had limited contact with the membrane. Almost all hyphae within the mycelium were inflated, which in some cases resembled basidia. Ear like structures were not formed in the other *hom2*^{con} transformants and inflated hyphae were much less abundant.

In the next step, the slowest growing transformant T4, as well as the fastest (T10) and an intermediate growing transformant (T3) were crossed with a compatible wild type strain. The resulting dikaryons showed less severe phenotypes when compared to T4, T3, and T10. However, they all grew slower than the wild type dikaryon and developed spore forming fruiting bodies immediately after transfer to the light at low CO_2 conditions. In contrast, the wild-type continued vegetative growth for 1-2 days and then started to form fruiting bodies.

Transformant T8 was crossed with a Δ*hom2* monokaryon. Siblings were

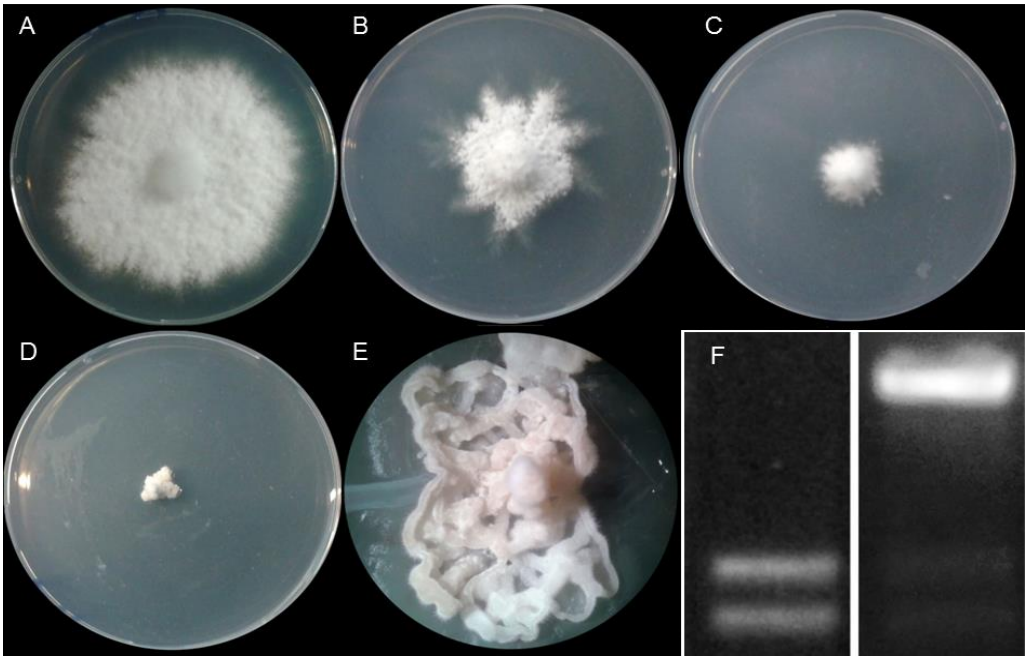

Figure 3 Six-day-old colonies of the wild type monokaryon (A) and different *hom2*^{con} strains (B-D), a close up of the ear-like structures formed by T4 (E) and relative *hom2* and *hom2*^{con} expression in wild-type (left) and strain T4 (right).

selected on nourseothricin to screen for the *hom2* deletion and on phleomycin to screen for the presence of *hom2*^{con} insertion. This Δ*hom2::hom2*^{con} monokaryon fruited similar to strain T8, showing that Hom2^{con} functions independent of the endogenous Hom2.

DISCUSSION

Spore dispersal will not be effective when *S. commune* forms its fruiting bodies within its substrates such as logs. It can thus be expected that this organism has developed mechanisms to sense its exposure to open air as a signal that fruiting bodies can be formed. CO_2 concentration and blue light, the environmental cues that trigger fruiting in *S. commune* are in fact very efficient indicators of open air exposure. Light maximally penetrates 200 µm in wood (Hon & Ifju, 1978), while CO_2 levels are between 7-21% inside wood and 0.04% in open air (Jensen, 1969). Blue light in *S. commune* is signaled via the white collar complex, consisting of the Wc-1 sensor and the Wc-2 transcription factor (Ohm *et al*., 2013). CO_2 is sensed via the cAMP-Pka pathway (Chapter 3). Here, it was shown that Hom2 is a target of this pathway. This homeodomain protein has previously been shown to be involved in early stages of mushroom formation (Ohm *et al.,* 2011) and in repression of vegetative growth (Chapter 2).

The fact that Δ*hom2*Δ*hom2* dikaryons grown at low or high CO_2 levels have a similar phenotype as wild-type colonies grown at high CO_2 or high cAMP levels (Ohm *et al.,* 2011; Chapter 3) was the first indication of a link between CO_2 sensing and Hom2. The Δ*hom2*Δ*hom2* dikaryons do not show an overall or local growth arrest (the latter indicated by asymmetrical growth of colonies) and no aggregates, primordia, and fruiting bodies are formed. The second indication for a link between CO_2 sensing and Hom2 was obtained by monitoring biomass formation in wild-type and Δ*hom2*Δ*hom2* dikaryons. Growth of wild-type colonies in the dark is reduced at low CO_2 levels (0.04%) when compared to high CO_2 levels (5%). In contrast, this effect of reduced growth rate at low CO_2 was not observed in the Δ*hom2*Δ*hom2* dikaryon. This suggested that Hom2 is a target for Pka. Indeed, 4 Pka phosphorylation motifs were found in Hom2. Orthologues of *hom2* in other mushroom forming Agaricomycetes also showed 3-5 Pka motifs, of which 3 are conserved.

A derivative of *hom2* was constructed in which all 4 Pka motifs were eliminated by replacing serine for alanine codons. Introduction of this derivative, called *hom2*^{con}, in a monokaryotic strain resulted in severe phenotypes. Colonies grew very slowly when compared to wild-type, albeit with variable extension rates. This is probably explained by ectopic integration of a variable number of copies of *hom2*^{con} in the different transformants. These data indicate that *hom2* in the non-phosphorylated state represses vegetative growth. The phosphorylated form of Hom2 resulting from Pka activity at high CO_2 levels would thus be inactive in growth repression. The inhibition of growth in the *hom2*^{con} strains was much more dramatic when compared to wild-type colonies grown at low CO_2. This suggests that only a fraction of *hom2* is dephosphorylated when grown in this condition. The *hom2*^{con} transformants still had an endogenous copy of *hom2.* Gene *hom2*^{con} was crossed in the Δ*hom2* background to exclude that the phenotype was the result of heterodimer formation or titration of the wild-type form. Indeed, a Δ*hom2::hom2*^{con} strain showed phenotypes similar to the *hom2*^{con} strains.

Wild type monokaryons do not show different growth rates at high and low CO_2 as opposed to dikaryotic strains. Therefore, it came to a surprise that introduction of *hom2*^{con} in the sterile monokaryon resulted in a growth effect. Transformants even fruited when exposed to light and low CO_2, thus resembling a dikaryon. This suggests that Hom2 is maintained in a phosphorylated state in the monokaryon both in low and high CO_2 conditions. Data also indicate that the phosphorylation state of Hom2 is the only inhibitory factor that suppresses fruiting in monokaryons in conditions favorable for mushroom formation in the dikaryon. These data also imply that the role of the mating type genes in sexual development is less directive than previously assumed. They would function in the establishment and maintenance of the dikaryotic state but not in the fruiting process itself. The fact that sporulation was not observed in monokaryotic fruiting bodies, in contrast to the *hom2*^{con} dikaryotic strains, stresses the importance of dikaryosis for this process.

Fruiting in monokaryotic and dikaryotic strains expressing *hom2*^{con} was still sensitive to high CO_2 and to 2.5 mM cAMP in the medium (Chapter 3). Apparently, there are other targets of PKA that are essential for the initiation of fruiting. Genes involved in signal transduction were enriched in the set of genes containing 4 or more RRXS boxes. This set includes 9 transcription factor genes and 5 serine-threonine

kinase genes and they could be targets for Pka and fulfill a role in fruiting together with Hom2.

ACKNOWLEDGEMENTS

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Supplementary Table 1 Enrichment of GO terms in *S. commune* genes containing ≥3 RRXS motifs.

Annotation term	Description	# proteins in study set with annotation term	# proteins in study set without annotation term	# other proteins with annotation term	# other proteins without annotation term	log odds ratio	p-value (corrected)
GO:0046578	Regulation of Ras protein signal transduction	7	232	13	15952	3.6	2.30E-06
GO:1902531	Regulation of intracellular signal transduction	8	231	22	15943	3.2	2.30E-06
GO:0051056|	Regulation of small GTPase mediated signal transduction	8	231	22	15943	3.2	2.30E-06
GO:0005085	Guanyl-nucleotide exchange factor activity	8	231	21	15944	3.2	2.30E-06
GO:0023051	Regulation of signaling	8	231	27	15938	3.0	4.18E-06
GO:0009966	Regulation of signal transduction	8	231	27	15938	3.0	4.18E-06
GO:0010646	Regulation of cell communication	8	231	27	15938	3.0	4.18E-06
GO:0048583	Regulation of response to stimulus	8	231	27	15938	3.0	4.18E-06
GO:0005086	ARF guanyl-nucleotide exchange factor activity	3	236	2	15963	4.6	0.003
GO:0032012	Regulation of ARF protein signal transduction	3	236	2	15963	4.6	0.003
GO:0050789	Regulation of biological process	18	221	394	15571	1.2	0.004
GO:0005089	Rho guanyl-nucleotide exchange factor activity	4	235	11	15954	3.2	0.004
GO:0005088	Ras guanyl-nucleotide exchange factor activity	4	235	11	15954	3.2	0.004
GO:0035023	Regulation of Rho protein signal transduction	4	235	11	15954	3.2	0.004

GO:0006470	Protein dephosphorylation	4	235	13	15952	3.0	0.006
GO:0016311	Dephosphorylation	4	235	14	15951	3.0	0.006
GO:0065007	Biological regulation	18	221	428	15537	1.1	0.006
GO:0004721	Phosphoprotein phosphatase activity	4	235	14	15951	3.0	0.006
GO:0050794	Regulation of cellular process	17	222	385	15580	1.1	0.006
GO:0008138	Protein tyrosine/serine/threonine phosphatase activity	3	236	11	15954	2.9	0.043
GO:0006793	Phosphorus metabolic process	13	226	311	15654	1.1	0.043
GO:0006796	Phosphate-containing compound metabolic process	13	226	310	15655	1.1	0.043

Chapter 5

The transcriptional regulator *c2h2* of *Agaricus bisporus* is involved in mushroom formation

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ABSTRACT

The Cys2His2 zinc finger protein gene *c2h2* of *Schizophyllum commune* is involved in mushroom formation. Its inactivation results in a strain that is arrested at the stage of aggregate formation. In this study, the *c2h2* orthologue of *Agaricus bisporus* was overexpressed in this white button mushroom forming basidiomycete using *Agrobacterium*-mediated transformation. Morphology, cap expansion rate, and total numbers and biomass of mushrooms harvested during culturing was not affected by over-expression of *c2h2*. However, the *c2h2* overexpression strains formed more class II mushrooms when compared to the parental wild-type strain and yield-per-day peaked one day earlier. These data and expression analysis indicate that C2H2 functions early in fruiting body development, while it also seems to have a role in selective tissues of young mushrooms. Data also indicate that *c2h2* is a target for breeding of commercial mushroom strains.

INTRODUCTION

The basidiomycete *Agaricus bisporus* is cultivated at a global scale for the production of white button mushrooms. These fruiting bodies have a relative high protein content and contain fibers, vitamins, minerals, and bioactive compounds. *A. bisporus* is grown on compost formed from wheat straw, horse or chicken manure, and gypsum. Upon colonization, the compost is topped with a non-nutritional casing layer needed for moisture and microbial flora (Bels-Koning, 1950; Flegg, 1956; Kalberer, 1987). Induction of mushroom formation depends on environmental signals. The volatile 1-octen-3-ol represses early development, high temperature (i.e. 25 °C instead of 18 °C) inhibits development from smooth to elongated primordia, while CO_2 impacts the number of fruiting bodies that are formed (Noble *et al.,* 2009; Eastwood *et al.,* 2013).

Development of *A. bisporus* mushrooms is a complex process (Kuës and Navarro-Gonzáles, 2015). It starts with aggregation of hyphae into hyphal knots (Umar and van Griensven, 1997b). These structures develop into 1-2 mm initials, also called primordia, that differentiate by forming cap and stem tissue (Umar and van Griensven, 1997b). Up to 10% of differentiated primordia develop into mushrooms (Noble *et al.,* 2003). Breaking of the veil of these fruiting bodies enables airborne dispersal of basidiospores that had been formed in the gill tissue within the cap.

Production conditions of white button mushrooms have been optimized with respect to yield and quality of the fruiting bodies (Straatsma *et al.,* 2013). However, the molecular mechanisms underlying mushroom formation are poorly understood. For instance, transcription factors involved in control of white button development have not been identified. Such regulatory proteins have been identified in the model organism *Schizophyllum commune* (Ohm *et al.,* 2010b; 2011; 2013). Formation of its fruiting bodies is induced by blue light and is repressed by high CO_2 (Perkins and Gordon, 1969; Niederpruem, 1963; Raudaskoski and Viitanen, 1982; Ohm *et al.,* 2013; Chapter 3). The blue light receptor complex consists of the sensor Wc-1 and the transcription factor Wc-2. Inactivation of *wc-1* and / or *wc-2* results in a blind phenotype not able to produce aggregates, primordia, and fruiting bodies (Ohm *et al.,* 2013). Strains in which the homeodomain gene *hom2* or the zinc finger transcription factor gene *fst4* are inactivated are also not able to produce aggregates (Ohm *et al.,* 2010b; 2011). In contrast, inactivation of the gene encoding the Cys2His2 zinc finger protein C2H2 results in a strain that does form aggregates but not primordia and fruiting bodies (Ohm *et al.,* 2011). Strains in which the genes are inactivated that encode the zinc finger protein Fst3, the GATA type zinc finger protein Gat1 or the homeodomain protein Hom1 form smaller fruiting bodies but in higher numbers (Ohm *et al.,* 2011). These proteins were proposed to play a role in repression of outgrowth of primordia into fruiting bodies or to play a role in expansion of the fruiting body.

Homologues of the *S. commune* transcription factors involved in fruiting body development have been identified in other mushroom forming fungi. Expression analysis in *A. bisporus, Laccaria bicolor,* and *Coprinopsis cinerea* suggests that mushroom development in the Basidiomycota follows a core regulatory program with species specific variations that explain differences in morphology and sensitivity to environmental signals (Ohm *et al.,* 2010b; Morin *et al.,* 2012; Plaza *et al.,* 2014). In this study, the *A. bisporus c2h2* homologue was over-expressed in the commercial strain A15. This resulted in accelerated rate of mushroom production but also in a lower mushroom quality. Experimental data indicate that C2H2 functions both early and late in mushroom development and that this protein is an interesting target for breeding of commercial strains.

EXPERIMENTAL PROCEDURES

Culture conditions and strains

The heterokaryotic *A. bisporus* strain A15 and its derivatives AT273-1 and AT273-5 that over-express *c2h2* were routinely grown at 25 ˚C on malt extract agar medium (MEA; 20 gr l^{-1} malt extract agar [BD biosciences, Franklin Lakes, USA], 2.1 gr l^{-1} MOPS, pH 7.0). Spawn substrate was produced by heating 75 gr *Sorghum* seeds in water of 100 ºC for 20 min, after which 24 gr kg^{-1} $CaSO_4$ and 6.87 gr kg^{-1} $CaCO_3$ was added. Spawn was colonized for 3 weeks at 25 ˚C using 2 1-week-old *A. bisporus* colonies as inoculum. Mushrooms were produced by inoculating boxes (40 cm width x 60 cm length x 22 cm height) containing 16 kg phase 2 compost (CNC, Milsbeek, The Netherlands) with 75 gr of spawn. Temperature was maintained at 25 °C with an air temperature of 22 °C. Relative humidity in the growth cell was kept at 95%, while CO_2 levels fluctuated between 1500 ppm and 2000 ppm. 10 boxes were inoculated per strain and randomly distributed in the growth cell. After 16 days, the compost in each box was topped with 7 kg casing layer (CNC, Milsbeek, The Netherlands). Growth was prolonged for 14 days before venting. 4 days prior to venting the casing was manually broken and mixed to create fast regenerative growth and a more equal distribution of *A. bisporus* in the casing layer. Venting resulted in a gradual decrease of compost and air temperature to 19 and 18 °C, respectively. Relative humidity and CO_2 levels decreased gradually to 85% and 1200 ppm, respectively. The first buttons were removed from the bed 9 days after venting.

Analysis of mushroom formation

Photos of the casing layer surfaces were taken in a fixed rig at 24 h intervals from venting until the start of the first flush. Emergence of mushrooms and growth rate of the caps was monitored using ImageJ (http://imagej.nih.gov/ij/). Harvesting of mushrooms was done by a professional picker as performed in commercial production. Prior to the flushes some buttons were removed to open up the space between developing buttons. Buttons with a diameter between 40 and 60 mm were always harvested, while buttons with a diameter of ≤ 40 mm were harvested from densely populated areas to provide more space, water, and nutrients to the remaining mushrooms, thereby ensuring optimal yield. Mushrooms were classified on basis of quality (class I or II) and size (size 40 [mushrooms with a cap ≤ 40 mm] and size 60

[mushrooms with a cap between 40-60 mm]). Yield per box was expressed as the biomass and the number of harvested mushrooms. Height and width of cap and stem were determined of 10 randomly selected mushrooms per box during the first peak day of the first flush. Dry weight of the mushrooms was assessed by drying 200 gr wet weight fruiting bodies at 100 °C.

Table 1 List of primers used in this study.

Primer name	Sequence
C2h2ABownF	CGCTTAATTAACCTGGCAAAAAAGTGAAC
C2h2ABownR	ATATGGCGCGCCACTACGTCGATGATCATG
McSpBH_F	GATCGTTAATTAAGAATTCAGATCTCAATTGGGCGCGCC
McSpBH_R	GGCGCGCCCAATTGAGATCTGAATTCTTAATTAAC

Over-expression of c2h2

Primer pair McSpBH_F/McSpBH_R (Table 1) was used to introduce PacI and AscI sites into pBHg (Chen *et al.,* 2000), creating pBHgPA. Gene *c2h2* of *A. bisporus* (ProteinID 230069, http://genome.jgi.doe.gov/Agabi_varbisH97_2) encompassing its coding region with 750 bp up- and downstream sequences was amplified by PCR using genomic DNA of *A. bisporus* A15, primer pair C2h2Abo7wnF/ C2h2ABownR (Table 1) and Phusion Hot Start II High-Fidelity DNA polymerase (Thermo Fisher Scientific, Waltham, USA). The amplicon contained PacI and AscI linkers at its 5' and 3' ends, respectively, enabling its introduction in pBHgPA that had been cut with PacI and AscI. The resulting plasmid pKS273 was transformed to *Agrobacterium tumefaciens* AGL-1 (Chen *et al.,* 2000). Transformation of *A. bisporus* A15 gills was performed as described (Romaine and Chen, 2005). Transformants were screened on MEA plates containing 25 µg ml^{-1} hygromycin, 200 µM cefotaxime, and 100 µg ml^{-1} moxalactum. Transformants were transferred to a second selection plate containing 40 µg ml^{-1} hygromycin, 200 µM cefotaxime, and 17 µg ml^{-1} tetracycline.

Whole genome expression analysis

A. bisporus strain A15 was harvested 9 days after venting at a commercial hand-picking grower (Maatschap van den Heuvel, de Rips, The Netherlands). Mycelium in

the casing layer was obtained as well as 1-2 mm initials, buttons that started cap-stipe differentiation (CS), buttons that showed interior tissue differentiation (DIFF), and young fruiting bodies (YFB). Due to the method of cultivation, all developmental stages were present on the casing bed. The buttons in cap-stipe stage were divided in cap and stipe using a scalpel. Similarly, buttons in early tissue differentiation and young fruiting bodies were divided into components of the stipe (skin, underlying tissue and center) and cap (skin, underlying tissue, gill tissue and veil). Samples were randomly taken from two distinct places in the casing bed and were immediately frozen in liquid nitrogen. The casing mycelium sample was broken in pieces and kept frozen with liquid nitrogen while harvesting mycelium using cooled tweezers. Samples were homogenized using the TissueLyser II (Qiagen, Düsseldorf, Germany) and RNA was purified using the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany). Quality was assessed by gel electrophoresis and sent to ServiceXS (Leiden, the Netherlands) for Illumina Next Generation Sequencing.

The RNA-Seq pipeline used the TRIMMOMATIC read trimmer version 0.32 (Bolger *et al.,* 2014) to remove the ILLUMINA adapters from the raw 125 bp paired end reads and reads with low quality regions. These filtered reads were aligned by STAR aligner version 2.4.0f1 (Dobin *et al.,* 2013) to the *A. bisporus* v3.0 genome (Sonnenberg, unpublished data). The size of the introns was limited to 1500 bp based on the largest intron sizes in the genome annotation provided by JGI DOE. Abundance estimation was calculated with Cufflinks version 2.1.1 (Trapnell *et al.,* 2012a), and differential expression tests were performed by Cuffdiff (Trapnell *et al.,* 2012b). Proteins annotated to contain a DNA-binding or regulatory protein domain in the InterPro annotation predictions provided by JGI DOE were defined as transcription factors.

RESULTS

Whole genome expression analysis

RNA composition of strain A15 was determined of casing mycelium, 1-2 mm initials, buttons during cap-stipe differentiation (CS), buttons showing early signs of tissue differentiation (DIF), and young fruiting bodies (YFB). CS buttons, DIF buttons, and YFBs were dissected into stipe and cap. Stipe and caps of DIF buttons and YFBs were subdivided into skin, underlying tissue, and central tissue (stipes) and skin,

underlying tissue, gill tissue, and veil tissue (caps). 875 and 707 genes were up- and down-regulated in initials when compared to casing mycelium (Figure 1A). In CS button stipes and caps 1194 and 1496 genes were up-regulated when compared to initials, while 1788 and 2225 genes were down-regulated. The number of genes that were up-regulated in DIF tissues ranged from 39 to 147, while 105 to 503 genes were down-regulated when compared to the CS tissues. The number of up-regulated genes

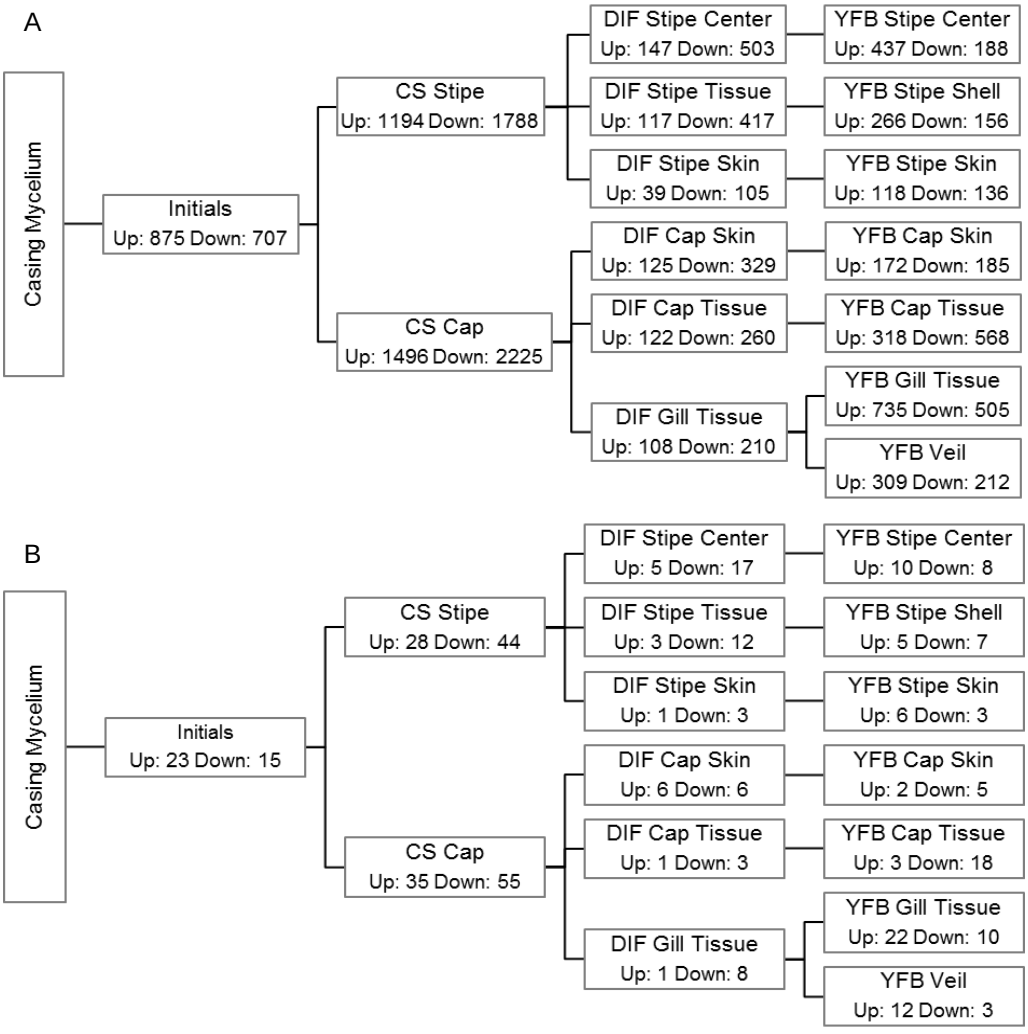

Figure 1 Number of total up- and downregulated genes (A) and up- and downregulated transcription factor genes (B) comparing initials, buttons in cap-stipe differentiation (CS), buttons showing early tissue differentiation (DIFF), and young fruiting bodies (YFBs) with the preceding developmental stage.

in YFB tissues ranged from 118 to 735 compared to the DIF tissues, while 136 to 568 genes were down-regulated.

The overall number of transcription factor genes that were up-regulated ranged from 1 to 35 when consecutive stages were compared, while the number of down-regulated regulatory genes ranged from 3 to 55 (Figure 1B). The most prominent changes were observed when initials and CS caps were compared (90 differentially expressed transcription factor genes), while only 4 transcription factor genes were differentially expressed in the transition from CS stipe to CS stipe skin and from CS cap to CS cap tissue (Figure 1B).

Expression of the *A. bisporus* orthologues of the blue light sensor gene *wc-1* and the transcription factor genes *wc-2*, *hom2*, *fst4*, *c2h2*, *fst3*, *gat1,* and *hom1* of *S. commune* (Morin *et al.,* 2012) was analysed. Transcript levels of *wc-2* and *c2h2* increased > 2 fold in initials compared to casing mycelium, while *hom1* levels decreased > 2 fold (Table 2; Supplementary Table 1). Expression of *wc-1* and *wc-2* was in general higher in the aerial structures when compared to the casing mycelium, in stipes when compared to caps, and in outer tissues when compared to inner tissues of the aerial structures. Genes *hom2* and *fst4* were ≥ 2 fold over-expressed when initials had developed in buttons. Like *wc-1* and *wc-2*, they were more highly expressed in stipes when compared to caps but in this case there was no difference between outer and inner tissues of the buttons and young fruiting bodies. Gene *c2h2* showed high expression at different stages of fruiting body development. Expression levels ≥ 4 fold were observed in initials, caps of CS buttons, gill tissue of DIFF buttons, and veil tissue of YFBs. Expression of *c2h2* was reduced ≥ 2 fold when compared to casing mycelium in YFBs in stipe and cap skin and in cap tissue. Increased (≥ 2 fold) levels of *fst3* were only observed in stipes of CS buttons and in stipe skin and tissue of DIFF buttons. Genes *gat1* and *hom1* showed the most aberrant expression. These genes were in general down-regulated when compared to casing mycelium. This effect was most prominent for *gat1.*

Over expression of c2h2 of A. bisporus results in faster production of mushrooms

Gene *c2h2* (protein ID 230069) of *A. bisporus* shares 79% identity with its homologue of *S. commune* (protein ID 1194000; http://genome.jgi.doe.gov/Schco3). Expression

construct pKS273 (see Material and Methods) encompassing *A. bisporus* gene *c2h2* was introduced in strain A15 using *A. tumefaciens* mediated transformation. This resulted in 10 transformants, 2 of which were picked for further analysis. qPCR showed a 2.5 and a 30 fold increased expression of *c2h2* in strains AT273-5 and AT273-1, respectively. Growth of these strains on MEA was similar to the parental strain.

Table 2 Fold changes in expression of orthologues of regulatory *S. commune* genes that are involved in mushroom formation during *A. bisporus* development. Expression was related to expression in the casing layer. Light and dark green shading indicates ≥ 2 fold and ≥ 4 fold reduced expression in the aerial structures. Light red, red, and dark red shading indicates ≥ 2 fold, ≥ 4 fold, and ≥ 10 fold increased expression in the aerial structures.

	wc-1	*wc-2*	*hom2*	*fst4*	*c2h2*	*fst3*	*gat1*	*hom1*
Casing Myc	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Initials	1.4	6.0	1.6	1.9	4.4	1.1	0.9	0.4
Buttons in cap-stipe differentiation								
Stipe	2.3	3.4	5.6	2.3	3.1	2.0	0.4	0.6
Cap	1.1	2.8	1.4	2.1	8.5	0.9	0.4	0.3
Buttons in early tissue differentiation								
Stipe Center	1.6	5.5	6.0	2.1	3.2	1.3	0.2	0.7
Stipe Tissue	3.4	11.2	6.0	2.1	1.2	2.2	0.2	0.9
Stipe Skin	2.7	8.2	4.7	2.0	1.6	2.0	0.4	0.9
Cap Skin	2,1	3.2	0.6	1.3	2.1	0.9	0.4	0.6
Cap Tissue	1.3	1.5	1.1	1.6	3.8	0.9	0.3	0.5
Gill Tissue	0.8	0.8	1.7	1.9	7.6	0.8	0.3	0.4
Young fruiting bodies								
Stipe Center	3.1	7.6	8.3	2.1	1.2	1.5	0.5	1.4
Stipe Shell	4.6	18.8	8.0	2.0	1.6	1.9	0.3	1.4
Stipe Skin	4.1	24.0	4.6	1.7	0.3	1.9	0..3	1.3
Cap Skin	3.7	8.2	0.5	0.9	0.4	1.0	0.4	1.1
Cap Tissue	2.8	2.3	1.0	0.8	0.1	1.0	0.3	0.8
Gill Tissue	3.8	9.1	1.4	1.3	3.0	1.4	0.4	1.1
Veil	2.3	3.3	1.7	1.8	6.7	1.4	0.4	0.7

Mushroom production of strains AT273-5 and AT273-1 was assessed in a semi-commercial setting. The first flush started 9 days after venting and progressed until day 14. The second flush took place between day 19 and day 22 (Figure 2). Mushrooms with a diameter of ≥ 40 mm were not formed at days 23-25. Biomass of mushrooms harvested at day 9-11 and at day 19-21 was higher for strains AT273-1 and AT273-5 when compared to A15 (Figure 2A). The latter strain produced more biomass at day 13 and 22, resulting in a similar total production of mushrooms for the 3 strains (Table 3). A similar result was found for the number of mushrooms that were

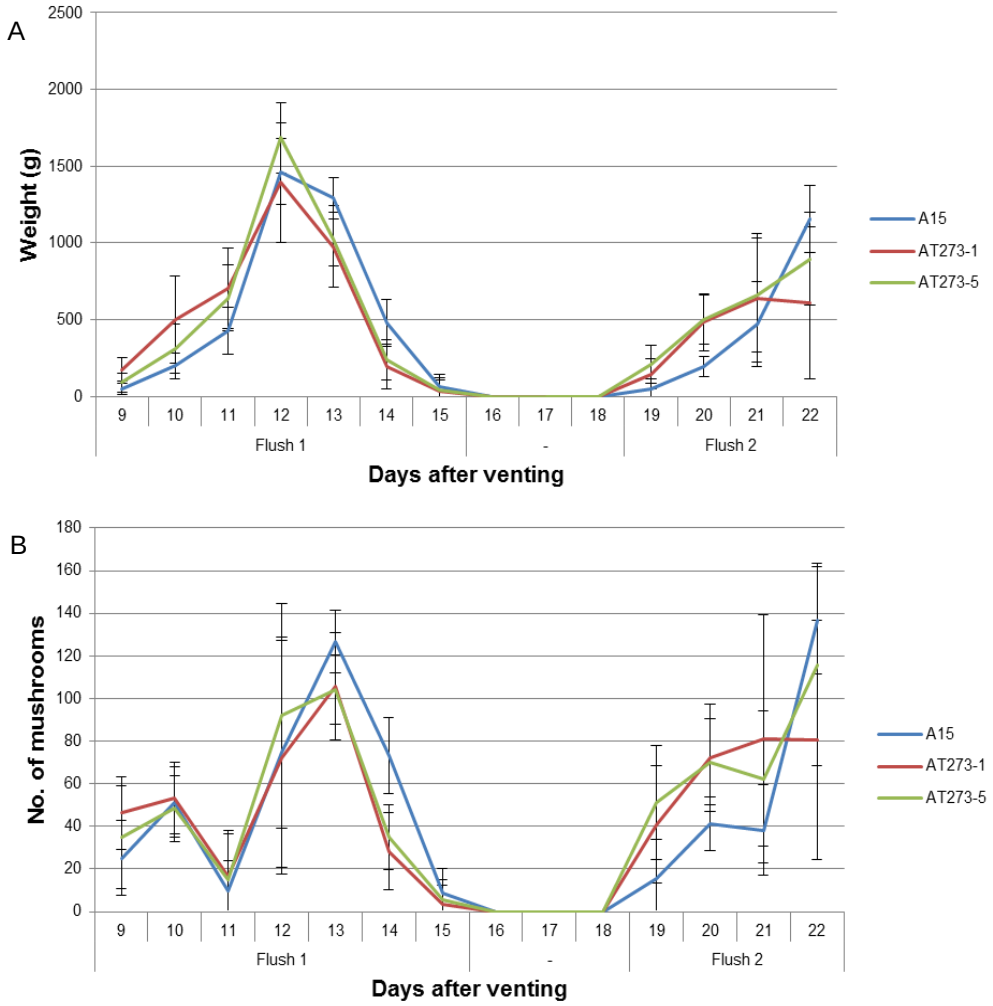

Figure 2 Average biomass (A) and number (B) of A15, AT273-1, and AT273-5 mushrooms picked per box during a 22-day period after venting (t = 0).

harvested each day (Figure 2B). Together, these data show that over-expression of *c2h2* accelerates development of mushrooms.

Harvested mushrooms were classified based on quality (class I and II) and size (size 40 [mushrooms with a cap ≤ 40 mm] and size 60 [mushrooms with a cap between 40-60 mm]) (Figure 3). During the first flush, A15 produced 11.4% of class I mushrooms. These numbers were 2% and 0.8% for AT273-1 and AT273-5, respectively. At the same time, A15 produced more size 40 mushrooms (57%), while

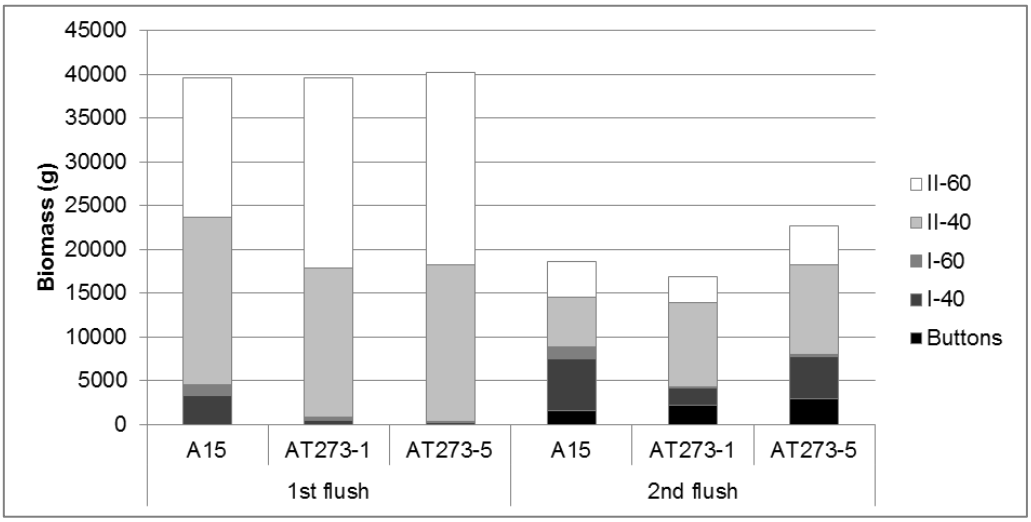

Figure 3 Quality and size composition of A15, AT273-1, and AT273-5 mushrooms harvested during two flushes.

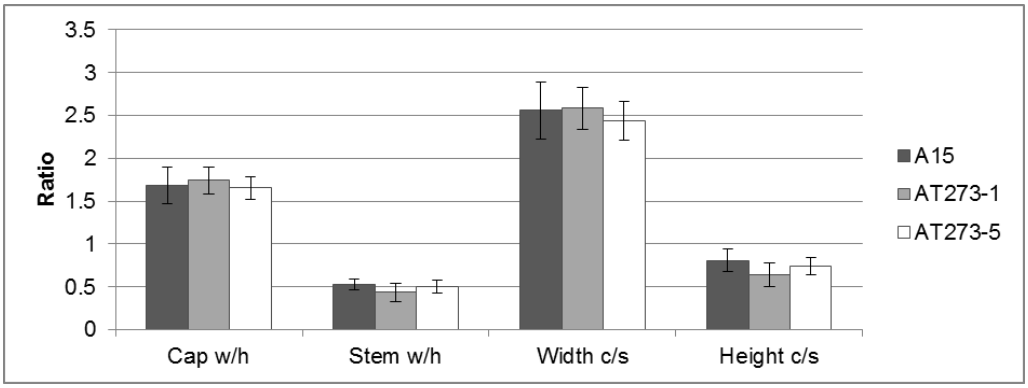

Figure 4 Ratios of width (w) and height (h) of caps and stems as well as ratios of widths of caps (c) and stems (s) and height of caps (c) and stems (s) of mushroom of A15, AT273-1, and AT273-5.

Table 3 Total weight and number of mushrooms per box of strains A15, AT273-1, and AT273-5 after 2 flushes.

	Weight (g)		Number of mushrooms	
Strain	Average	Standard deviation	Average	Standard deviation
A15	4198	200	600	115
AT273-1	4210	439	573	149
AT273-5	4324	236	635	91

the *c2h2* overexpressing strains produced more size 60 mushrooms (56 and 55%, respectively). Ratios between the height of the stem and the width of the cap as well as the width and height of the stem versus width and height of the cap were similar (Figure 4). A relative dry weight of 8% was found for the mushrooms of all 3 strains picked at day 12 and 13 (Figure 5). A15 also produced more class I mushrooms in the second flush when compared to AT273-1 and AT273-5 (39% versus 12 % and 23 %). All strains produced more size 40 mushrooms in this flush (62% for A15 versus 69 and 67% for AT273-1 and AT273-5). Relative dry weight at day 22 amounted between 6.2 and 6.7% for the three strains. Together, these data show that overexpression of *c2h2* affects quality of the mushrooms and promotes size in the first flush.

Mushroom formation was monitored by analyzing photos taken in 24 h intervals. Cap expansion was similar for the 3 strains (Figure 6B). The number of buttons emerging from the casing was not significantly different between the three strains but there was a trend that the *c2h2* over-expression strains showed accelerated button emergence (Figure 6A).

DISCUSSION

Formation of mushrooms is a highly complex developmental process (Kues, 2000). After a submerged mycelium has been formed, hyphae escape the substrate to grow into the air. These hyphae form aggregates with a diameter < 1 mm. They result from a single hypha that branches intensely or arise from branches of neighboring aerial hyphae that grow towards and alongside each other. The dark-grown aggregate *of C. cinerea* can develop into different structures depending on light. Continuation of growth in the dark results in formation of sclerotia, while a 12h day-night cycle induces

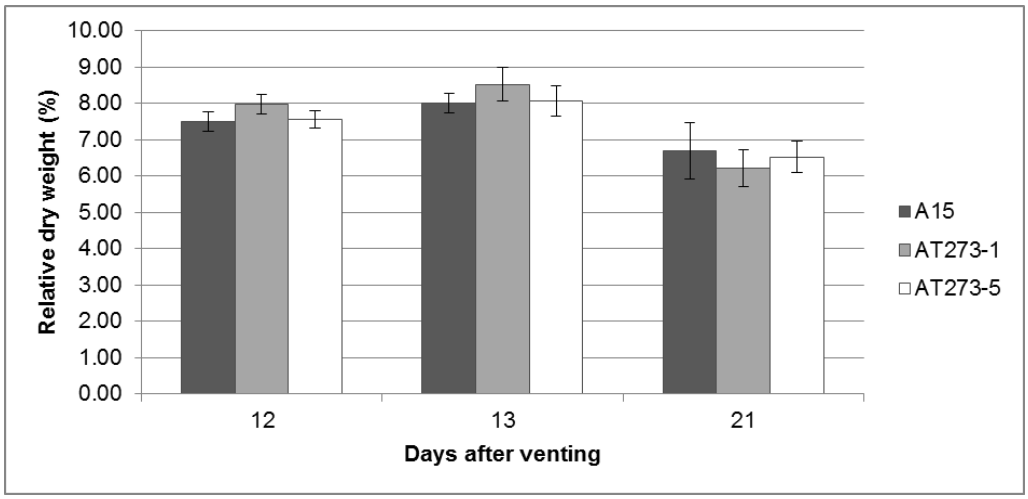

Figure 5 Dry weight of A15, AT273-1 ,and AT273-5 mushrooms harvested 12, 13, and 21 days after vent-off.

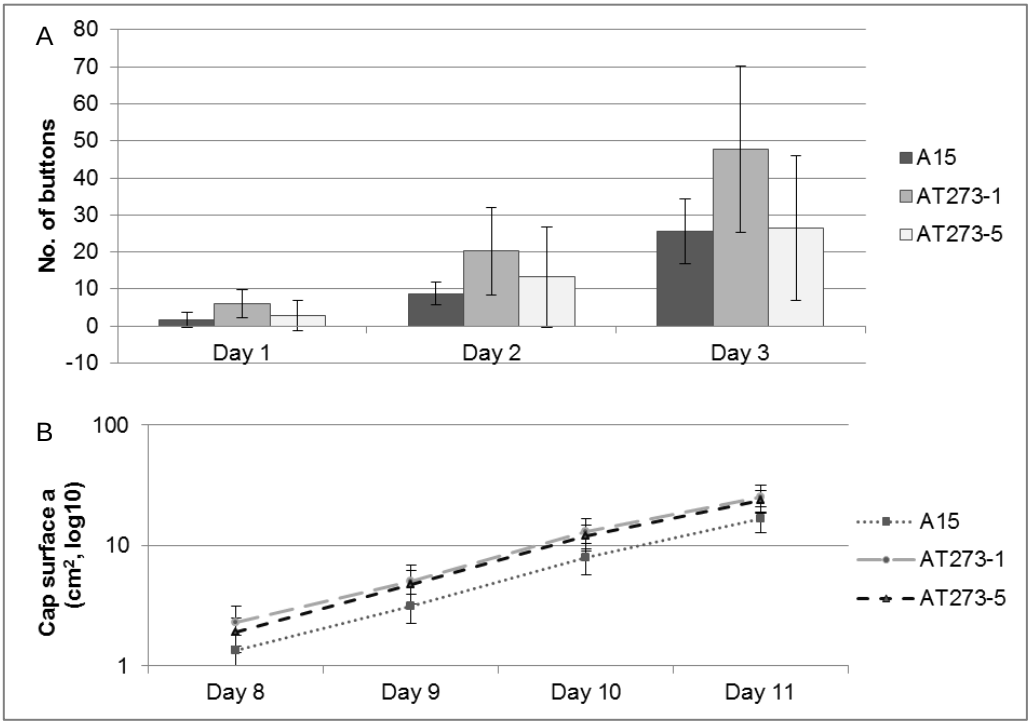

Figure 6 Number of A15, AT273-1 and AT273-5 buttons that had emerged from the casing soil 1, 2, and 3 days after venting (A) and expansion of A15, AT273-1, and AT273-5 mushroom cap size in time (B).

development of initials. Initials, or primordia, are the first fruiting body specific structures and can be selectively stained with Janus green (Sánchez and Moore, 1999). The switch from aggregate to primordia can therefore be considered key in development. The fact that *c2h2* of *S. commune* is involved in the switch from aggregates to primordia (Ohm *et al.,* 2011) makes it a gene of high interest for fruiting body development. Here the *c2h2* orthologue of *A. bisporus* was over-expressed resulting in accelerated mushroom formation and reduced quality of the fruiting bodies.

Expression of *c2h2* was studied during development of fruiting bodies starting at initiation of aerial growth. Gene *c2h2* was ≥ 4 fold up-regulated in initials of *A. bisporus* when compared to the casing mycelium. Up-regulation was also found in very young buttons (buttons with cap and stipe differentiation; CS buttons) and in young buttons with tissue differentiation (DIFF buttons). Up-regulation of *c2h2* was particularly found in cap tissue. Expression of *c2h2* was reduced or ≤ 2 fold up-regulated in the tissues of young fruiting bodies (YFBs) with the exception of veil and gill tissue. These expression data indicate that *c2h2* functions in aerial structures early in fruiting body development, while it also seems to have a role in selective tissues of young mushrooms.

Gene *c2h2* of *A. bisporus* was over-expressed in strain A15. Two transformants were selected for phenotypic analysis. These strains, called AT273-1 and AT273-5, displayed 30 fold and 2.5 fold increased *c2h2* expression, respectively. Phenotypes of these strains were similar, indicating that a few fold increased expression of *c2h2* is sufficient to obtain a full effect of overexpression. Morphology, cap expansion rate, and total numbers and biomass of mushrooms harvested throughout culturing were not affected by overexpression of *c2h2*. However, formation of mushroom with a cap size ≥ 4 cm was accelerated. Biomass and number of mushrooms were increased on day 9 to11 (first flush) and 19 to 21 (second flush) in the transformants when compared to A15. On the other hand, A15 produced more biomass and mushrooms on day 13 and 22. The fact that expansion rate of mushrooms was similar between AT273-1 and AT273-5 and A15 implies that accelerated mushroom formation is caused early in development at the level of outgrowth of initials. This is supported by quantitative analysis of the number of buttons that had emerged from the casing soil 1, 2, and 3 days after venting. A trend

was observed that the transformants had formed more of these developmental structures when compared to A15. Accelerated mushroom formation in AT273-1 and AT273-5 was accompanied by reduced quality of the harvested mushrooms. This may be explained by the expression profile of *c2h2* in wild-type *A. bisporus.* High expression is needed in early stages of development, while reduced expression in young fruiting bodies ensures mushroom quality. Thus, it is proposed that for an optimal effect *c2h2* should be over-expressed early in mushroom development but not at later stages.

Apart from *c2h2, wc-1, wc-2, hom2, fst4, fst3, gat1,* and *hom1* play a role in *S. commune* fruiting body development (Ohm *et al.,* 2010b; 2011; 2013). These genes have homologues in other mushroom forming fungi (Ohm *et al.,* 2010b). It is difficult to compare the expression study performed in this work with those of Ohm *et al.* (2010b), Morin *et al.* (2012), and Plaza *et al.* (2014). Culturing conditions and genetic background of the mycelium used as reference expression was not similar and in some studies whole cultures were used for RNA extraction, while in other studies pure developmental stages were used. For instance, in this work RNA from a fertile casing mycelium was used as reference for differential expression, while Plaza *et al.* (2014) used fertile vegetative mycelium from a culture grown between complete medium and a cellophane membrane. Ohm *et al.* (2010b, 2011, 2013) compared whole cultures of a sterile monokaryotic vegetative mycelium with whole cultures of the fertile dikaryon at different developmental stages. In contrast, we here used pure developmental structures and tissues. This makes that comparative transcriptomics will only result in trends in this case. Therefore, we restrict comparison of *c2h2, wc-1, wc-2, hom2, fst4, fst3, gat1,* and *hom1* expression at different stages of *A. bisporus* and *S. commune* fruiting development (Ohm *et al.,* 2010b, 2011, 2013, Chapter 2). Expression of *c2h2* in *S. commune* is highest in primordia and mature fruiting bodies (Chapter 2), which is in agreement with the findings in *A. bisporus*. The genes encoding the blue light sensing complex components Wc-1 and Wc-2 are also most highly expressed in primordia and fruiting bodies of *S. commune.* This agrees with the finding that the *A. bisporus* homologues were more highly expressed in aerial structures when compared to casing mycelium, in stipe tissue when compared to cap tissue and in outer tissues of the aerial structures when compared to inner tissues. *A. bisporus* does not require blue light to produce mushrooms. Yet blue light sensing is probably required to induce

genes that are involved in light-related DNA damage repair (e.g. photolyase) and in conversion of highly photoreactive porphyrins that are formed during heme synthesis (ferrochelatase).. Expression of *hom2* of *S. commune* is similar in vegetative mycelium and aerial structures until the stage of mature fruiting bodies where expression drops, while expression of *fst4* and *fst3* is more or less constitutive. Expression profiles of the latter genes of *A. bisporus* is similar but that of *hom2* is different. Gene *hom2* was ≥ 2 fold over-expressed when initials had developed in buttons. Like *wc-1* and *wc-2*, expression of *hom2, fst4,* and *fst3* were more highly expressed in stipes when compared to caps but no huge differences were observed between outer and inner tissues of the buttons and young fruiting bodies. Genes *gat1* and *hom1* of *S. commune* are most highly expressed in late stages of mushroom development, although their up-regulation is restricted. These genes have a different expression profile in *A. bisporus.* They were in general down-regulated when compared to casing mycelium. This effect was most prominent for *gat1.* It may thus be that their role in *S. commune* and *A. bisporus* is different. Together, these data support the view that mushroom development in the Basidiomycota follows a core regulatory program with species specific variations that may explain differences in morphology and sensitivity to environmental signals.

ACKNOWLEDGEMENTS

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Supplementary Table 1 Expression levels (FPKM) of orthologues of regulators of mushroom formation in *S. commune* during development of *A. bisporus.*

	wc-1	*wc-2*	*hom2*	*fst4*	*c2h2*	*fst3*	*gat1*	*hom1*
Casing Myc	64	139	50	120	45	74	284	438
Initials	88	827	82	233	196	84	250	166
Buttons in cap-stipe differentiation								
Stipe	145	474	281	289	140	155	113	250
Cap	71	382	69	249	384	65	119	150
Buttons in early tissue differentiation								
Stipe Center	104	772	301	251	143	93	50	312
Stipe Tissue	214	1558	302	251	55	166	69	378
Stipe Skin	174	1145	234	242	70	152	118	386
Cap Skin	137	448	32	155	94	63	123	276
Cap Tissue	86	212	55	192	172	69	95	211
Gill Tissue	51	111	83	234	343	61	86	179
Young fruiting bodies								
Stipe Center	197	1050	416	286	52	113	135	618
Stipe Shell	297	2620	405	246	73	138	96	627
Stipe Skin	264	3335	232	198	13	139	77	585
Cap Skin	236	1136	25	105	18	75	105	472
Cap Tissue	181	328	51	99	6	75	72	351
Gill Tissue	242	1267	69	161	137	101	116	492
Veil	149	462	86	221	302	98	115	296

Chapter 6
Summary and General discussion

Mushrooms are of great value as a food source. For instance, the European Union produces one billion kilogram of *Agaricus bisporus* mushrooms, also known as white button mushrooms, of which 25% originates from the Netherlands. With a sales of € 300 million annually, this mushroom represents the third most important “vegetable” crop in the Netherlands after tomato and paprika. Mushrooms are considered a good source of digestible protein with contents higher than most vegetables and only somewhat less than meat products and milk. Moreover, they contain vitamins, minerals, and bioactive compounds such as anti-cancer polysaccharides. The market for mushrooms is predicted to increase in the future in view of the increasing demand of high quality food with an increasing world population. *Agaricus bisporus* is difficult to study under laboratory conditions. For instance, it has a relatively long life cycle of 6 weeks and does not fruit on defined medium. In contrast, *Schizophyllum commune* has a relatively short life cycle of 7 days and fruits on defined synthetic media. Moreover, transformation procedures and inactivation of genes are relatively well established. This is why *S. commune* is used as a model system for mushroom development.

Mushrooms are mainly formed by basidiomycetes. These fruiting bodies produce sexual spores that are dispersed. Upon germination, these spores give rise to a vegetative mycelium that colonizes organic substrates or that interact with plants (mycorrhiza such as the fly agaric) or animals (e.g. *Cordyceps*, that is parasitic on insects and other arthropods). Heterothallic basidiomycetes such as *S. commune* fuse with a compatible mating partner (i.e. a partner with different mating type loci) to form a fertile heterokaryotic mycelium that is capable of forming fruiting bodies. Secondary heterothallic basidiomycetes such as *A. bisporus* contain compatible nuclei in the sexual spores and thus do not have to fuse to form a fertile mycelium. Environmental factors such as O_2 and CO_2 levels, light, temperature, humidity, volatiles, pH, salinity, and availability of nutrients play a role in initiation of mushroom formation by a fertile mycelium (Madelin, 1956, Manachère, 1980; Kües and Liu, 2000; Eastwood *et al.,* 2013; Pelkmans *et al.,* 2016; Chapter 1). Formation of white button mushrooms by *A. bisporus* is repressed by 1-octen-3-ol and high CO_2 levels, while low temperature stimulates mushroom formation. On the other hand, fruiting body development is responsive to blue light in *S. commune*, while also in this case high CO_2 levels repress fruiting (Perkins, 1969; Raudaskoski and Yli-Mattila, 1985, Ohm *et al.,* 2013;

Eastwood *et al.,* 2013). Fruiting body development starts with aggregation of aerial hyphae. These aggregates develop into primordia that further develop into mushrooms with different tissues. For instance, basidia are formed in the gill tissue giving rise to basidiospores. Initiation of fruiting in *S. commune* is preceded by a switch from symmetrical to asymmetrical vegetative colony due to local repression of growth.

Signaling of favorable environmental conditions leads to transcription factor activity. A set of *S. commune* transcription factors and a blue light sensor have been identified that are involved in fruiting body development (Ohm *et al.,* 2010b; 2011; 2013). Deletion of the blue light sensor gene *wc-1* and the transcription factor genes *wc-2* or *hom2* results in symmetrical colonies that do not form aggregates, primordia or fruiting bodies. Inactivation of *fst4* represses aggregate formation, while strains in which *c2h2* is inactivated do form aggregates but not primordia. On the other hand, deletion strains of *fst3*, *gat1* or *hom1* do form fruiting bodies, but they are smaller and present in higher numbers. Homologues of these genes have been identified in *Laccaria bicolor* (bicoloured deceiver), *Coprinopsis cinerea* (ink cap mushroom), and *A. bisporus* (Ohm *et al.,* 2010b; Morin *et al.,* 2013; Plaza *et al.,* 2014). Comparison of expression of these homoloques suggests that mushroom development in basidiomycetes follows a core regulatory program with species specific variations that explain differences in morphology and sensitivity to environmental signals (Ohm *et al.,* 2010b; Morin *et al.,* 2012; Plaza *et al.,* 2014). Gene *bril* of *S. commune* also has been reported to be involved in fruiting. However, in Chapter 2 it is shown that this strain can produce mushrooms albeit at slower rate.

The aim of this thesis was to further characterize the regulatory network of fruiting body development in *S. commune* and to link this network to environmental signalling. Furthermore, I started to modulate mushroom formation in *A. bisporus* using the knowledge obtained in *S. commune*.

Environmental signaling

Fruiting body development is often, if not always, the result of a combination of environmental conditions (Pelkmans *et al.,* 2016; Chapter 1). *S. commune* grows within a solid substrate witnesses darkness and is exposed to high CO_2 levels. The latter is the result of a high rate of aerobic respiration by *S. commune* combined with

slow diffusion into the environment due to the compactness of the substrate. At the moment *S. commune* hyphae reach the surface of the substrate, they will be exposed to light and to low CO_2. These conditions are indicative for a favorable environment enabling formation of aerial reproductive structures. In this Thesis it was shown that CO_2 and blue light not only impact fruiting initiation but also later stages development. By transferring colonies from low to high CO_2 conditions it was shown that all developmental stages are repressed by this compound (Chapter 3). Blue light was shown to be at least essential for aggregation of aerial hyphae and for development of primordia (unpublished data). Mushroom development in *C. cinerea* involves at least five light sensitive phases (Kües, 2000; Lu, 2000). Light is required for the formation of initials, as well as the development of primordia and karyogamy. However, it represses hyphal knot formation and completion of meiosis. The impact of light and CO_2 on different stages of fruiting may enable the fungus to halt development when environmental conditions change, for instance when the fruiting body becomes covered with litter.

S. commune and *C. cinerea* contain functional copies of the blue light sensing White Collar complex. Deletion of genes encoding components of this complex, i.e. *wc-1* and *wc-2,* results in a blind phenotype (Kamada *et al.,* 2010; Ohm *et al.,* 2013). Blue light is not essential for fruiting in *A. bisporus*. Yet expression of homologues of *wc-1* and *wc-2* in *A. bisporus* is regulated on tissue level (Chapter 5). Most likely, blue light sensing is required to induce genes involved in light-related DNA damage repair (photolyase) and in conversion of highly photoreactive porphyrins that are formed during heme synthesis (ferrochelatase).

CO_2 sensing has so far been mainly studied in plant and human pathogens. In these organisms the cAMP-Pka signaling pathway is used. In Chapter 3 it was addressed whether this pathway is also used for CO_2 sensing in *S. commune*. The genome of this basiodiomycete contains all components of a CO_2-cAMP-Pka signaling pathway. This pathway starts with the conversion of CO_2 in bicarbonate by carbonic anhydrase. The bicarbonate activates adenylase cyclase. As a result, cAMP is formed that releases two regulatory subunits from the two catalytic subunits of the Pka complex. This enables the catalytic subunits to phosphorylate RRXS/T motifs in target proteins. Phosphorylation can influence protein stability, protein-protein interactions, protein localization and, in the case of transcription factors, their ability to bind DNA

(Whitmarsh and Davis, 2000). Levels of cAMP, and thereby Pka activity, can be controlled within the cell by phosphodiesterase (PDE) activity. Protein location prediction programs indicate that bicarbonate production takes place in the cytosol, while cAMP production and degradation as well as Pka activation and phosphorylation of target protein take place within the nucleus.

Gene *sca2* encoding the cytosolic carbonic anhydrase was the only gene of the CO_2-cAMP-PKA signaling pathway that was differentially expressed when low and high CO_2 conditions were compared. This indicates that transcriptional regulation of CO_2 signaling only occurs at the beginning of this pathway. Constitutive expression of the other components makes sense considering the fact that the cAMP-PKA pathway is also involved in signaling of other environmental conditions. For instance, Ras increases cAMP levels in *S. cerevisae* as a response to carbon source levels (Tamanoi, 2011). Indeed, Sac1 of *S. commune* has LRR, PP2C, CYCc and putative Ras interaction domains that would integrate different inputs from the environment and, as a result, would give a go or no go for fruiting to take place. Concentration or the nature of the carbon source could be such an input apart from CO_2. This is indicated from the findings that transfer of colonies to a medium without glucose abolishes fruiting, while transfer to a plate with high glucose levels increases fruiting incidence.

Addition of cAMP to the medium resulted in symmetrical colonies that do not form aggregates, primordia or fruiting bodies (Chapter 3). Thus, high cAMP levels repress fruiting body formation. This finding is supported by the fact that overexpression of the phosphodiesterase gene *pde2* resulted in increased fruiting even under repressing levels of exogenous cAMP or CO_2. On the other hand, repression of Pde2 activity by IBMX reduced mushroom formation even under low CO_2 conditions (Chapter 3). Together, this set of data show a clear link between high CO_2 and cAMP levels and repression of fruiting and thus concludes the debate whether cAMP would have a repressing or inducing role in fruiting of *S. commune* (Yamagishi *et al.,* 2002; 2004; Schwalb, 1974; 1978; Yli-Mattila, 1987; Uno and Ishikawa, 1971). I propose that CO_2 sensing in *A. bisporus* is similar to that of *S. commune.* The white button mushroom fungus also has all components of a functional CO_2-cAMP-Pka pathway. Thus, identification of an inhibitor of *A. bisporus* adenylyl

cyclase or a cAMP antagonist would enable spatial and temporal control of mushroom production in a commercial setting.

Pka phosphorylates proteins with a RRXS/T motif(s). Hom2 of *S. commune* contains 4 of these motifs and was therefore proposed to be a target for Pka (Chapter 4). Therefore, a derivative of *hom2* was created in which the serine codons within the Pka motifs were replaced for alanine codons. Introduction of this *hom2*^{con} derivative in a wild-type *S. commune* monokaryon resulted in colonies with reduced colony extension. This indicated that *hom2* in the non-phophorylated state represses vegetative growth. Thus, Pka activity at high CO_2 levels would lead to an inactive phosphorylated Hom2 form resulting in unaffected growth within the substrate. The inhibition of growth in the *hom2*^{con} strains was much more dramatic when compared to wild-type colonies grown at low CO_2. This suggests that only a fraction of *hom2* is dephosphorylated when grown in this condition. This fraction may be (slightly) different between hyphae and could explain why colonies of a dikaryon in the light and at low CO_2 show irregular growth. Some areas would show repression of growth due to a relatively low phosphorylation state of Hom2, while growth in other areas is not affected due to a relatively high phosphorylation state.

The monokaryotic *hom2*^{con} strains not only showed a growth effect but even fruited (albeit spores were not formed) when exposed to light and low CO_2, thus resembling a dikaryon (Chapter 4). This suggests that Hom2 is maintained in a phosphorylated state in the monokaryon and that this state is the only inhibitory factor that suppresses fruiting in such strains in conditions favorable for mushroom formation in the dikaryon. Data also indicate that the role of the mating type genes in sexual development is less directive than previously assumed. The mating type genes would function in the establishment and maintenance of the dikaryotic state, but not in the fruiting process. The mating types would also be important for sporulation. Basidiospores were not observed in monokaryotic *hom2*^{con} fruiting bodies, but were formed in *hom2*^{con} dikaryotic strains. Together, Hom2 is proposed to be a main switch in mushroom formation. Ortologues of Hom2 were identified in the genomes of other mushroom forming basidiomycetes. All predicted orthologues have 3-5 RRXS boxes suggesting that Hom2 fulfills a similar role in other mushroom forming fungi.

Fruiting in monokaryotic and dikaryotic *hom2*^{con} strains was sensitive to high CO_2 and high cAMP levels (Chapter 4) as observed in wild-type strains (Chapter 3).

Apparently, there are other targets of PKA that are essential for the initiation of fruiting. I propose the existence of a target of Pka that acts at a later stage than Hom2 at the moment aggregate formation is initiated (Figure 1). This protein may be an activator or a repressor and the role of Pka would be to repress or activate this factor, respectively.

High CO_2 levels also impact expression of other regulators involved in mushroom formation

Transcription factor genes were identified that are either up- (18 genes) or down- (18 genes) regulated under high CO_2 conditions (Chapter 3). These genes may encode regulators of mushroom formation. Indeed, genes *wc-2*, *c2h2*, and *hom1* were among these down-regulated genes (Figure 1). The fact that *wc-2* is down-regulated under high CO_2 conditions (Chapter 3), while components of the CO_2-cAMP-Pka pathway are not affected by light conditions (unpublished data) indicates that CO_2 signaling functions upstream of light regulation. This is supported by the finding that fruiting structures are never formed in the light under high CO_2 conditions but that these structures are occasionally formed in the dark under low CO_2 conditions.

Down-regulation of *hom1* by high CO_2 levels is interesting considering the fact that this gene is also down-regulated by Gat1 and Fst3 and up-regulated by Wc-2 and Hom2 (Figure 1). The opposite effects of high CO_2 and Hom2 is expected since the former results in an inactive form of the latter. Similarly, high CO_2 results in lower levels of *wc-2* mRNA and, thus, also in this case an opposite effect of high CO_2 and *wc-2* on *hom1* expression can be expected. Gene *c2h2* is also up-regulated by Wc-2 and Hom2 (Figure 1) and this is also in line with its down-regulation by CO_2. The fact that *c2h2* of *S. commune* is involved in the switch from aggregates to primordia (Ohm *et al.,* 2011) makes it a gene of high interest to study fruiting body development in *A. bisporus*. Therefore, its *c2h2* orthologue was over-expressed in the commercial strain A15. Morphology, cap expansion rate, and total numbers and biomass of harvested mushrooms was not affected by overexpression of *c2h2*. However, formation of mushrooms was accelerated (Chapter 5). Accelerated mushroom formation was accompanied by reduced quality of the harvested mushrooms. These phenotypes can be explained considering expression of *c2h2* in wild-type *A. bisporus.* This gene is up-regulated in initials, during cap stipe differentiation, and during tissue development. In

contrast, it is down-regulated in tissues of young fruiting bodies with the exception of veil and gill tissue. Overexpression of *c2h2* results in high mRNA levels throughout development. High expression is needed in early stages of development, which would explain the phenotype of accelerated mushroom formation upon over-expression of c2h2. On the other hand, over-expression of *c2h2* in late stages may impact

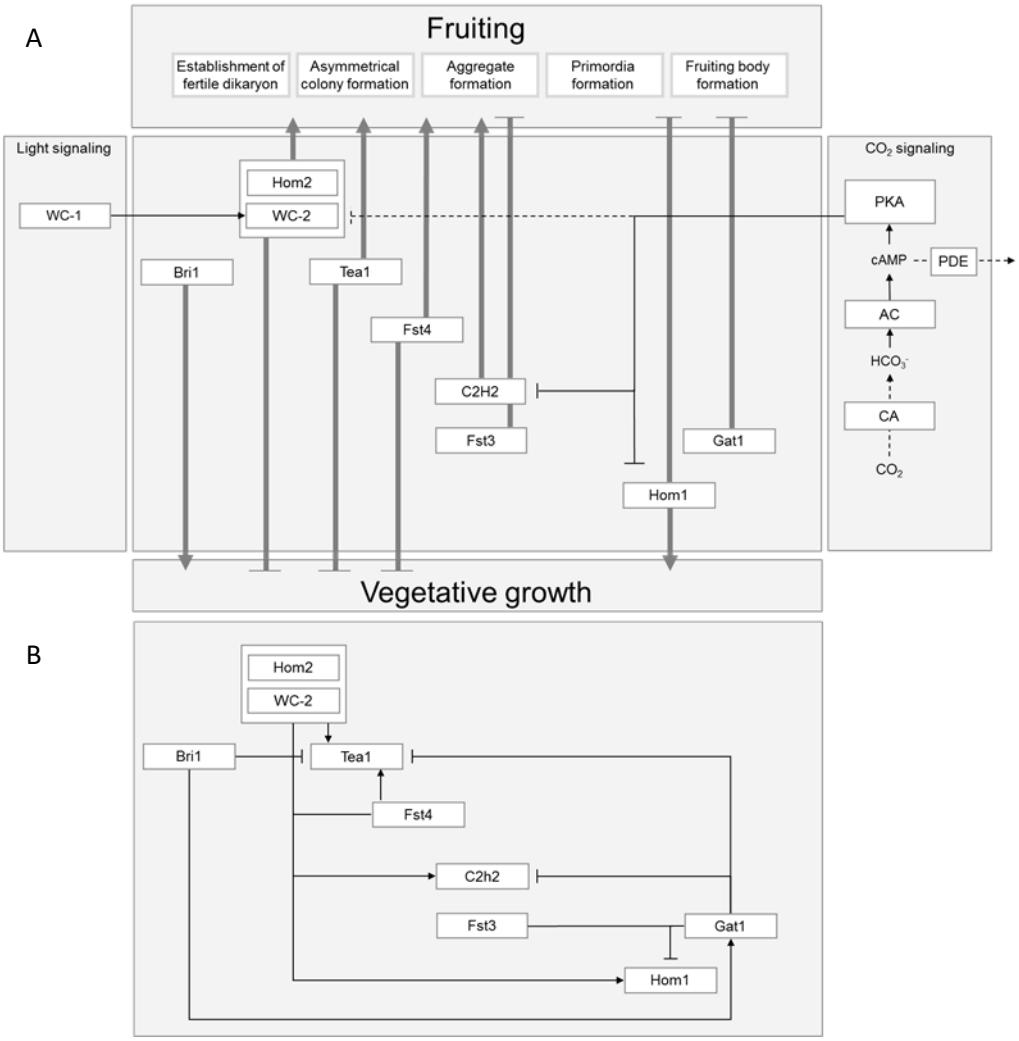

Figure 1 Model for the role of transcription factors involved in regulation of vegetative growth and fruiting body development and the link to environmental signaling (A) and the interaction between these transcription factors (B). Dotted line from Pka to Wc-2 represents a significant but <2-fold effect of CO_2 on Wc-2. See text for details.

mushroom quality considering the low expression of this gene in wild-type mushrooms. The over-expression studies of *c2h2* in *A. bisporus* and the deletion studies of this gene in *S. commune* support the view that mushroom development in basidiomycetes follows a core regulatory program with species specific variations that explain differences in morphology and sensitivity to environmental signals (Ohm *et al.,* 2010b; Morin *et al.,* 2012; Plaza *et al.,* 2014).

Biomass formation and mushroom formation

Previously, the role of the *S. commune* transcription factor genes *wc-2*, *hom2, fst4*, *brlI*, *c2h2*, *fst3*, *gat1,* and *hom1* in fruiting body formation has been reported (Ohm *et al.,* 2010b; 2011; 2013). Chapter 2 and 4 describe that part of these transcription factors are also involved in regulation of vegetative growth (Figure 1). Hom1, that is involved in late stages of development, was found to stimulate growth of the mycelium in the substrate. In contrast, Hom2, Fst4, and Wc-2 that are involved in early stages of fruiting repressed vegetative growth. Notably, the Δ*hom2*Δ*hom2*Δ*fst4*Δ*fst4* formed more biomass than Δ*hom2*Δ*hom2* and Δ*fst4*Δ*fst4* in liquid shaken cultures. This indicates that Fst4 and Hom2 represent different branches of the repression pathway of vegetative growth. A Δ*tea1*Δ*tea1* strain also formed more biomass when compared to the wild-type. Moreover, it was severely affected in mushroom formation (Chapter 2). The phenotype of this transcription factor gene is in line with its expression profile. Gene *tea1* was down-regulated in Δ*hom2*Δ*hom2,* Δ*fst4*Δ*fst4,* Δ*wc-1*Δ*wc-1,* and Δ*wc-2*Δ*wc-2*, while it was up-regulated in Δ*fst3*Δfst3, Δ*hom1*Δ*hom1*, and Δ*gat1*Δ*gat1* (Figure 1). These data indicate that Tea1 functions downstream of Wc-1, Wc-2, Hom2, and Fst4. However, blue light and / or CO_2 signaling are not channeled via this protein because Δ*tea1*Δ*tea1*does occasionally form fruiting structures, whereas this is not observed in Δ*hom2*Δ*hom2*, Δ*wc-1*Δ*wc-1*, and Δ*wc-2*Δ*wc-2*. The results described in Chapter 2 represent a direct link between repression of vegetative growth and induction of sexual reproduction. A higher growth rate as observed in the Δ*hom2*Δ*hom2*, Δ*fst4*Δ*fst4,* and Δ*wc-2*Δ*wc-2* may be of interest for the biotech industry that uses fungi to produce proteins, organic acids or other metabolites. It may also be of interest for the mushroom industry to create a dual-inoculant system (Woolston *et al.,* 2011) employing a fast colonizing feeding mycelium not able to fruit in combination with a strain that is less effective in colonization but produces high yields of high

quality mushrooms. In this concept the mycelia of these strains should fuse for instance in the casing layer.

Repression of vegetative growth by a reproduction pathway has also been observed in *Aspergillus* where repression of vegetative growth is linked to asexual development (Krijgsheld *et al.,* 2013). This link involves the activity of FlbA that regulates the Gα-subunit of a heterotrimeric G-protein (Yu *et al.,* 1996). Inactivation of *flbA* results in a strain that cannot initiate asexual development and, as a consequence, grows throughout the colony instead of growing at the periphery only. Notably, *S. commune* has a homologue of *flbA* called *thn*. Inactivation of *thn* results in a strain unable to form fruiting bodies (Fowler and Mitton, 2000). This suggests that similar signaling pathways are involved in the decision to stop vegetative growth and to invest in reproduction in ascomycetes and basidiomycetes.

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Nederlandse Samenvatting

Paddenstoelen zijn van grote waarde als voedingsbron. Ze bevatten hogere hoeveelheden eiwit dan de meeste groentes en slechts enigszins lager dan vlees- en melkproducten. Daarnaast bevatten ze vitamines, mineralen en bioactieve stoffen zoals polysachariden met antikanker eigenschappen. De Europese Unie alleen al produceert 1 miljard kilogram champignons, waarvan de Nederlandse bijdrage 25% bedraagt. De markt voor paddenstoelen zal in de toekomst groter worden door een toenemende vraag naar hoogwaardige voeding om de groeiende wereldbevolking te onderhouden. Om deze reden zullen nieuwe manieren gevonden moeten worden om de efficiëntie van de productie van champignons en andere paddenstoelen te verhogen. Moleculair onderzoek kan hier een bijdrage aan leveren. Echter, de champignon, *Agaricus bisporus*, is moeilijk te onderzoeken in het laboratorium. De schimmel groeit relatief langzaam ten opzichte van andere soorten en vormt geen paddenstoelen onder deze omstandigheden. Verder zijn essentiële moleculaire technieken zoals transformatie en deletie van genen nog niet voldoende ontwikkeld. Dit in tegenstelling tot *Schizophyllum commune* (het waaiertje) welke een korte levenscyclus heeft, paddenstoelen vormt op een gedefinieerd medium en waarvoor moleculaire technieken zijn ontwikkeld. Om deze reden wordt deze schimmel gebruikt als model organisme voor paddenstoelvorming.

Paddenstoelen worden vooral gevormd door schimmels uit de groep Basidiomycota. Deze vruchtlichamen vormen seksuele sporen die verspreid worden. Kieming van de sporen leidt tot groei van een mycelium dat organische substraten kan koloniseren of een interactie kan aangaan met planten of dieren. Bij heterothallische basidiomyceten fuseren compatibele partners, waaruit een vruchtbare kolonie ontstaat die paddenstoelen kan vormen. Secundair heterothallische basidiomyceten, zoals de champignon, bevatten in het mycelium reeds compatibele kernen en hoeven dus geen compatibele partner meer te vinden.

Omgevingsfactoren zoals zuurstof, koolstofdioxide, licht, temperatuur, vochtigheid, vluchtige stoffen, pH, zoutgehalte en aanwezigheid van voedingstoffen spelen een rol in de start van paddenstoelvorming. In het geval van de champignon hebben 1-octen-3-ol en hoog CO_2 een remmende werking op paddenstoelvorming, terwijl een lage temperatuur het proces stimuleert. Een hoog CO_2 niveau heeft ook een remmende werking in *S. commune*, terwijl blauw licht cruciaal is voor het vormen van paddenstoelen. Wanneer de omstandigheden gunstig zijn, zal de schimmel

starten met het vormen van paddenstoelen. Luchthyfen uit het mycelium bundelen samen en vormen aggregaten. Vervolgens ontwikkelen deze zich bij aanhoudende gunstige condities tot primordia en uiteindelijk worden hieruit paddenstoelen gevormd. In de lamellen van de paddenstoel ontwikkelen zich basidia (steeltjes), waaruit basidiosporen worden geproduceerd, waarmee de levenscyclus rond is.

Informatie over de status van de omgeving wordt in de schimmel vertaald naar activiteit van transcriptie factoren. Enkele van deze genen, en hun rol in paddenstoelvorming, zijn in het verleden al geïdentificeerd. Deletie van zowel de blauw licht sensor *wc-1* als transcriptie factor *wc-2* leidt tot symmetrische kolonies die geen aanzet doen tot het vormen van vruchtlichamen. Hetzelfde fenotype is geobserveerd voor de deletie van *hom2*. Wanneer *fst4* wordt uitgeschakeld stopt de vorming van aggregaten, terwijl het verwijderen van *c2h2* resulteert in het onvermogen om primordia te vormen. Het uitschakelen van *fst3*, *hom1* of *gat1* heeft als gevolg dat kleinere paddenstoelen worden gevormd, maar in grotere aantallen. Homologen van deze genen zijn aanwezig in *Laccaria bicolor* (de tweekleurige fopzwam), *Coprinopsis cinerea* (de wortelende inktzwam) en *A. bisporus*. Analyse van de expressiepatronen van de homologen in deze soorten suggereert dat er een basisprogramma bestaat voor de regulatie van paddenstoelvorming.

Het doel van dit proefschrift was om de genetische regulatie van paddenstoelvorming in *S. commune* verder in kaart te brengen en dit te verbinden met het waarnemen van omgevingsfactoren. Daarnaast zijn de eerste stappen gezet in het modificeren van paddenstoelvorming in *A. bisporus* met behulp van de verkregen kennis in *S. commune*.

Signalering van omgevingsfactoren

S. commune groeit in de natuur op dood hout. Dit betekent dat de schimmel het substraat in groeit en hierbij in het donker en bij hoog CO_2 groeit. Wanneer het oppervlak wordt bereikt wordt blauw licht gesignaleerd en daalt de CO_2 concentratie dramatisch. Wanneer ook voldoende biomassa gecreëerd is, zullen deze omstandigheden leiden tot het opstarten van paddenstoelvorming. In dit onderzoek is aangetoond dat het CO_2 niveau in *S. commune* tijdens latere stadia gunstig moeten blijven om de ontwikkeling voort te zetten (Hoofdstuk 3).

CO_2 wordt doorgaans in organismen gesignaleerd via cAMP-Pka signalering. Zowel *S. commune* als *A. bisporus* bevatten alle componenten die nodig zijn voor deze signalering, wat duidt op conservering van het waarnemen van CO_2. Deze start in de cel met het omzetten van CO_2 tot bicarbonaat. Dit proces wordt gekatalyseerd door carbonic anhydrase. Bicarbonaat stimuleert de activiteit van adenylyl cycase, wat verantwoordelijk is voor cAMP productie. Dit signaalmolecuul bindt aan twee regulatie eenheden van Protein Kinase A (Pka), waardoor deze de twee katalytische eenheden loslaten. Vervolgens kan de katalytische eenheid eiwitten, zoals transcriptiefac met een RRXS/T motief fosforyleren. Als laatste zorgt phosphodiesterase (PDE) voor afbraak van cAMP. Met behulp van software was voorspeld dat productie en afbraak van cAMP, alsmede Pka fosforylatie, in de kern van de cel plaatsvinden (Hoofdstuk 3). Alle genen coderend voor de componenten kwamen constitutief tot expressie, behalve *sca2*. Expressie van dit gen coderend voor carbonic anhydrase was onder invloed van de CO_2 concentratie. Dit laat zien dat transcriptionele regulatie van CO_2 waarneming alleen in het begin van de signalering voorkomt. Wanneer extern cAMP werd aangeboden aan *S. commune* dan groeide de kolonie symmetrisch en was het niet in staat aggregaten, primordia of paddenstoelen te vormen. Overexpressie van *pde*2 daarentegen resulteerde in een kolonie die in staat was zelfs bij hoog CO_2 paddenstoelen te vormen (Hoofdstuk 3). Dit fenotype kon teniet worden gedaan door toevoeging van de Pde remmer IBMX. Hiermee is een duidelijk verband aangetoond tussen de remmende werking van hoge CO_2 en cAMP niveaus in *S. commune*.

Transcriptiefactor Hom2 bevat vier RRXS/T motieven en is daarmee een mogelijk doel voor fosforylering door Pka. Om dit te onderzoeken is in Hoofdstuk 4 een versie van Hom2 gecreëerd waarin de RRXS/T motieven zijn veranderd naar RRXA, wat zou leiden tot een niet-fosforyleerbare versie van het eiwit. Deze *hom2*^{con} transformanten vertoonden inderdaad verminderde vegetatieve groei en konden zelfs als monokaryon vruchtlichamen vormen wanneer het mycelium werd blootgesteld aan blauw licht en laag CO_2. De gevoeligheid van *hom2*^{con} voor hoog CO_2 suggereert dat een tweede gen nodig is in de cAMP-Pka signalering van CO_2. Samengevat speelt Hom2 een sleutelrol in de aanzet tot paddenstoelvorming. Er zijn homologen gevonden van Hom2 in andere paddenstoelvormende basidiomyceten, onder andere *A. bisporus*. Deze bevatten ook de RRXS boxen en dit suggereert een soortgelijke rol van Hom2 in andere paddenstoelvormende schimmels.

Hoge CO_2 niveaus hebben een effect op de expressie van regulatoren betrokken bij paddenstoelvorming

Door middel van genexpressie analyse zijn 36 genen geïdentificeerd die respectievelijk hoger (18) en lager (18) tot expressie kwamen wanneer de CO_2 niveaus hoog waren in *S. commune* (Hoofdstuk 3). De transcriptiefactors *wc-2*, *c2h2* en *hom1* bevonden zich onder de set van genen met verminderde expressie. Het feit dat de componenten van de cAMP-Pka signalering niet in expressie veranderen onder hoog CO_2, terwijl *wc-2* dit wel doet, suggereert dat blauw licht signalering ondergeschikt is aan CO_2 signalering.

Deletie van het CO_2-gevoelige gen *c2h2* in *S. commune* resulteert in een stop van primordia ontwikkeling. De *c2h2* ortholoog van *A. bisporus* werd tot overexpressie gebracht in de commerciële stam A15. Morfologie, groei van de kap en de totale opbrengst in hoeveelheden en biomassa veranderden niet als gevolg van *c2h2* overexpressie. Echter, het had wel een versnelde paddenstoelvorming tot gevolg (Hoofdstuk 5), bijgestaan door een vermindering in kwaliteit. Expressie van *c2h2* in *A. bisporus* komt hoog tot expressie in initials, gedurende differentiatie van de kap en stam en gedurende de ontwikkeling van weefsels. Dit is dus in tegenstelling tot de overexpressie stam, waar expressie hoger is, wat het fenotype kan verklaren. Dit overexpressie onderzoek in *A. bisporus* en het deletie onderzoek in *S. commune* van *c2h2* steunt ons idee dat paddenstoelontwikkeling in basidiomyceten een basisprogramma volgt, waarbij specifieke variaties per soort resulteren in een verschil in morfologie en de gevoeligheid van signalering van omgevingssignalen.

Biomassa vorming en paddenstoelvorming

In hoofdstuk 2 wordt beschreven hoe Hom1, betrokken bij latere ontwikkelingsstadia, groei van mycelium op substraat stimuleerde. Dit is in tegenstelling tot de transcriptiefactoren Hom2, Fst4 en Wc-2 die betrokken zijn bij de vroegere stadia van paddenstoelvorming. Deze stammen vertoonden hogere vegetatieve groei. De deletiestam waarin zowel *hom2* als *fst4* uitgeschakeld zijn, vertoonde zelfs hogere biomassa productie dan de *hom2* en *fst4* deletiestammen apart. Dit toont aan dat Hom2 en Fst4 via verschillende wegen repressie van vegetatieve groei bewerkstelligen. Het gen *tea1* werd in hoofdstuk 2 geïdentificeerd

als een transcriptie factor wier expressie positief werd beïnvloed door *wc-1*, *wc-2*, *hom2* en *fst4,* terwijl *fst3*, *hom1* en *gat1* expressie van *tea1* onderdrukten. Ondanks het feit dat Tea1 stroomafwaarts ligt van Wc-1, Wc-2, Hom2 en Fst4, is deze toch in staat vruchtlichamen te produceren. Dit gebeurt echter zonder regelmaat en op random posities. De resultaten in Hoofdstuk 2 geven daarmee een relatie tussen remming van vegetatieve groei en initiatie van seksuele reproductie.

Curriculum vitae

Jordi Franciscus Pelkmans was born on November 16th, 1985 in Gilze, The Netherlands. He followed his secondary education at the Koning Willem II college in Tilburg, The Netherlands and graduated in 2004 with a Gymnasium-level diploma. In September of 2006 he started his Bachelor in Biology at Utrecht University, followed by a Master in Molecular and Cellular Life Sciences at Utrecht University. As part of his studies he did an internship at the Molecular Microbiology group of the Department of Biology at Utrecht University under the supervision of C. de Bekker. His performed his second internship at Dyadic NL in Wageningen under supervision of Dr. V. Joosten and Dr. H. Visser. Jordi obtained his MSc diploma in August 2011. In September 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 *Schizophyllum commune* and *Agaricus bisporus* was financially supported during this period by Technologiestichting STW and is described in this thesis.

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