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Fruiting body initiation in the basidiomycete *Coprinus cinereus*

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to my husband

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Summary

The basidiomycete *Coprinus cinereus* initiates fruiting body formation in the dark by localized intense branching in the vegetative aerial mycelium. The resulting hyphal complex is called a primary hyphal knot and is about 30 µm in diameter (chapter 3). The primary hyphal knot consists of numerous short hyphae that may twist around each other. It may arise from a single hypha or from two or more neighboring hyphae. Primary hyphal knots have two developmental potentials. When a light signal is given, they transform into compact aggregated secondary hyphal knots, which are the first fruiting body specific structures. In an alternating light/dark rhythm, secondary hyphal knots undergo the whole developmental processes, leading the differentiated primordia to mature fruiting bodies. However, when light is absent, primary hyphal knots develop into multicellular resting structures, called sclerotia.

Two galectins, Cgl2 and Cgl1, β-galactoside binding lectins, are expressed synchronously to the formation of primary and secondary hyphal knots, respectively. Expression of Cgl2 increases during primordium development up to the stage of karyogamy and meiosis (corresponding to approximately 5-8 mm sized primordia). High expression of Cgl1 correlates with hymenium differentiation. At the postmeiotic stage, both galectin genes are not expressed. The temporal and spatial expression patterns of the *cgl* genes provide us with molecular markers to follow the early processes during fruiting body formation (chapter 3).

Specific mutations at both mating-type loci enable *C. cinereus* homokaryon AmutBmut (*A43mut B43mut pab-1*) to develop mature fruiting bodies without mating to another strain. We made use of this self-compatibility of homokaryon AmutBmut to generate a collection of developmental mutants. Mutants defective in forming primary hyphal knots (*pkn*) or secondary hyphal knots (*skn*) were identified (chapter 2). Examination of galectin expression in these mutants indicates that Cgl2 expression and primary hyphal knot formation are two independent but synchronous events (chapter 3).

Mutant 6-031 (*A43mut B43mut skn pab-1*) is developmentally arrested at the transition state from primary to secondary hyphal knots. This defect is caused by a single mutation in a recessive allele (*skn1*). Using a SIB-selection transformation procedure, cosmid 40-5A was isolated from a *C. cinereus* genomic DNA library that complemented the defect. The responsible complementing gene (*cfs1*) on this cosmid encodes a protein highly similar to bacterial cyclopropane fatty acid synthases, a

specific family of S-adenosyl-methionine (SAM) dependent C-methyltransferases. In *E. coli*, this enzyme catalyzes the formation of a cyclopropane ring by transferring a methyl group from SAM to a *cis*-double bond in the unsaturated fatty acid chains of membrane phospholipids, thereby modifying the membrane characteristics. The *cfs1* allele of mutant 6-031 carries a T to G transversion, resulting in an amino acid substitution (Y441D) in the C-terminus of the gene product. Computer programs predict that this region spans the membrane and contributes to enzymatic catalysis. The mutant *cfs1* allele was unable to complement the fruiting deficiency in strain 6-031, suggesting an essential role of gene *cfs1* in fruiting body initiation in *C. cinereus* (chapter 4).

Riassunto

Il basidiomicete *Coprinus cinereus* inizia la formazione del corpo fruttifero al buio attraverso un'intensa ramificazione localizzata nel micelio vegetativo aereo. Il complesso ifale risultante è chiamato nodo ifale primario ed ha un diametro di circa 30 µm (capitolo 3). Il nodo ifale primario è costituito da numerose ife corte che possono avvolgersi l'una all'altra. Può originarsi da un'ifa singola o da due o più ife vicine. I nodi ifali primari hanno due potenzialità di sviluppo. Quando viene fornito un segnale luminoso, si trasformano in aggregati compatti detti nodi ifali secondari, che sono le prime strutture specifiche di fruttificazione. In presenza di cicli giorno/notte, i nodi ifali secondari iniziano l'intero processo di sviluppo, che porta i primordi differenziati a corpi fruttiferi maturi. In assenza di luce, invece, i nodi ifali primari si sviluppano in strutture multicellulari di conservazione, chiamate sclerozi.

Due galectine, Cgl2 e Cgl1, lectine leganti β-galattoside, sono espresse sincronicamente al momento della formazione dei nodi ifali primari e secondari, rispettivamente. L'espressione di Cgl2 aumenta durante lo sviluppo del primordio fino allo stadio di cariogamia e meiosi (corrispondente a primordi della dimensione di circa 5-8 mm). Un elevata espressione di Cgl1 è correlata con la differenziazione dell'imenio. Entrambi i geni delle galectine non sono espressi allo stadio postmeiotico. I pattern di espressione temporale e spaziale dei geni *cgl* ci forniscono un marcatore molecolare per seguire i processi precoci durante la formazione del corpo fruttifero (capitolo 3).

Mutazioni specifiche ad entrambi i *loci* dei fattori riproduttori mettono in grado l'omocarion AmutBmut di *C. cinereus* (*A43mut B43mut pab-1*) di sviluppare corpi fruttiferi maturi senza accoppiamento con un altro ceppo. Abbiamo utilizzato l'autocompatibilità dell'omocarion AmutBmut per generare una collezione di mutanti dello sviluppo. Sono stati identificati mutanti difettivi della formazione dei nodi ifali primari (*pkn*) o dei nodi ifali secondari (*skn*) (capitolo 2). L'esame dell'espressione delle galectine in questi mutanti indica che l'espressione di Cgl2 e la formazione dei nodi ifali primari sono due eventi indipendenti ma sincroni (capitolo 3).

Il mutante 6-031 (*A43mut B43mut skn pab-1*) è arrestato nello sviluppo a livello della transizione da nodi ifali primari a secondari. Questo difetto è causato da una mutazione singola in un allele recessivo (*skn1*). Utilizzando una procedura di trasformazione che prevede una selezione di tipo SIB, da una libreria di DNA genomico di *C. cinereus* è stato isolato il cosmide 40-5A che complementa il difetto. Il gene responsabile della

complementazione (*cfs1*) su questo cosmide codifica una proteina molto similare alle sintetasi batteriche di acidi grassi ciclopropanati, una specifica famiglia di C-metiltransferasi, dipendenti dalla S-adenosil-metionina (SAM). In *E. coli*, questo enzima catalizza la formazione dell'anello del ciclopropano mediante il trasferimento di un gruppo metilico dalla SAM ad un doppio legame in *cis* nelle catene di acidi grassi insaturi dei fosfolipidi di membrana, modificando di conseguenza le caratteristiche della membrana. L'allele *cfs1* del mutante 6-031 porta una transversione da T a G, risultante nella sostituzione di un amminoacido (Y441D) nel terminale carbossilico del prodotto proteico. Studi sulle banche dati indicano che questa regione attraversa la membrana e contribuisce alla catalisi enzimatica. L'allele mutante *cfs1* non ha la capacità di complementare il difetto di fruttificazione nel ceppo 6-031, suggerendo un ruolo essenziale del gene *cfs1* nell'iniziazione del corpo fruttifero in *C. cinereus* (capitolo 4).

Chapter 1

Introduction

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1 Introduction

1.1 Mushrooms and their applications

Fungi are eukaryotic, heterotrophic, absorptive organisms that develop a rather diffuse, branched, tubular body and reproduce by spore production (Kendrick, 1992). The number of fungal species estimated on earth is 1.5 million, of which only about 69,000 species are described (Hawksworth, 1991), and 10,000 species are mushrooms (Kendrick, 1992). A mushroom is a macrofungus with a distinctive fruiting body that can be either hypogeous (underground) or epigeous (above ground), large enough to be seen with the naked eye and to be picked up by hand (Hawksworth *et al.*, 1995). Most mushrooms belong to basidiomycetes, others are ascomycetes. The natural habitats of mushrooms include wood, litter and dung, which can be efficiently converted into the biomass of fruiting bodies (Poppe and Hofte, 1995). The ability of degrading lignocellulose waste (Whiteford and Thurston, 2000) entitle mushrooms as scavengers of forestry and agriculture. Based on their applications, mushrooms are divided into four categories: edible mushrooms, medicinal mushrooms, poisonous mushrooms and non-defined mushrooms. Data from 1775 studied species suggest that half of the described mushroom species are primely edible, approximately 18% are of medicinal value and 10% are poisonous (Chang, 1993). Some mushrooms are only either edible (*e.g. Agaricus bisporus*) or medicinal (*e.g. Ganoderma*, *Coriolus*), while others possess both properties (*e.g. Auricularia*, *Flammulina*, *Grifola*, *Hericium*, *Lentinus*, *Pleurotus* and *Tremella*) or none of them (Chang, 1996).

Fruiting body is a general term for spore-bearing organ, which in higher basidiomycetes is commonly known as mushroom. Fruiting bodies are rich in proteins (Casalicchio *et al.*, 1975), carbohydrates, fibres, unsaturated fatty acids, vitamins and minerals, but low in calories (Miles and Chang, 1997), low in nucleic acids and free of cholesterol (Bano and Rajarathnam, 1988). The nutritional profile makes mushrooms a very good dietary food. Besides being a culinary delicacy, some mushrooms were used as medical and tonic attributes in traditional remedies, particularly in China (Ying *et al.*, 1987), Japan, Korea and Russia (Wasser and Weis, 1999), where the practice of using mushrooms as medicine had been rooted in the very ancient past. At the present time, searching for medicinal substances from mushrooms and studying the medicinal value of these substances have become a matter of great interest. A number of bioactive substances have been identified from mycelia (Haak-Frendscho *et al.*, 1993; Kawagishi

et al., 1996), culture medium and/or fruiting bodies (Kidd, 2000; Kües and Liu, 2000; Wasser and Weis, 1999). These substances can stimulate the synthesis of nerve growth factor, modulate the immune system, lower the blood pressure, reduce the level of blood glucose and lipids, inhibit inflammation, inhibit the growth of cancer cells, microbes, fungi and viruses (Jong and Donovick, 1989; Kües and Liu, 2000; Wasser and Weis, 1999).

With the recognition of their values in nutrition and medicine, the demand for mushroom production is increasing all over the world. In fact, the world production of cultivated mushrooms has expanded tenfold in the last fourty years (Kües and Liu, 2000). Most mushrooms are produced for dietary purpose, while others are used to extract mushroom-derived products. A number of pharmaceutical β-D-glucans and proteoglycans derived from mushrooms are used in cancer treatment, *e.g.* ABM from *Agaricus blazei*, Befungin from *Inonotus obliquus*, Lentinan from *Lentinula edodes*, Schizophyllan from *Schizophyllum commune*, Polysaccharide-K (PSK) and Polysaccharide-peptide (PSP) from *Coriolus versicolor* (Kidd, 2000; Wasser and Weis, 1999). For less defined dietary supplements but with potential therapeutic value, a new term, "mushroom nutriceuticals", was coined. Nutriceuticals are extractable from either the mycelium or the fruiting body and embody both nutritional and medicinal features (Chang and Buswell, 1996). Small companies were founded in recent years to produce and sell such products, *e.g.* dietary supplements made from fruiting bodies of *Grifola frondosa* (http://www.ellesworth.com/). In addition, aromatic flavors used as natural food additives can be extracted from mushrooms, which serve as an alternative to plants in aroma and flavour industry (Lomascolo *et al.*, 1999). The last but not the least application of mushrooms is their function as ornamentals in floristic arrangements (Poppe and Heungens, 1991).

The broad application in biotechnology has made a notable impact on mushroom production and on the diversity of cultivated species. The record of mushroom cultivation can be dated back to 600 A. D. with *Auricularia auricula* in China. For the worldwide most produced mushroom *A. bisporus*, cultivation was first achieved in France in the seventeenth century (Chang, 1993). However, the major achievement in mushroom cultivation was made in the past twenty years (Chang, 1993), including one report on the successful cultivation of the golden chanterelle *Cantharellus cibarius* (Danell and Camacho, 1997) and two reports on the formation of immature fruiting bodies in three mycorrhizal *Boletus* spp. (Granetti, 1990; Yamanaka *et al.*, 2000). In

total, 38 species from 26 genera so far can be standardly cultivated (Chang, 1993; Danell and Camacho, 1997). Only the productions of 11 species have reached commercial scales (Kües and Liu, 2000).

A major hindrance in mushroom cultivation is the poor understanding of their developmental processes, especially at the initial stage. Mushroom formation is a highly organized process, which requires coordination among genetic, physiological and environmental factors. Up to now, information regarding fruiting body development mainly comes from studies on a few species, such as *Coprinus cinereus* and *Schizophyllum commune* (Kües, 2000; Moore, 1998; Wessels, 1993; Wessels, 1994). For most other species, less is known about their biology. In most cases, including *A. bisporus*, the suitable effective tools to study developmental processes are still missing (Stoop and Mooibroek, 1999), unlike in *C. cinereus* and *S. commune* (Walser *et al.*, 2001).

1.2 *Coprinus cinereus* and *Schizophyllum commune* are model organisms for studying development in basidiomycetes

Although neither of edible (Arora, 1986) nor of medicinal value, *C. cinereus* (Schaeff. ex Fr.) S. F. Gray sensu Konr. (Pinto-Lopes and Almeida, 1970-1971) has served for almost a century as a model organism to study developmental processes in basidiomycetes (Kües, 2000). The fungus has a relatively short life cycle, *i.e.* two weeks, and can be readily cultivated in the laboratory both on natural and artificial media (Moore and Pukkila, 1985). It is accessible to classical genetics and molecular biology techniques (Pukkila, 1993; Walser *et al.*, 2001) and has well established transformation systems (Binninger *et al.*, 1987; Cummings *et al.*, 1999; Granado *et al.*, 1997).

C. cinereus is a saprophytic fungus and grows on dung in nature (Ulje and Noordeloos, 1999). The scientific name indicates both its food source (from *kopro* (Gr.) = dung) and its color (from *cinis* (Lat.) = ash). In the past, this fungus has been isolated under various names making the analysis of old literature difficult and uncertain (Kües, 2000). For example, *C. cinereus* was studied in details by Lewis, Day and numerous other workers under the name *C. lagopus* and *C. lagopus* sensu Buller (Pinto-Lopes and Almeida, 1970-1971). Isolates named as species *C. delicatulus* Apinis, *C. fimetarius* L.ex Fr., *C. fimetarius L.* Fr. var. *macrorhizus* Pers. Ex Fr. and *C. macrorhizus f.*

microsporus Hongo are reported to be interfertile with *C. cinereus* (Kemp, 1975; Kemp, 1985).

Early systematic work in the Coprinaceae was based exclusively on morphological characters (Ulje and Noordeloos, 1997; Ulje and Noordeloos, 1999). The deliquescence of gills at maturity into inky-looking fluid plus the black spore print are the main diagnostic features of *Coprinus* (Arora, 1986; Buller, 1909). Due to these unique morphological and developmental characters, *Coprinus* was generally regarded as a monophyletic genus. Only recently, this concept was strongly challenged by cladistic data obtained from sequences encoding large-subunit rDNA genes (Hopple and Vilgalys, 1994; Hopple and Vilgalys, 1999; Moncalvo *et al.*, 2000) and the internal transcribed spacer regions (ITS) of rDNA genes (Park *et al.*, 1999a; Park *et al.*, 1999b). Different phylogenetic analysis based on these sequences strongly suggests that with the exclusion of two species *Coprinus comatus* (type species) and *Coprinus sterquilinus*, the genus *Coprinus* is monophyletic (Hopple and Vilgalys, 1999).

Although the current nomenclatural hierarchy (botanical) for *C. cinereus* is shown below (Hawksworth *et al.*, 1995), based on data discussed above, reclassification and renaming are requested (Redhead *et al.*, 2001).

Domain:	Eukaryota
Kingdom:	Fungi
Phylum (division):	Basidiomycota
Class:	Basidiomycetes
Order:	Agaricales
Family:	Coprinaceae
Genus:	*Coprinus*
Species:	*Coprinus cinereus*

C. cinereus is a heterothallic (self-incompatible) basidiomycete. It has a haplo-dikaryotic life cycle (Fig. 1) showing an alternation between two different mycelial states, the sterile monokaryon and the fertile dikaryon. The monokaryon is a primary mycelium containing genetically identical haploid nuclei in every hyphal cell (Hawksworth *et al.*, 1995) and arises from the germination of a binucleate basidiospore or of a uninucleate oidium. The monokaryotic hyphae have simple septa with dolipores (the barrel-shaped pore in the hyphal septum of basidiomycetes), but are devoid of

clamp cells. Mating between two compatible monokaryons gives rise to a dikaryon, a specific heterokaryon. Hyphal fusion is not followed immediately by nuclear fusion. Therefore, the dikaryon is binucleate, containing two different haploid nuclei in each hyphal cell, one from each mating partner. Clamp cells are found above the hyphal dolipore septa, which contribute to the establishment and maintenance of the binucleate state in the dikaryon (Casselton and Economou, 1985; Kües, 2000). Although both monokaryons and dikaryons are capable of indefinite vegetative growth, dikaryons are predominantly found in nature (Bensaude, 1918; Brunswik, 1924; Miles, 1996).

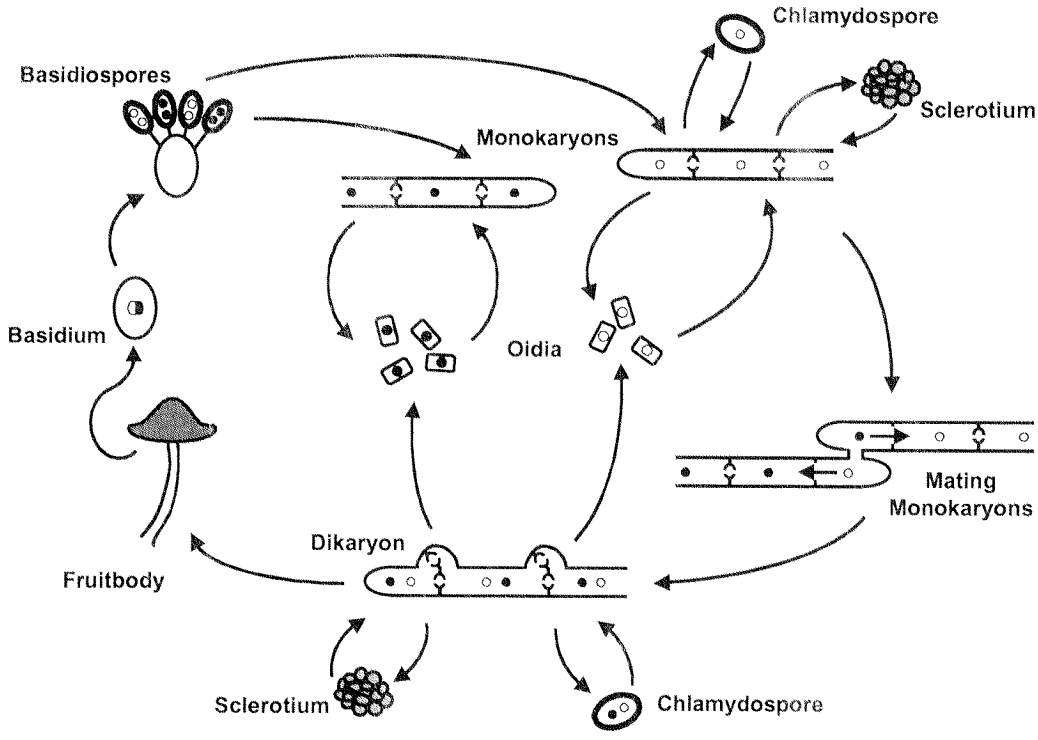

Figure 1. The life cycle of *C. cinereus* [from (Kües, 2000)]

Monokaryon and dikaryon have various developmental potentials and considerable flexibility in forming several differentiated structures. Both mycelial states can form haploid mitotic oidia, chlamydospores and multicellular sclerotia (Kües, 2000; Kües *et al.*, 2000b). The unicellular oidia are uninucleate, haploid and rod-shaped asexual spores, which are mainly formed in aerial mycelium on the specialized oidiophores and occasionally in submerged mycelia (Brodie, 1931; Brodie, 1936; Polak *et al.*, 1997; Polak *et al.*, 2000). Chlamydospores are mitospores found in submerged mycelia, usually having variable forms with condensed cytoplasm enclosed by thick cell wall

(Anderson, 1971; Bensaude, 1918; Esser, 2000; Kües *et al.*, 1998). Sclerotia are globular perennial multicellular resting structures formed under severe environmental conditions in aerial and submerged mycelia (Waters *et al.*, 1975a). Since sclerotia are formed as alternatives of fruiting bodies on dikaryons (Moore, 1981), their structures are described in the following in more detail.

A developing aerial sclerotium has four layers of cells. The outmost layer is made of thin-walled dead hyphal cells that are loosely attached to the second layer (rind or cortex). The rind cells are thick-walled and impregnated with dark-brown melanic pigments. No dolipore is observed in the septa of rind cells. An intermediate layer between the rind and the central core (medulla) consists of cells destined to form either rind cells or medullary cells in the final stages of maturation. The central medulla is a mass of bulbous thick-walled cells with oval or irregular shapes, which very much resemble chlamydospores. In the developing sclerotium, these inflated cells are randomly interspersed by thin-walled cells. Dolipore septa are present in the transverse walls between two thin-walled cells and between thin- and thick- walled cells (Volz and Niederpruem, 1970; Waters *et al.*, 1972; Waters *et al.*, 1975a; Waters *et al.*, 1975b). The color of the aerial sclerotium becomes dark-brown upon maturity by the melanization of rind cell walls (Waters *et al.*, 1975b) [for review see (Kües, 2000)]. Different from the aerial sclerotia, submerged sclerotia are pale brown and of irregular shape. Essentially, a submerged sclerotium is a mass of loosely organized submerged inflated cells (medulla) enclosed by a thick-walled rind devoid of living cytoplasm (Waters *et al.*, 1975a).

It is the dikaryon but not the monokaryon of *C. cinereus* that differentiates under suitable light and temperature conditions into fruiting bodies composed of different tissue types with distinct functions. The mode of fruiting body development in *C. cinereus* is angiocarpic (enclosed hymenium during differentiation) (Reijnders, 1963). The early fruiting processes in *C. cinereus* include the formation of hyphal knots, fruiting body initials and differentiated primordia. The occurence of karyogamy within the basidium, a specific cap cell, initiates the maturation processes. In parallel to the formation of haploid meiotic basidiospores in a young fruiting body, the rapid stipe elongation and cap expansion lead to the formation of a fully developed mature fruiting body (Fig. 2). In the final stage, gills of mature fruiting bodies autolyze progressively to liberate all basidiospores into inky liquid (section 1.3.1) [for review see (Kües, 2000)].

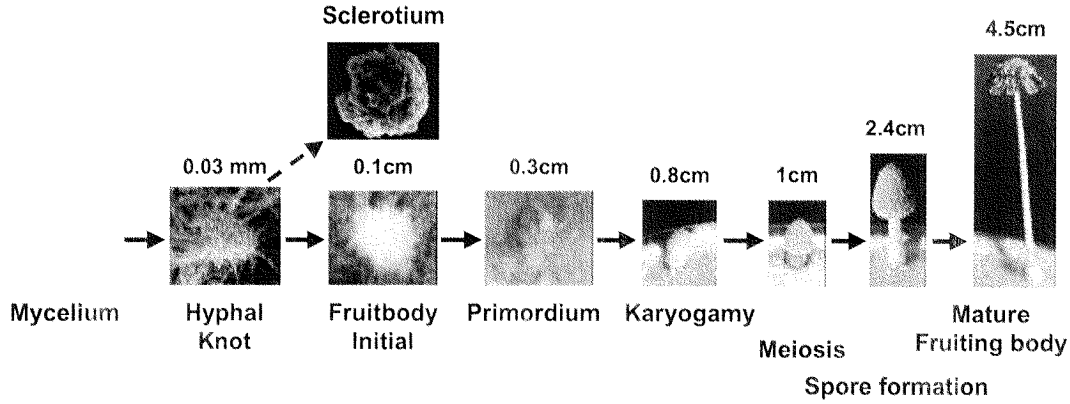

Figure 2. The pathway of fruiting body development in *C. cinereus* [modified from (Boulianne *et al*, 2000)].

The other model organism of higher basidiomycetes, *Schizophyllum commune* Fr., is a wood-inhabiting fungus, belongs to the order Schizophyllales (Hawksworth *et al.*, 1995) and also fruits readily on artificial media under laboratory conditions (Niederpruem and Wessels, 1969; Wessels, 1993). Like *C. cinereus*, *S. commune* alternates during its life cycle between monokaryotic and dikaryotic mycelia, but produces chlamydospores (Kendrick and Watling, 1979) as the only mitotic reproductive structure (Brodie, 1936; Raper and Miles, 1958). The fan-shaped fruiting bodies (Alexopoulos and Mims, 1979) are formed on the dikaryotic thallus (Niederpruem and Wessels, 1969). The mode of fruiting body development in *S. commune* is gymnocarpic (exposed hymenium during differentiation) (Reijnders, 1963). The early fruiting stages include the formation of hyphal aggregates and tiny concave primordia (van der Valk and Marchant, 1978). On the apical surface of the primordium, the hymenium extends radially and produces gills continuously (Niederpruem and Wessels, 1969). The differentiation of gills in association with the basidiospore formation leads to the formation of mature fruiting body (Leonard and Dick, 1968). The increase in size of *S. commune* fruiting bodies is by hyphal proliferation, not by hyphal inflation (Wessels, 1992).

Since most mushrooms have well developed caps and stipes, the fruiting pathway of *C. cinereus* rather than *S. commune* may reflect a more general picture of mushroom development in higher basidiomycetes. In contrast to *S. commune*, the highly synchronous basidial development and the synchronous nuclear division in the basidium (Lu, 1967) make *C. cinereus* particularly suitable to comparative cytological studies (Pukkila and Casselton, 1991).

1.3 Fruiting body development in basidiomycetes

The fruiting body is the most conspicuous emergent structure during the life cycle of higher Basidiomycetes. The majority of species including *C. cinereus* and *S. commune* is heterothallic (Webster, 1980) with fruiting bodies usually forming on the dikaryon (Kües, 2000; Wessels, 1993; Wessels, 1994). Occasionally, the haploid monokaryon of heterothallic species can also form fruiting bodies. Such monokaryotic fruiting can be induced by mutations in certain genes leading to self-fertilism (homothallism), by unusual environmental or nutritional conditions, by mechanical injury and by fruiting inducing substances (section 1.4). Few natural homothallic species such as *Coprinus heptemerus* Lange & Smith (Lamoure, 1989; T.Y. James, personal communication), *Bolbitius vitellinus* (Pers.: Fr.) Fr. and *Lepiota procera* (Scop.: Fr.) Sing. (Lamoure, 1989) fruit and produce normal basidiospores without mating to another strain of the same species (primary homothallism) (Miles, 1996). In others, *e.g. A. bisporus*, the basidiospores mostly contain two haploid nuclei of different mating type. The germinated dikaryotic mycelium proceeds to form fruiting bodies without mating (secondary homothallism) (Raper and Raper, 1972). In *Armillaria mellea* (Vahl ex Fr.) Kummer (Ullrich and Anderson, 1978) and most other *Armillaria* spp. (Guillaumin *et al.*, 1991), the haploid basidiospores germinate to form haploid homokaryons. Compatible matings permit the formation of transient dikaryotic cells, but the two haploid nuclei soon fuse in hyphal tip cells to produce a diploid nucleus. The uninucleate diploid clampless mycelium is the persistent vegetative form (Anderson and Kohn, 1992). Fruiting bodies develop on such diploid mycelium. Within the fruiting body, the dikaryotic clamp-bearing cells are only found in the gill tissue (Ullrich and Anderson, 1978).

1.3.1 Fruiting body development in *C. cinereus*

The formation of fruiting bodies initiates the sexual reproduction of *C. cinereus*. Once a dikaryotic thallus is established, the fungal colony is ready to commence fruiting body morphogenesis upon environmental stimuli (section 1.4.2). The initial morphological change is to turn a loose mycelial network into a three-dimensional aggregated structure. Attempts have been made to record the cytological changes occuring in this initial event, but only several morphological changes at steady states are documented. A real-time documentation following a single hypha has not yet been achieved. In consequence, the nomenclatures in previous literature regarding subsequent

stages in fruiting body initiation and maturation are unclear. Different terms have been used to describe the same developmental structure. Sometimes, the same term refers to different developmental stages. This situation often causes confusion and makes comparative reading very difficult. Table 1 gives an overview on how different authors defined the fruiting stages and an attempt to bring these studies into an agreement. By reading the original literatures, at the first sight, few similarities are found among different descriptions of these morphological events. Because of differences in strains, length and intensity of light illumination, medium composition and temperature, fruiting structures varied greatly in size and their time of emergence was also different from study to study. In addition, the definitions of a "day" and of developmental stages are quite variable among different authors (a "day" can begin at midnight or at the start of the light cycle, the stages are defined by the beginning or by the end of a morphological event). Therefore, size and time are not perfect criteria to distinguish one developmental structure from the other, unless within a carefully defined system (strain, light conditions, temperature, humidity and growth conditions). However, when brought into a correct time scale using light-controlled events as reference points between different authors, the consecutive steps in fruiting pathway correlate surprisingly well (Table 1).

1.3.1.1 Defining developmental stages as landmarks in fruiting body formation

Kamada and coworkers divided fruiting body initiation into three stages starting with the undifferentiated dikaryotic mycelium as stage 0. The first fruiting specific structures referred to as hyphal knots and primordia are formed in stages I and II, respectively (Takemaru and Kamada, 1972). The term hyphal knot is defined as fruiting body initials of 0.2-0.5 mm in diameter in which no histological differentiation into the primordial pileus and stipe is discernible (Kamada and Tsuru, 1993; Muraguchi *et al.*, 1999). Such hyphal knots appear after light illumination (Morimoto and Oda, 1973). Kamada's light dependent hyphal knots are clearly different from the hyphal knots described by the groups of Moore and Kües (Kües, 2000; Moore *et al.*, 1979) (Table 1). The hyphal knots of these authors are localized highly branched structures, which are formed in darkness and repressed by light (Boulianne *et al.*, 2000; Kües *et al.*, 1998; Moore and Jirjis, 1976; Waters *et al.*, 1975b).

The term "hyphal knot" was originally introduced by Reijnders in a broad sense. He described a hyphal knot as a bundle of hyphae that continue to adhere to one another during the expansion of tissues. Reijnders's hyphal knot consists of a central induction

Table 1. Developmental course of fruiting body formation in *C. cinereus* – definition of specific stages and their hallmarks

Characteristics		Terms defined by								
		Kamada & coworkers (1-5)	Morimoto & Oda (5-7)	Matthews & Niederpruem (8, 9)	Moore & coworkers (5, 10-18)	Kües & coworkers (19-21)	Lu & coworkers (5, 22-25)		McLaughlin (5, 26-28)	This study
Regular hyphal growth and branching Glycogen accumulated in submerged mycelium[29] Light independent		**Stage 0** **dikaryon**	**Mycelial growth** 5-6 days				**Mycelial growth*** 5 days			
Localized switch of growth pattern Intense localized hyphal branching Prior to fruiting body initiation and sclerotium formation Light repressed				**Stage I lattice**	**Hyphal knot** (hyphal tuft)	**Hyphal knot** (primary nodule) 200 µm				**Primary hyphal knot** (primary nodule)
Fruiting body specific Hyphal aggregation Loose cellular organization Change in cell wall structure Light induced		**Stage I** **hyphal knot** 0.2-0.5 mm day 4	**Stage 1*** **primordium** < 1mm day 6-7	**Stage II** **primordium** 0.2-0.5 mm 24-30 h	**Stage 0** **carpophore initial** 0.2-0.7 mm	**Initial** (secondary nodule) 0.2-0.7 mm				**Secondary hyphal knot** (secondary nodule, initial)
Compact organization First cellular differentiation Regional cell proliferation Transfer of glycogen into fruiting structure Polysaccharides in basal region				**Stage III** **primordium** ca. 0.7 mm						
First differentiation of cap and stipe tissues Enclosed in veil Polysaccharide deposits in basal cells Light independent	Repression of unlimited stipe cell proliferation (dark stipe) by light	**Stage II *** **primordium** ≥1 mm to 14-16 mm up to day 8		**Stage IV** **primordium** ≥1 mm	**Stage 0** **carpophore initial** 0.8-2 mm	**Initial** 0.8-2 mm	Stage 0* -64 h	**Day 0*** **primordial bud**	Day -3	**Stage 0** **primordium**
Formation of gill rudiments Light dependent			**Stage 2*** **primordium** 2-5 x 5-7 mm day 8		**Stage 1** **primordium** 2-9 mm	**Stage 1** **primordium** 2-6 mm				**Stage 1** **primordium** pre-karyogamy
Formation of primary gills and gill cavities								**Day 1***		

Formation of secondary gills Formation of probasidia and facial cystidia			**0-hr stage** day 8					**Stage 1* primordium** -16 h prekaryogamy (meiotic S-phase)	**Day 2**		
Polysaccharides deposits in base of stipe, subhymenium and facial cystidia Transformation of some probasidia into cystesia Formation of paraphyses Minor stipe elongation by cell proliferation											
Basidium maturation	Light dependent (16-6 h before karyogamy)		**Phase 1***						**Day 3**		
	Light delays or hinders karyogamy and meiosis (10-15 h period, strain dependent)		**Phase 2***								
	Karyogamy (0 h)	**Stage III** day 9		**Stage 3*** day 9 meiosis I completed, ± 12 h after karyogamy						**Day –2** early morning	
Prophase I of meiosis Basidia and cystidia enlarge	Dark phase needed to enter metaphase I (strain dependent)		**Phase 3***			**Stage 2 primordium** 6-15 mm 6-7.5 h after karyogamy	**Stage 2 primordium** 6-10 mm	**Stage 2*** 0-16 h after karyogamy, end of meiosis		**Day –2** evening to midnight	**Stage 2 primordium** post-karyogamy
	Light accelerates (strain dependent)		**Phase 4***						**Day 4**	**Day –1** 1st 12 h	
Metaphase I	Basidia, facial cystidia and 75% of paraphyses fully developed Polysaccharides deposits mostly in subhymenium, cystidia and paraphyses First gill separation Veil still intact but becoming more or less free Beginning of stipe elongation by cell inflation Light independent		**Phase 5***	**Stage 4** day 10 meiosis II finished ± 14 h, sterigmata formation ± 15 h after karyogamy							
Anaphase I											
Meiosis II											

Sterigmata formation All paraphyses fully developed Veil raptures Some stipe elongation by cell inflation Cap still closed	Light independent					**Stage 3 immature fruit** 1-2 cm, 4 h after meiosis	**Immature fruiting body** 1-2 cm	**Stage 3** 8-10 h after meiosis			**Immature fruiting body**
Basidiospore formation Carbohydrates transferred to immature basidiospores											
Nuclear migration into basidiospores Post-meiotic mitosis Some stipe elongation by cell inflation						**Stage 4 immature fruit** 1.5-4.5 cm, 6-12 h after meiosis	**Immature fruiting body** 1.5-4.5 cm	**Stage 4**		**Day –1** 2nd 12 h	**Immature fruiting body**
Basidiospore pigmentation Polysaccharides disappeared from subhymenium Cap begins to open Formation of marginal cystidia Rapid stipe elongation by cell inflation											
Final stipe elongation by cell inflation Major cap expansion and gill separation						**Stage 5 mature fruit** 2-10 cm	**Mature fruiting body** 4-7 cm	**Stage 5**		**Day 0**	**Mature fruiting body**
Cap autolysis				**Stage 5** day 11							

[1](Takemaru and Kamada, 1972), [2](Kamada *et al.*, 1978), [3](Kamada and Tsuru, 1993), [4](Muraguchi *et al.*, 1999), [5](Pukkila *et al.*, 1984), [6](Morimoto and Oda, 1973), [7](Morimoto and Oda, 1974), [8](Matthews and Niederpruem, 1972), [9](Matthews and Niederpruem, 1973), [10](Stewart and Moore, 1974), [11](Waters *et al.*, 1975b), [12](Jirjis and Moore, 1976), [13](Moore *et al.*, 1979), [14](Rosin and Moore, 1985b), [15](Rosin and Moore, 1985a), [16](Hammad *et al.*, 1993a), [17](Chiu and Moore, 1993), [18](Moore, 1995), [19](Kües *et al.*, 1998), [20](Kües, 2000), [21](Boulianne *et al.*, 2000), [22](Lu, 1974), [23](Lu, 1991), [24](Lu, 2000), [25](B.C. Lu, personal communication), [26](McLaughlin, 1973), [27](McLaughlin, 1974), [28](McLaughlin, 1982), [29](Madelin, 1960), *light influence has been demonstrated by the author(s).

hypha and a community of surrounding hyphae that differentiate in concert. Such hyphal knots are frequently found in various tissues of primordia and fruiting bodies, *e.g.* in stipe, veil and pileus (Reijnders, 1977).

Matthews and Niederpruem divided the initial steps leading to fruiting body formation into four stages. Stage I is the formation of hyphal lattices, which are derived from localized interaction between secondary and tertiary branches of dikaryotic hyphae. The up-growth of aerial cells specifically from lattices followed by a general increase in cell density leads to the formation of primordia (Stage II) (Matthews and Niederpruem, 1972). The morphological events occuring in these two stages clearly resemble the formation of hyphal knots in studies by Moore and coworkers (Waters *et al.*, 1975b). In Stage III of Matthews and Niederpruem, primordia undergo regional cell growth and primitive tissue organization. The size of Stage III primordia can be up to 0.7 mm in diameter, in which insoluble polysaccharide and protein are uniformly distributed (Matthews and Niederpruem, 1973). The Stage III primordia correlate to the Stage I hyphal knots of Kamada, to the Stage 0 "carpophore initials" of Moore and to the initials of Kües (Table 1). According to Matthews and Niederpruem, in Stage IV hyphal aggregates differentiate into visible cap and stipe tissues, in which cells have distinct morphology and staining properties (Matthews and Niederpruem, 1973). Primordia formed in Stage IV correlate to the Stage II primordia of Kamada (Takemaru and Kamada, 1972).

Moore and his coworkers studied the formation of aerial sclerotia, multicellular hyphal resting bodies that form alternatively to the fruiting body initials (Moore, 1981) when a light signal is absent (Boulianne *et al.*, 2000). Moore used a term "hyphal tuft" (Waters *et al.*, 1975b) or "hyphal knot" (Moore, 1981) to name the structure that serves as a precursor for both fruiting body initials and sclerotia. Eventually, a hyphal tuft (or hyphal knot) is a prosenchyma (Greek *pros*=toward, *enchyma*=infusion, meaning approaching or almost a tissue) that is formed after aggregated hyphal growth (Moore, 1995). A fruiting body initial less than 0.2 mm is solely an aggregation of hyphae, while the differentiation of stipe and pileus is evident in a primordium of 0.8 mm. The shape of a mushroom is clearly visible in a primordium of 1.2 mm (Moore *et al.*, 1979).

How to solve this terminological confusion in the initial steps of fruiting body development? Clémençon defined two types of distinctive nodules in the initial stages during fruiting body formation of *Psilocybe cyanescens, Lyophyllum tylicolor* and *A. bisporus* (Clémençon, 1997). According to him, a nodule is a monocentric and knot-like

structure of great morphogenetic potential and importance. Clémençon divides such nodules into primary and secondary types. A primary nodule appears first and is homoiomerous (*i.e.*, composed only of one type of vegetative hyphae). Further growth of the primary nodule leads to the formation of the secondary nodule, which is heteromerous (*i.e.*, having a compact core surrounded by loose hyphae). The entire primary nodule and the core of the secondary nodule are made of dense interwoven hyphae, with a denser prosenchyma in the secondary nodule (Clémençon, 1997).

If we apply Clémençon's definition of nodules to *C. cinereus*, the dark dependent hyphal knot of Moore and Kües would correspond to the primary nodule and could be termed as primary hyphal knot. The light dependent fruiting body initial, *i.e.* the hyphal knot of Kamada, would instead correspond to the secondary nodule and could therefore be termed as secondary hyphal knot (Table 1). When kept in dark, the primary hyphal knots are formed on aerial dikaryotic mycelia (for further details see section 1.3.1.2 and chapter 3). Under suitable environmental conditions (light, temperature and humidity), the primary hyphal knot becomes more compact and forms the secondary hyphal knot. A secondary hyphal knot is a globular aggregation of undifferentiated hyphal mass. The differentiation within the secondary hyphal knot leads to the formation of primordia, in which all the structural tissues of cap and stipe are discernible. The terminology following the differentiated primordia until the mature fruiting bodies is relatively consistent (Table 1). The size of primordia ranges about 1-2 mm to 0.8-1 cm. When primordia reach about 0.8-1 cm in size (stage 1 and 2), karyogamy occurs, quickly followed by meiosis. At the end of meiosis, basidiospore formation begins in the immature and slowly elongating fruiting bodies. In parallel to the basidiospore maturation, stipes rapidly elongate and caps expand, leading to the formation of mature fruiting bodies [for review see (Kües, 2000)]. The step from karyogamy to mature fruiting bodies takes place approximately within 35 hours (Pukkila *et al.*, 1984). The duration from karyogamy to metaphase I is the same in all strains studied, but the initiation of karyogamy is influenced by strain background and light intensity (Lu, 2000). Once the fruiting bodies matured, gill tissues start to autolyze. Within a few hours, basidiospores are released into inky-colored fluid (Buller, 1931; Kües, 2000).

1.3.1.2 More details on primary and secondary hyphal knot formation

It has been proposed that the sexual development of *Coprinus* is monocentric, meaning that a basidiocarp can arise from a single cell (Buller, 1931). A leading aerial

hypha produces secondary and tertiary branches. Branches of neighboring hyphae possibly merge by anastomosis (hyphal fusion) to form a distinguishable lattice. The lattice serves as an active center to produce abundant aerial hyphae, many of which have a globular or inflated morphology. The continuous coherent growth (Gregory, 1984) of those aerial hyphae in a self restricted manner results in a round aggregation of tightly woven dikaryotic hyphae. The time required to produce such aggregations from the hyphal lattices is about 24-30 h (Matthews and Niederpruem, 1972). The cellular structure formed at this stage is now referred as primary hyphal knot (Table 1).

The primary hyphal knot has two potentials for further development. The fate to develop into a secondary hyphal knot or a sclerotium is determined by environmental factors such as light, temperature, carbon and nitrogen supplies (Moore *et al.*, 1979). Morphological and genetic evidence show that fruiting bodies and aerial sclerotia are of the same origin (Waters *et al.*, 1975b) and share a common pathway of initiation (Moore, 1981). With the presence of light and ambient (ca. 25°C) temperature, primary hyphal knots are transformed into secondary hyphal knots. A secondary hyphal knot is a mass of prosenchymatous tissue resulting from the aggregated growth of one or more hyphae and their branches (Moore, 1995).

The secondary hyphal knot is the first fruiting body specific structure, ranging from 0.2 to 0.5 mm in diameter. A 0.2 mm secondary hyphal knot is a spherical structure having a core surrounded by a coat. Cells of the compact core are heavily branched, short and outwardly directed. These cells will differentiate into the cap and stipe. The coat consists of numerous large vacuolated cells, which will become the veil. The morphology and organization of cells in a 0.5 mm secondary hyphal knot is very similar to those of the 0.2 mm secondary hyphal knot, except that the cellular collective is more compact and little intercellular space is left (Lu, 1974; Matthews and Niederpruem, 1973; van der Valk and Marchant, 1978). Anastomosis commonly occurs between parallel hyphae in the core, but is absent between large hyphae of the outer layer. The densely packed cells of the core are rich in glycogen (Matthews and Niederpruem, 1973) and are covered with large amounts of amorphous mucilaginous material (van der Valk and Marchant, 1978). The mucilage possibly aids the adhesion of hyphae prior to fusion. The core hyphae have dolipore septa, which are usually surrounded by an additional hemispherical membranous cap. The outer cap appears to be occasionally perforated, but usually it is continuous with parenthesome, or with the endoplasmic

reticulum. Such outer cap has not been seen in the vegetative mycelium of *C. cinereus* or in the primordium of *S. commune* (van der Valk and Marchant, 1978).

The helical arrangement of chitin microfibrils is a potential marker to monitor the histological transition from the primary into the secondary hyphal knot. Chitin microfibrils are arranged randomly in vegetative mycelium, but helically in the secondary hyphal knot of 0.5 mm in diameter. Both types of arrangement can be found in 0.1-0.2 mm secondary hyphal knots (Kamada and Tsuru, 1993). This finding suggests that the onset of the helical arrangement of chitin microfibrils has begun in 0.1-0.2 mm secondary hyphal knots. Thus, it would be very interesting to examine the arrangement pattern of chitin microfibrils in primary hyphal knots.

To distinguish the primary hyphal knot from the secondary hyphal knot or *vice versa*, the differential expression of two fruiting body specific galectins can be used as molecular markers. Two galectins, Cgl1 and Cgl2, β-galactoside sugar binding lectins, were isolated from a wild-type dikaryon (Cooper *et al.*, 1997) and an *Amut Bmut* homokaryon of *C. cinereus*. In the *Amut Bmut* homokaryon, the expression of Cgl2 and Cgl1 is synchronous to the formation of primary and secondary hyphal knots, respectively (Boulianne *et al.*, 2000) (see chapter 3).

1.3.1.3 Formation of primordia

The cellular differentiation within secondary hyphal knots leads to the formation of primordia. Since the primordium of *C. cinereus* forms within a prosenchymatous matrix, such mode of carpogenesis is termed endocarpy (Clémençon, 1997). The development of different parts of the fruiting body, stipe, cap and hymenium, initiates more or less at the same time (isocarpous) (Reijnders, 1986). The hymenium arises internally, but is later exposed by rupture of the outermost tissue layer (veil) during further growth (hemi-angiocarpous) (Reijnders, 1979).

In primordia of 0.7 mm in diameter, organization and regional development of cells begin to be evident. In the upper third of the aggregation (the pre-pileus region) is an endogenous mass of densely packed cells. In the central region (pre-stipe), cells appear to have taken a vertical orientation. Randomly oriented cells are found in the lower third of the primordium. The apex of the developing pileus and the cells below the stipe are rich in glycogen (Matthews and Niederpruem, 1973). At this stage of early internal differentiation, the whole complex is enclosed by a marginal veil made of hyphae which originate from the top of the pileus (Matthews and Niederpruem, 1973). Veil cells are

large, septate and multinucleate and lack clamp connections (Lu, 1974). Already at a slightly larger size, inside 0.8 mm primordia, tissues that are demarcated into presumptive stipe and pileus are visible. At this stage, polysaccharide (glycogen) is most evident in the stipe base and along the edge of the pileus (Moore *et al.*, 1979). When primordia reach a size of 1.0-1.2 mm, the presumptive stipe, the subhymenium (a region between stipe and pileus), the pileus and the basal region are clearly visible. Ridges made up of small closely packed cells are seen on the subhymenium (Lu, 1991; Rosin and Moore, 1985b). The deposition of glycogen at the stipe base and edges of the pileus is now accentuated (Moore *et al.*, 1979). The internal structure of a primordium is uniformly solid until gills and gill cavities begin to arise, possibly by a process of programmed cell death (Lu, 1991). Well developed gill lamellae and a distinct gill cavity are seen in primordia of 1.5 to 3 mm. Dense glycogen deposits can be detected in cells of the basal bulb of the stipe (Moore *et al.*, 1979).

1.3.1.4 Maturation of primordia (pre- and post-karyogamy)

Gill formation. The subhymenium has two domains, an inner gill domain and an outer gill cavity domain. Gill differentiation is initiated before the formation of annular gill cavities, resulting in two types of gills, the primary and secondary gills (Rosin and Moore, 1985b). In a transverse section of the cap, gills are formed as vertical plates arranged radially around the stipe (Moore, 1995). Primary gills are formed first. A 1.0-1.2 mm primordium only contains primary gills, which are connected with the pileus at their outer edge and with the stipe at their inner edge (Rosin and Moore, 1985b). Gills develop outwards, away from the stipe (Moore, 1987). Vertical ridges of small closely packed cells arise in a region corresponding to the boundary between the stipe and pileus tissues. These groups of cells become chromophilic, elongated and form two radially arranged rows of oblong cells. These paired rows represent layers of poorly differentiated hymenia and are separated by an annular gill cavitiy. At this stage, the hymenium is discontinuous over the inner edge of primary gills (Rosin and Moore, 1985b). Gill cavities may be carved out by degenerating cells from the outer domain. The cell degeneration is associated with multivesicular structures, which contain electron-dense material and vacuoles containing fibrous residual bodies (Lu, 1991). The gill cavity elongates centrifugally towards the outer surface of the pileus. A formative element responsible for the outward progression of the gills has been postulated and termed as "the gill organizer"(Moore, 1995). A gill organizer is thought to bifurcate, to

be located in the outmost tissues of the gill cavity, and to move radially outwards to penetrate into undifferentiated pileus tissues. Since the fruiting body cup enlarges outwardly by the enlarged circumferences, the distance between two gill organizers increase. This enlarged distance between the two gill organizers allows a new organizer to arise in between them. When a new gill organizer emerges, the margin of a new secondary gill is formed. Unlike the primary gills that are connected to the stipe, the secondary gill has a free edge. Continued outward migration of the parent and daughter gill organizer generate together a secondary gill between them (Moore, 1995). Secondary gills have an enclosed trama, in which the hymenium is continuous over their inner edge (Moore, 1987).

Hymenium differentiation. The hymenium is made up of dikaryotic hyphae (Lu, 1974). In the young hymenium (2-6 mm primordia), a palisade of tightly packed club-shaped cells (probasidia) is dispersed among much larger inflated cells (cystidia) (Rosin and Moore, 1985a). In *C. cinereus*, both binucleate basidia and binucleate facial cystidia (Chiu and Moore, 1993) originate from the terminal cells of tramal hyphal branches (Horner and Moore, 1987). The formation of a facial cystidium inhibits the formation of other ones in the same hymenium within a radius of about 30 μm. As a result, only about 8% of these tramal hyphal branches become facial cystidia, and the rest become probasidia. In addition, the inhibition effect keeps the facial cystidia uniformly distributed over the gill surface (Horner and Moore, 1987). From the earliest stage during hymenium differentiation, facial cystidia are inserted into the opposite hymenium. As facial cystidia extend across the gill cavity, they contact probasidia in the opposing hymenium. Consequently the contacted probasidia change their cytoplasmic morphologies, generating a new cell type called "cystesium" (Horner and Moore, 1987). While probasidia proceed to karyogamy and meiosis, binucleate sterile paraphyses act as spacing elements and arise as branches of cells immediately beneath basidia (Buller, 1931; Chiu and Moore, 1993). Paraphyses are inserted into the basidial layer. Once paraphyseal insertion commences, basidial numbers do not increase any more. About 75% of the paraphyseal population is inserted at the end of meiosis, the rest at later stages of development (Rosin and Moore, 1985a). Therefore, the differentiation of hymenium follows a defined temporal sequence: probasidia and facial cystidia first, paraphysis and cystesia second (Moore, 1995). Of these four differentiated cell types, only basidia are committed to meiosis and basidiospore formation (Chiu and Moore,

1988b). The facial cystidia project across the gill cavities and hold adjacent gills apart, thus providing space for basidiospore development (Moore, 1995).

Basidium and basidiospore development. A basidium of *C. cinereus* arises from a terminal cell of a tramal hyphal branch (Horner and Moore, 1987). It enlarges while being filled with cytoplasm. The cytoplasm of a young basidium (probasidium) is relatively simple, containing numerous ribosomes, a few vacuoles, mitochondria and limited endoplasmic reticulum. Later in the prefusion stage, the basidial apex enlarges, glycogen is evident at the basidial base, and free ribosome and lipid droplets accumulate (McLaughlin, 1982). During the 10-hour period before karyogamy (Lu, 1972), probasidia are the only cells irreversibly committed as meiocytes to complete meiosis and sporulation. In contrast to facial cystidia and paraphyses, the majority of excised probasidia only arrest, but do not revert to hyphal growth. Once meiosis is initiated, the maturation of basidia is an autonomous endotropic process (Chiu and Moore, 1988b). During maturation, basidia continue to enlarge, while karyogamy and subsequent meiosis occur within. Although, in general, the basidia at the bottom of the gills are slightly more advanced in their development than those at the apex, about 70-80% of basidia are synchronous in their meiotic processes (Lu, 1967).

In a young basidium, a pair of haploid nuclei lies near the mid-region of the cell, one from each monokaryotic parent. At pre-fusion stage, the chromatin appears diffused when observed under the light microscope (Lu, 1967). As the binucleate basidium develops, the two nuclei come in close contact and fuse (karyogamy) to form a single large dumbbell-shaped nucleus; subsequently, the diploid nucleus becomes oval-shaped (Kimura and Takemaru, 1955). After nuclear fusion, individual chromosomes become visible. Two homologous chromosomes align in parallel, and pairing occurs by the formation of the synaptonemal complex. Crossing-over may take place at this stage. Spindle microtubules (Lu, 1967) participate in the following two meiotic divisions. After the completion of the second meiotic division, a tetrad of nuclei, equally distant from each other, is formed (Kimura and Takemaru, 1955) and the apical growth of the basidium stops (McLaughlin, 1973).

From the end of meiosis, it takes only about 18 h to complete the events leading to the discharge of basidiospores (Pukkila *et al.*, 1984). First, these four meiotic nuclei become more compact and elongated, migrating towards the tip of the basidium. Simultaneously, four sterigmata begin to project near the apex of the basidium (Kimura

and Takemaru, 1955), which are the renewed tip growth at four restricted loci on the basidial apex (McLaughlin, 1973). The position and pattern of sterigmata are not fixed early in basidial development, but rather determined at or just before their appearance on the basidia (McLaughlin, 1982). Sterigmata develop as blunt protuberances, then become tapered and curved as they enlarge (McLaughlin, 1977). It is not clear whether the sterigmal wall is a continuation of the basidial wall (McLaughlin, 1982). Ribosomes and other organelles are lacking at the sterigmata tip apex (McLaughlin, 1973), but vesicles derived from Golgi cisternae and containing carbohydrates are present at the growing apex (McLaughlin, 1972) and appear to fuse with the plasma membrane, implying a role in transporting wall materials (McLaughlin, 1973). The processes of basidiosporogenesis can be divided into four stages: inception, asymmetric growth, equal enlargement and elongation (McLaughlin, 1977). The cytoplasm becomes more and more complex as the development of a basidiospore progresses (Sundberg, 1978).

The first stage, inception, is characterized by the spherical enlargement of the sterigmata apex to form a more or less spherical spore initial, which grows more or less vertical (parallel to the longitudinal axis of the sterigmata). At the apex on the adaxial side of the basidiospore initials, an electronopaque structure (the hilar appendix body) appears near the plasma membrane and adjacent to the future site of the hilar appendix, persisting until the spores are matured. This hilar appendix body predicts the site of the hilar appendix that may be involved in discharging basidiospores (see below). The basidiospore wall at this stage is three-layered and thin, enclosing a simple cytoplasm (McLaughlin, 1977; McLaughlin, 1982).

In the second stage (asymmetric growth), the sterigmata change the orientation of growth from the vertical to the oblique/longitudinal axis, resulting in a greater increase in length than in width. The hilar appendix appears ajdacent to the hilar appendix body. The wall of basidiospores thickens and becomes six-layered. Small (30-60 nm) and big vesicles (60-100 nm) appear in the cytoplasm (McLaughlin, 1977; McLaughlin, 1982). Microtubules extend from the basidium into the sterigmata, suggesting that they may function as a cytoskeleton in the asymmetric growth or may direct the movement of vesicles during sterigmata development (McLaughlin, 1973).

In the third stage (equal enlargement), the sphere of a basidiospore enlarges equally in length and in width. At the beginning of this stage, a cylindrical layer of rough endoplasmic reticulum appears at the lower part of the basidiospore. Wall thickens only at the spore base where peripheral endoplasmic reticulum is present, suggesting the role

of those rough ER in forming enzymes for wall synthesis or expansion. The hilar appendix body projects further into the spore wall. The spore wall still has six layers, but the outer three layers are fused. The cytoplasm becomes more complex. Numerous Golgi vesicles are scattered throughout the basidiospore (McLaughlin, 1974). Microtubules are abundant in the sterigmata and in the lower end of the spores, but not associated with the hilar appendix body. Microbodies and mitochondria appear (McLaughlin, 1977; McLaughlin, 1982) and the spore wall begins to be pigmented (McLaughlin, 1974).

In the last elongation stage, the orientation of growth shifts from the oblique/longitudinal back to the vertical axis, thus basidiospores elongate considerably with little or no increase in width. The peripheral ER disappears at the beginning of this stage. As the spore elongates, the third wall layer disrupts and a pore cap develops at the spore apex. Wall growth takes place in this stage. Microtubules are distributed throughout the spore, often more or less oriented to its long axis and curved at the apex under the pore cap. A fully enlarged spore is 9-10.5 μm long and 5.5-6.5 μm wide (McLaughlin, 1977; McLaughlin, 1982). Nuclei migrate into the spores at this stage (McLaughlin, 1977).

Nuclei undergo a post-meiotic mitosis, giving rise to the mature binucleate basidiospore, but it is not clear whether before or after entering the basidiospore (Chiu and Moore, 1993; McLaughlin, 1977; Raju and Lu, 1970). Mature basidiospores are ballistically discharged by "Buller's droplet" which is formed 6 h before at the hilar appendix (Buller, 1922; McLaughlin *et al.*, 1985). In a phase of early enlargement, the droplets attain a diameter of about 0.5-1.0 μm and persist for a period of time. The second enlargement is very rapid, achieving 80-100 fold increase in volume at the point of spore discharge (McLaughlin *et al.*, 1985). Droplets at the hilar appendix on discharged spores had volumes of 1.66-24.1 μm^3 and their contents are mainly ice-like (McLaughlin and Beckett, 1987). Besides this mode of spore discharge in *C. cinereus*, the major release of spores occurs probably via autolysis of the cap (Iten and Matile, 1970; Kües, 2000).

Stipe development and stipe elongation. The stipe of *C. cinereus* contains both narrow and inflated hyphae. Narrow hyphae are seen to be branched or to be fused laterally with other narrow hyphae. No branching or fusion is seen between inflated hyphae. Both narrow and inflated hyphae can be found in 3 mm primordia. In a pre-

meiotic (likely prekaryogamic) primordium (< 6 mm) in which the lumen is not yet developed, the central region of the stipe is exclusively occupied by narrow hyphae. The proportion of narrow hyphae decreases with time, implying that some of them become inflated as primordia develop. With the increase in stipe diameter, a lumen develops centrally throughout most of the stipe. In well-developed stipes (70 mm) of the mature fruiting body (lumen is fully developed), narrow hyphae are particularly found in coating the outside of the stipe and fringing the central lumen. Narrow hyphae also fill the regions at the extreme bases and apices of elongating stipes. In addition, narrow hyphae are interspersed between the inflated hyphae that are situated between the mid-cortex and the lumen. Inflated hyphae appear to be evenly distributed regardless of the primordial age or position within the stipe (Hammad *et al.*, 1993b).

In *C. cinereus*, stipes of the pre-meiotic stage (3-8 mm primordia) probably only proliferate, but do not elongate, since the length/width ratios of stipe cells increase only little (Hammad *et al.*, 1993a). Stipe elongation is however mainly a post-meiotic event. The most rapid stipe elongation starts 8 h after karyogamy (*i.e.* the end of meiosis) and lasts for approximately 5 h (prior to spore discharge and pileus autolysis) (Hammad *et al.*, 1993a). The stipe does not elongate equally over its whole length. The upper mid-region is the most active zone of extension (Cox and Niederpruem, 1975). The middle zone elongates 8.4-fold in 15 h during fruiting body maturation (Kamada and Takemaru, 1977). Rapid stipe elongation of *C. cinereus* is an endotrophic autonomous process, since stipes from primordia greater than 5 mm (pre-karyogamy) can elongate without a requirement for connection with the cap or the parental mycelium (Cox and Niederpruem, 1975; Gooday, 1974) and the presence of exogenous nutrients or water (Gooday, 1975). However, although not dependent, the stipe elongation is significantly enhanced by the presence of the cap (Hammad *et al.*, 1993a). Rapid stipe elongation is mainly the result of an intercalary elongation of inflated cells situated between the mid-cortex and the lumen. The greatest cell expansion was seen in the inflated cells situated between the mid-cortex and the lumen, not at the periphery of the stipe (Hammad *et al.*, 1993b). Within these inflated cells, the cytoplasm is restricted to the cylindrical periphery by a vacuole (Kamada, 1994; Moore *et al.*, 1979). During stipe cell elongation, the cell width remains almost constant (Kamada and Takemaru, 1977).

Chitin is a major component of the *C. cinereus* stipe. Chitin microfibrils are predominantly found in the walls of stipe cells in a transverse orientation both before and after elongation (Gooday, 1975). During stipe elongation, the chitin content of the

stipe increases at the expense of proteins and glucans rather than glycogen (Gooday, 1975). New chitin microfibrils are dispersed among the pre-existing transverse microfibrils (Gooday, 1975).

Cap expansion and autolysis. Cap expansion correlates temporally and proportionally with the rapid stipe elongation (Kües, 2000). Most changes in the shape of the cap depend on cell expansion (Moore, 1998; Moore *et al.*, 1979). At the time of basidiospore pigmentation (ca. 8 h before the basidiospore discharge) (Iten and Matile, 1970), hymenial cells become vacuolated (Iten and Matile, 1970) and glycogen disappears from the subhymenium tissues (McLaughlin, 1974). As basidiospores mature (about 14 h after karyogamy), the expansion of different cell types in the cap begins immediately, as well as inflation of cells in the stipe (Hammad *et al.*, 1993a). Unlike formerly developed facial cystidia, multinucleate marginal cystidia arise from gill trama when primary gills pull away from the central stipe as the cap starts to expand (Chiu and Moore, 1993; Lu, 1974). Soon after the first basidiospore discharge, autolysis initiates (Iten and Matile, 1970) to make space for the liberation of basidiospores from the inner cap regions (Moore, 1998). Like the pigmentation of the spore walls, the autolysis of gills proceeds from the edge of the gill closest to the stipe towards the outer edge of the cap and from the cap margin towards the apex (Rosin and Moore, 1985b). Autolysis is caused by cell wall degradation by chitinases (Iten and Matile, 1970) and glucanases (Miyaka *et al.*, 1980). NADP-linked glutamate dehydrogenase is most active at the end of fruiting body development and possibly acts in metabolic control of autolysis (Stewart and Moore, 1974). The production of these three enzymes is not synchronous in time and space. The production of NADP-linked glutamate dehydrogenase starts during meiosis (Miyaka *et al.*, 1980) and increases its activity only in the pileus during fruiting body maturation (Stewart and Moore, 1974). The production of chitinases and glucanases is observed after the completion of meiosis (Miyaka *et al.*, 1980). Chitinases are located in vacuoles of gill tissues and synthesized about 2 h before spore release (Iten and Matile, 1970). Autolysis affects all pileus tissues, but not the outer veil cells (Kües, 2000).

1.3.2 Fruiting body development in other basidiomycetes

Schizophyllum commune: *S. commune* Fr. (Schizophyllales, Schizophyllaceae) (Hawksworth *et al.*, 1995) commonly know as "split gills" (Kendrick, 1992) is a

heterothallic species with mating governed by a tetrapolar breeding system (Raper and Miles, 1958) (section 1.4.1). The progression of morphological events leading to the formation of fruiting structures in *S. commune* can be divided into five stages (Leonard and Dick, 1968). Stage I involves the local aggregation and upgrowth of randomly oriented hyphae (van der Valk and Marchant, 1978). Such hyphal aggregates have no defined regular shape (Leonard and Dick, 1968) and possibly correspond to the primary hyphal knots of *C. cinereus*. It is not known whether in *S. commune* such hyphal aggregates arise from one or more hyphae. In stage II, hyphal growth becomes directed to the apical center of the aggregates. The apical cells are relatively short, while cells at the periphery are much longer (Wessels, 1992), resulting to the formation of a "hyphal tuft" with regular subspherical to cylindrical appearance (Volz and Niederpruem, 1969). The "hyphal tuft" of *S. commune* resembles the secondary hyphal knot of *C. cinereus*. The structure of stage III primordia of *S. commune* is similar to that of Stage II, but with a macroscopically visible apical pit (Volz and Niederpruem, 1969). The macroscopically visible gills are formed in Stage IV, and stage V is the expansion of the gilled surface (Leonard and Dick, 1968). Very different from the hemi-angiocarpic mode of *C. cinereus*, the hymenium development in *S. commune* is gymnocarpic (exposed hymenium) due to its formation at the apical surface of the primordium (van der Valk and Marchant, 1978). The hymenium develops early on the surface of the apical cavity. Folding of the hymenium produces the gills. Young gills form as palisade on the hymenial surface (Volz and Niederpruem, 1969). The cavity is then widened by lateral expansion of the hymenium, while gills are continuously formed. The edge of the hymenium folds and produces the typical split gills. The unilateral enlargement transforms the cup-shaped young fruiting bodies into fan-shaped mature fruiting bodies (Wessels, 1992). The differentiation of the hymenium gives rise to basidia. A basidium of *S. commune* arises as a proliferation from a terminal hymenial hypha and enlarges while nuclei are undergoing karyogamy and meiosis (Ehrlich and McDonough, 1949). Four sterigmata appear at the end of meiosis, while small vacuoles appear in the proximal regions of the basidia. With vacuoles increasing in number and in size, four haploid nuclei migrate individually into sterigmata. Following the movement of the basidial cytoplasm and the haploid nucleus into the basidiospore, an electrogen-dense hilum arises in the hilar region delimiting the basidiospore and sterigma. Soon after a post-meiotic mitosis, the basidiospore becomes binucleate and a hilar-appendix is formed at the dorsal side of the sterigma. At the end, basidiospores are ballisticaly

discharged, probably by the formation of a "Buller's droplet" (Wells, 1965). Different from *C. cinereus* gills, no stipe elongation nor cap autolysis occurs during fruiting body maturation in *S. commune*. In fact, *S. commune* only possesses a very underdeveloped stipe that contains thick-walled cells adjacent to collapsed filaments (Niederpruem and Wessels, 1969). In particular, fruiting bodies have stipes only when grown on an horizontal surface, whereas they are sessile when grown on inverted or verticle surfaces (Volz and Niederpruem, 1969). The fruiting bodies of *S. commune* are very long lasting and can shed basidiospores upon wetting after being kept dry for years (Ainsworth, 1962).

Agaricus bisporus: *A. bisporus* (Lange) Imbach (Agaricales, Agaricaceae) (Hawksworth *et al.*, 1995), the button mushroom (Royse and May, 1993), is a secondary heterothallic basidiomycete with a bipolar breeding system (Raper and Raper, 1972; Xu *et al.*, 1993). This organism predominantly produces basidia with only two spores that contain two haploid nuclei of different mating types and germinate to self-fertile heterokaryotic mycelia (Elliott, 1985; Miles, 1996). The cells of the vegetative mycelium are multinucleate (ca. 6-25 nuclei per cell). Septal clamp cells have never been observed and there is no evidence for nuclear migration, nuclear pairing or conjugate nuclear division (Raper and Raper, 1972).

The culture substrate used for the commercial production of fruiting bodies of *A. bisporus* is normally based on non-sterile composted plant residues. However, *A. bisporus* (Lange) Sing. formed 1-2 mm primordia in axenic artificial media (Wood, 1976). The early fruiting body development includes the formation of hyphal strands from isolated hyphae and the development of primordia from the strands (Angeli-Papa and Eyme, 1978). These early events can be divided into four stages. In stage 1, some hyphae grow simultaneously in the same direction and fuse to thicker hyphae. A thick hypha consists of two or several hyphae fused sidewise and have an average diameter of 6.7 μm. The surface of these thick hyphae is ornamented with calcium-rich small hairy structures. Thin layers of mucilaginous webbing materials extend between individual thick hyphae. Stage 2 is characterized by the consolidation through multihyphal aggregation of numerous hyphae into structures usually called strands (or cords). Within a strand, some hyphae appear to be devoid of contents, while neighboring hyphae are filled with cytoplasm. A hyphal strand branches occasionally and these branches increase in length without any special orientation. This loss of polarity in growth of

ramifications, together with their lack of adhesion, is the first sign of fruiting body formation. In stage 3, the microscopic primordial initials are formed, which first appear as more compact masses in already dense mycelial areas. These *A. bisporus* initials look like the secondary hyphal knots of *C. cinereus*. Numerous short branches are observed in primordial initials and primordial hyphae appear more turgid than their surrounding counterparts. In stage 4, these initials appear to recruit hyphal contents from surrounding mycelia, resulting in a localized thinning of mycelium around them (Angeli-Papa and Eyme, 1978; Heckman *et al.*, 1989). At the end of stage 4, a round-shaped primordium with little differentiated hyphal tissues is formed, which is enclosed by a universal veil. In the base of these primordia are vertical hyphae and irregularly interwined hyphae (veil tissue) are found at the apex (Angeli-Papa and Eyme, 1978; Umar and Van Griensven, 1997). Later, when primordia reach a size of ca. 10 mm, hyphae with parallel orientation can be found in the region where subsequently the upper part of the stipe develops. At this stage, cap tissue also becomes visible. Due to the differentiation of cap tissue, the gills become visible (De Groot *et al.*, 1997). Morphogenetic cell death occurs during the formation of cavities between the stipe-hymenium junction (Umar and Van Griensven, 1997), as well as during the earlier mycelial strand formation (Umar and Van Griensven, 1998). When primordia reach 15-20 mm in size, the shape of the *A. bisporus* fruiting body is clearly visible (Angeli-Papa and Eyme, 1978). Like *C. cinereus*, the last stages of fruiting body maturation involve rapid expansion of the cap and gills and rapid elongation of the stipe. Unlike *C. cinereus*, the rapid stipe elongation in *A. bisporus* occurs in the uppermost region by non-polar cell growth and cell division (Craig *et al.*, 1977).

Flammulina velutipes: *F. velutipes* (Agaricales, Tricholomataceae) (Hawksworth *et al.*, 1995), the enokitake of Japan (Kendrick, 1992), is a tetrapolar basidiomycete that grows and fruits readily in culture. The mode of fruiting body development is angiocarpic (Reijnders, 1963) and is initiated in the aerial mycelium by the formation of the "knot of hyphae", beset with "aspergilloid hyphae". Each of the aspergilloid hyphae has a slightly swollen apex, from where needle-shaped or feathery crystals radiate. The knot of hyphae in *F. velutipes* resembles the primary hyphal knot of *C. cinereus*. It develops into a shaft that consists of narrow and mostly parallel hyphae bound together in a common slime. Cystidia develop at the apex of this shaft, each secreting a droplet of liquid. As the shaft enlarges, a distinct pileus is formed at the upper part of the

primordium. At this stage, the whole primordium is densely covered with cystidia, particularly on the young cap, and the aspergilloid hyphae are no longer seen. Cystidia persist and increase in number during later development (Ingold, 1980).

Lentinus edodes: *L. edodes* (Berk) Sing (Agaricales, Tricholomataceae) (Hawksworth *et al.*, 1995) is the shiitake of Japan and the Xiang-gu of China (Kendrick, 1992). The origin of the *L. edodes* fruiting body may be multicellular, since two parental types of mtDNA were once recovered from tissues and basidiospores of a single fruiting body arised from the junction zone between the two mating colonies (Fukuda and Fukumasa-Nakai, 1996). At the beginning of fruiting body development, the whole originally fluffy vegetative mycelium develops into a tight aggregated mat (called semi-condensed mycelium). Progressive mycelial condensation results in the formation of a thick membrane-like mycelium (called condensed mycelium). Small protuberances (immature fruiting bodies) are formed on the condensed mycelium and they grow up into mature fruiting bodies (Takagi *et al.*, 1988). To my knowledge, the cellular events in fruiting body development of *L. edodes* are generally poorly described, except for the post-meiotic nuclear behaviour. In the basidia, the four haploid nuclei, formed after karyogamy and meiosis, migrate individually into the basidiospores. After a post-meiotic mitosis, the basidiospores become binucleate. Unlike *C. cinereus*, four basal nuclei of *L. edodes* migrate back into the basidium. As a result, the mature basidium bears four uninucleate basidiospores and contains four residual nuclei that later degenerate (Murakami and Takemaru, 1985).

***Pleurotus* spp.:** *Pleurotus ostreatus* (Jacq. Ex. Fr.) Kummer (Poriales, Lentinaceae) (Hawksworth *et al.*, 1995) is known as the oyster mushroom (Kendrick, 1992). The mode of fruiting body formation is gymnocarpic (Reijnders, 1963). Like in the case of *L. edodes*, literature on the cellular processes of fruiting body development is scarce. In several *Pleurotus* spp., the post-meiotic nuclear division occurs in the basidium, resulting in the formation of eight nuclei. Four nuclei migrate into the basidiospores and the other four nuclei degenerate in the basidium (Clémençon, 1997).

***Volvariella* spp.:** *Volvariella volvacea* (Bull ex Fr.) Sing. (Agaricales, Pluteaceae) (Hawksworth *et al.*, 1995) is commonly known as the paddy straw mushroom (Kendrick, 1992). It has long been considered as a primary homothallic species with

model fungus *C. cinereus* (section 1.3.1). Although fruiting body development appears not to follow an exact cellular scheme in different basidiomycete species, the initial steps in most cases are relatively similar with the formation of hyphal knots, which sometimes are obviously separated into two substages, the primary and the secondary hyphal knots. Later tissue differentiation in various species seems to follow different temporal and, most importantly, spatial routes, giving rise to very different appearances of fruiting bodies. Nevertheless, it is likely that the basic molecular processes are the same among different species. Therefore, unraveling the molecular basis in the model fungus *C. cinereus* should help to understand fruiting body development and its regulations in other mushrooms. Most fortunately, this fungus is readily accessible to a range of molecular and classical genetic techniques (Walser *et al.*, 2001).

1.4 Regulation of fruiting body development

The development of fruiting bodies is a highly organized process, which requires coordination among genetic, environmental and physiological factors. In *C. cinereus*, mating-type genes have been shown to be the master regulators of fruiting body initiation. Mutations in mating-type genes and in other genes acting in the *A* mating-type pathways can result in self-compatibility (section 1.4.1). Light and nutritional depletion are the two primary stimuli for inducing fruiting (sections 1.4.2 and 1.4.3). Light has both inducing and inhibiting effects on the development of fruiting bodies. Neither stimulus is effective, unless the mycelium is physiologically competent (section 1.4.4).

1.4.1 Genetic regulation

1.4.1.1 Mating-type genes as master regulators

Many heterothallic basidiomycetes including *C. cinereus* have a tetrapolar breeding system, in which mating incompatibility is determined by two unlinked mating-type loci *A* and *B* (Casselton and Kües, 1994). Tetrapolar refers to the fact that four different types of basidiospores can be produced from meiosis in respect of mating-type specificity (AxBx, AxBy, AyBx and AyBy, where x and y represent different mating-type specificities). Both the *A* and the *B* locus contain several multiallelic genes. Different combinations between these genes determine the estimated 12,000 mating types in natural populations. A successful mating between two monokaryons requires

that the two mating partners differ at least in one mating-type gene at each locus [for review see (Casselton and Olesnicky, 1998; Hiscock and Kües, 1999)]. The establishment and maintenance of a stable dikaryon are primarily governed by the mating-type genes (Tymon *et al.*, 1992) [for review see (Casselton and Olesnicky, 1998; Kües, 2000)]. Since fruiting bodies usually develop on a dikaryotic thallus, mating-types genes ultimately also control fruiting initiation [for review see (Kües and Liu, 2000)]. This postulate has been confirmed when monokaryons were shown to initiate fruiting body development upon transformation with cloned *A* and cloned *B* mating-type genes (Kües *et al.*, 1998; Tymon *et al.*, 1992; U. Kües and M. Klaus, unpublished results). In particular, heterologous *A* genes induce primary hyphal knot formation in transformed monokaryons and these primary hyphal knots develop into secondary hyphal knots upon light induction (Kües *et al.*, 1998). Heterologous *B* genes have also been shown to promote the formation of primary hyphal knots, but development does not continue even in light. However, in transformants carrying both heterologous *A* and *B* genes, fruiting body development up to maturation has been observed. Moreover, initiation of fruiting in these transformants is more regular, indicating that the coordination between *A* and *B* mating type genes is an important factor for regulating fruiting body initiation (U. Kües and M. Klaus, unpublished data).

The *A* mating type locus contains three to four paralogous pairs of multiallelic genes (Pardo *et al.*, 1996) that encode two types of homeodomain proteins (HD1 and HD2) differing in the sequence of their DNA-binding domains (Kües *et al.*, 1994a). When mating cells fuse, HD1 and HD2 of different specificity are thought to heterodimerize via the N-terminal domains in order to generate a transcription factor that promotes the regulated expression of genes required for dikaryosis (Banham *et al.*, 1995). Heterodimerization brings together potential trans-activation domains at the C terminus of the HD1 protein and the essential HD2 DNA-binding domain (Asante-Owusu *et al.*, 1996). Unlike the HD1 proteins, HD2 proteins have no nuclear localization signals (Kües *et al.*, 1994a; Tymon *et al.*, 1992), suggesting that heterodimerization also regulates the entry of the active transcription factor complex into the nucleus (Spit *et al.*, 1998).

The multiallelic *B* mating type locus contains three paralogous families of genes, but they encode a large family of lipopeptide pheromones with a consensus CaaX (C = cysteine, a = aliphatic amino acid, X = any amino acid) motif for isoprenylation at their C-terminal ends, and pheromone receptors with 7 transmembrane domains,

respectively. Each family consists of two pheromone genes and one receptor gene (Halsall *et al.*, 2000; O'Shea *et al.*, 1998). For regulating development, pheromones of one *B* mating-type specificity have to be recognized by pheromone receptors of another *B* mating-type specificity (Olesnicky *et al.*, 1999; Olesnicky *et al.*, 2000).

Special mutations in both mating-type genes of *C. cinereus* result in the formation of *Amut Bmut* homokaryons, which bypass the control in development by mating-type genes and therefore form normal fruiting bodies without mating to another strain (Haylock *et al.*, 1980; Swamy *et al.*, 1984). In a *C. cinereus* mutant defective in the *A* mating-type locus (*Amut*), an inframe fusion between an *HD2* and an *HD1* gene within the same *A* locus constitutively switched on *A*-controlled development (Kües *et al.*, 1994b). In a mutant defective in the *B* mating-type locus (*Bmut*), a single amino acid change (Q229P) in the transmembrane domain VI of receptor $Rcb2^{6m}$ confers a self-compatible mating phenotype (Olesnicky *et al.*, 1999). In other cases, mutations altered the binding specificities of pheromones and pheromone receptors (Olesnicky *et al.*, 2000). Similar mutations have been shown to exist in mating-type mutants of *S. commune* (Fowler *et al.*, 1998; Kothe, 1999) that are also able to initiate fruiting body development without mating to another strain (Raper *et al.*, 1965)

1.4.1.2 Genes involved in fruiting body development

RNA populations fluctuate during fruiting body development. In *S. commune*, 5% of mRNA specifically accumulated and some proteins were only synthesized during primordium formation (De Vries and Wessels, 1984; Hoge *et al.*, 1982). In *C. cinereus*, the transcription of 7% mRNA showed both temporal and spatial specificities during fruiting body morphogenesis (Yashar and Pukkila, 1985). Such fluctuation is caused by the differential transcription of developmentally regulated genes, which are either specifically expressed or induced during fruiting. This group of genes is not tightly clustered in genomes of *C. cinereus* (Yashar and Pukkila, 1985), *S. commune* (Mulder and Wessels, 1986) and *A. aegerita* (Salvado and Labarere, 1991). Several approaches have been employed to clone genes acting in fruiting body development. For model organisms such as *C. cinereus*, large collections of developmental mutants are available (Cummings *et al.*, 1999; Muraguchi *et al.*, 1999; U. Kües, *et al*, in prep). Genes corresponding to the mutations can be isolated from specific mutants by DNA complementation if obtained by traditional mutagenesis techniques (UV, X-ray, chemical or spontaneous), and by plasmid rescue or specific PCR techniques in case of

REMI-mutants. When information about functions of genes is available in other related organisms, PCR with degenerative primers becomes a powerful tool, *e.g.* in the cloning of *ras* genes from *L. edodes* (Hori *et al.*, 1991) and *C. cinereus* (Bottoli *et al.*, 1999; Ishibashi and Shishido, 1993), the adenylate cyclase gene *cac* from *C. cinereus* (Bottoli, 2001) and the chitin synthase gene *chs1* from *A. bisporus* (Sreenivasaprasad *et al.*, 2000). For species such as *A. bisporus* and *L. edodes* that only recently became amenable to genetic manipulations (de Groot *et al.*, 1998; Hirano *et al.*, 2000), RNA-fingerprinting by arbitrarily primed PCR (RAP-PCR) (Leung *et al.*, 2000) and expressed sequence tag (EST) analysis (Ospina-Giraldo *et al.*, 2000) provided a simple and sensitive method to study differential gene expression and gene isolation. Molecular analysis of differentially expressed genes in their hosts may become possible in the future, since more efficient transformation systems have now been described for both species (Chen *et al.*, 2000).

Table 2 compiles cloned genes from basidiomycetes that are shown or thought to play a role in fruiting body development. Only very few of these genes are specifically transcribed in fruiting body tissues, for example the galectin genes of *C. cinereus* and some hydrophobin genes and laccase genes of other basidiomycetes (Table 2). Analysis of a number of *C. cinereus* mutants with specific defects in fruiting body development showed that genes such as *eln2* (Muraguchi and Kamada, 2000) and *cfs1* (see chapter 4) are indeed transcribed also in vegetative mycelium, indicating that transcription alone can not be the only criteria to decide the function of a gene in fruiting.

Undisputed is the role of hydrophobins in fruiting body development. Hydrophobins are small, moderately hydrophobic proteins that have molecular masses around 10 kDa. Although different hydrophobins share little amino-acid homology, the spacing pattern between seven or eight conserved cystein residues is well conserved (Ng *et al.*, 2000) [for review see (Wessels, 1997)]. These cystein residues are important for the general structures and functions. Multiple hydrophobins are common to basidiomycetes and are shown to be involved in different biological processes. The temporal and spatial transcription patterns indicate that different hydrophobins contribute to the surface hydrophobicity of morphologically different structures including fruiting bodies [for review see (Kershaw and Talbot, 1998; Wessels, 1997)]. On the basis of similarity in hydropathy patterns and the solubility characteristics of assemblage, hydrophobins are divided into two classes. Class I is insoluble in SDS, class II is soluble [for review see (Wessels, 1997)]. Most fruiting body specific hydrophobins, HypA/ABH1 and

Table 2. Genes (potentially) involved in fruiting body development in higher basidiomycetes

(putative) Function	Gene	Organism	Transcriptional pattern	Reference
Substrate utilization / nutritional regulation				
Laccase	not named	*A. bisporus*	highest before fruiting body development	1
Hexose transporter	*LeStr*	*L. edodes*	high in vegetative mycelium after cold treatment and in primordia	2
Fructose 1,6-bisphosphatase	*LeFbp*	*L. edodes*	high in mature fruiting bodies	2
α subunit of mitochondrial ATP synthase	*AtpD*	*A. bisporus*	high before primordium formation	3
Adenylate cyclase	*cac*	*C. cinereus*	constitutive	4, 5
Signal transduction				
B mating-type pheromones and receptors	*B* genes	*C. cinereus*	not known	6, 7
	B genes	*S. commune*	pheromone genes are highly transcribed at 12-24 h after mating (during the period of nuclear migration)	8
Small G-protein	*Cc ras*	*C. cinereus*	constitutive	4, 5, 9
	Le ras	*L. edodes*	constitutive	10
	ras1	*S. commune*	not known	50
Transducin α subunit	*cpgα1*	*C. congregatus*	specific to hyphal tip cells	11
Adenylyl-cyclase-associated protein, CAP	*cap*	*L. edodes*	equal in all stages in fruiting body development	48
CAP-interacting protein	14-3-3 gene	*L. edodes*	not known	49
Mitogen-activated protein kinase	*LeMapK*	*L. edodes*	high in primordia and young fruiting bodies (after karyogamy)	2
Transcriptional regulation				
A mating-type homeodomain transcription factors	*A* genes	*C. cinereus*	constitutive	12
	A genes	*C. bilanatus*	not determined	13
	A genes	*S. commune*	constitutive	14, 15, 16
Transcription factor with a HMG domain	*pcc1*	*C. cinereus*	high in dikaryon and in *A*on or *B*on monokaryons	17
Transcription factor	*LeNotI*	*L. edodes*	high in primordia	2
Novel with zinc-finger and zinc-cluster	*priA*	*L. edodes*	high in primordia and young fruiting bodies (after karyogamy)	2, 18
Transcription factor	*LeJun*	*L. edodes*	high in primordia and young fruiting bodies (after karyogamy)	2
DNA binding protein with zinc-cluster	*priB*	*L. edodes*	high in primordia	19, 47

Cell division / wall synthesis				
Cyclin B	*LeClb* (2.5 kb)	*L. edodes*	all stages in fruiting body development	2
	LeClb (2.5/2.2 kb)	*L.. edodes*	high in mature fruiting bodies	2
Chitin synthase	*chs1*	*A. bisporus*	all stages in fruiting body development	20
Hyphal aggregation				
Galectin	*cgl1*	*C. cinereus*	specific to primordia (high at karyogamy-pachytene)	21, 22, 23
	cgl2	*C. cinereus*	specific to primordia (high at karyogamy-pachytene)	21, 22, 23
Aggregation factor with SET domain and Phd finger (transcription factors?)	*mfbA*	*L. edodes*	specific to mature fruiting bodies (high in stipe)	24, 25
Hydrophobin	*HypB/ABH2*	*A. bisporus*	high in small primordia	26
	HypA/ABH1	*A. bisporus*	specific in veils of primordia and mature fruiting bodies	26, 27, 28
	Aa-Pri2	*A. aegerita*	specific to primordia	29
	Lehyd1	*L. edodes*	specific to primordia	30
	Fbh1	*P. ostreatus*	specific to mature fruiting bodies	31
	Sc4	*S. commune*	high in primordia	32
	Sc7	*S. commune*	specific to mature fruiting bodies	33
Secondary hyphal knot formation				
Cyclopropane fatty acid synthase	*cfs1*	*C. cinereus*	increase during fruiting and high in primordia in meiosis	51
Primordial tissue formation				
Cytochrome P450	*CypA*	*A. bisporus*	high before primordium formation	34
Cytochrome P450	*eln2*	*C. cinereus*	constitutive	35
Asp-hemolysin	*Aa-Pri1*	*A. aegerita*	specific to primordia and young fruiting bodies after karyogamy	36
O-methyltransferase	*ich1*	*C. cinereus*	specific to the pileus	37
Meiosis / DNA recombination				
Chromosomal pairing	*CoLIM15*	*C. cinereus*	high at 4-5 h after karyogamy	38

Novel	*rad9*	*C. cinereus*	high at 6 h after karyogamy	39
Rad51	*rad51*	*C. cinereus*	induced by γ-radiation, high at 6 h after karyogamy	40
Meiotic recombination protein	*mre11*	*C. cinereus*	high at 6 h after karyogamy	41
Type II topoisomerase	*spo11*	*C. cinereus*	high at 6 h after karyogamy	42
Fruiting body maturation				
Laccase	*lac1*	*L. edodes*	high in mature caps	43
	lac2	*L. edodes*	high in mature caps	43
Mitochondrial biogenesis				
β subunit of mitochondrial processing peptidase	*β-MPP*	*L. edodes*	high in primordia to mature fruiting bodies	2
Protein degradation				
Ubiquitin	*LeUbi*	*L. edodes*	high in primordia	2
Others				
Drug efflux pump	*LeDep*	*L. edodes*	high in vegetative mycelium after cold treatment	2
unknown	*EMAa-1 to EMAa-8*	*A. aegerita*	specific to primordia	44
unknown	*fds*	*F. velutipes*	all stages of fruiting body development, *F. velutipes* specific	45, 46
unknown	not named	*L. edodes*	high in primordia to young fruiting bodies after karyogamy	2
unknown	not named	*L.edodes*	high in mature fruiting bodies	2

1(Ohga *et al.*, 1999), 2(Leung *et al.*, 2000), 3(De Groot *et al.*, 1997), 4(Bottoli *et al.*, 1999), 5(Bottoli, 2001), 6(O'Shea *et al.*, 1998), 7(Halsall *et al.*, 2000), 8(Vaillancourt *et al.*, 1997), 9(Ishibashi and Shishido, 1993), 10(Hori *et al.*, 1991), 11(Kozak *et al.*, 1995), 12(Richardson *et al.*, 1993), 13(Kües *et al.*, 2000a), 14(Yang *et al.*, 1995), 15(Shen *et al.*, 1996), 16(Wu *et al.*, 1996), 17(Murata *et al.*, 1998), 18(Kajiwara *et al.*, 1992), 19(Endo *et al.*, 1994), 20(Sreenivasaprasad *et al.*, 2000), 21(Charlton *et al.*, 1992), 22(Cooper *et al.*, 1997), 23(Boulianne *et al.*, 2000), 24(Kondoh *et al.*, 1995), 25(Kües and Liu, 2000), 26(De Groot *et al.*, 1999), 27(Lugones *et al.*, 1996), 28(De Groot *et al.*, 1996), 29(Santos and Labarere, 1999), 30(Ng *et al.*, 2000), 31(Penas *et al.*, 1998), 32(Schuren and Wessels, 1990), 33(Schuren *et al.*, 1993), 34(De Groot *et al.*, 1997), 35(Muraguchi and Kamada, 2000), 36(Fernandez Espinar and Labarere, 1997), 37(Muraguchi and Kamada, 1998), 38(Nara *et al.*, 1999), 39(Seitz *et al.*, 1996), 40(Stassen *et al.*, 1997), 41(Gerecke and Zolan, 2000), 42(Celerin *et al.*, 2000), 43(Zhao and Kwan, 1999), 44(Salvado and Labarere, 1991), 45(Azuma *et al.*, 1996b), 46(Azuma *et al.*, 1996a), 47(Miyazaki *et al.*, 1997), 48(Zhou *et al.*, 1998), 49(Zhou *et al.*, 2000), 50(Accession number AF268471), 51(chapter 4).

HypB/ABH2 of *A. bisporus* (De Groot *et al.*, 1999; De Groot *et al.*, 1996; Lugones *et al.*, 1998), Aa-Pri2 from *A. aegerita* (Santos and Labarere, 1999), Sc4 and Sc7 of *S. commune* (Schuren *et al.*, 1993; Schuren and Wessels, 1990), Lehyd1 and Lehyd2 of *L. edodes* (Ng *et al.*, 2000) belong to the class I hydrophobins. Only Fbh1 of *P. ostreatus* belongs to the class II hydrophobins (Penas *et al.*, 1998). Immuno-localization studies suggest that Sc4 of *S. commune* as well as HypA/ABH1 of *A. bisporus* are probably responsible for the hydrophobicity of the fruiting body surface and line air channels within fruiting bodies (Lugones *et al.*, 1996; Lugones *et al.*, 1999).

In *C. cinereus*, two galectins (Cgl1 and Cgl2) that specifically bind to β-galactoside carbohydrates (Cooper *et al.*, 1997) may be involved in fruiting body specific hyphal aggregation (Boulianne *et al.*, 2000). Expression of Cgl2 and Cgl1 is synchronous to the formation of primary and secondary hyphal knots, respectively (Boulianne *et al.*, 2000). Expression of galectins and formation of primary and secondary hyphal knots are under the regulation of *A* mating-type genes, light and nutrition (Bottoli, 2001; Boulianne *et al.*, 2000; Kües *et al.*, 1998) (see chapter 3), indicating a link between them. However, gene *cgl1 and cgl2* are transcribed highest in primordia at the time period from karyogamy to early pachytene (Charlton *et al.*, 1992). This high expression is thought to prepare the primordia for the rapid stipe elongation, which probably requires large amount of galectins to keep the elongating cells of the stipe attached to each other (Boulianne *et al.*, 2000) (see chapter 3).

Other proteins being discussed to act in the structure of fruiting bodies include a putative hemolysin Aa-Pri1 of *A. aegerita* (Fernandez Espinar and Labarere, 1997) and MfbA of *L. edodes* (Kondoh *et al.*, 1995), possibly representing aggregation factors. The presence of a hydrophobic N-region might allow the Aa-Pri1 protein to bind to specific membrane receptors and this could cause aggregation amongst hyphae in order to form the primordia of *A. aegerita* (Fernandez Espinar and Labarere, 1997). A short peptide with a RGD motif of *L. edodes* MfbA protein has been shown to aggregate *S. commune* hyphae (Yasuda *et al.*, 1997), but the general structure of the protein includes a cysteine-rich SET domain and a Phd finger that are typical for transcription factors (Kües and Liu, 2000).

Extracellular laccases (EC 1.10.3.2), a type of copper-containing polyphenol oxidase (Thurston, 1994), might also contribute to the structure of fruiting bodies by oxidative polyphenolization of cell wall components to strengthen cell-to-cell adhesion (Zhao and Kwan, 1999). Moreover, laccases can play a role in pigmentation of the fruiting bodies

and/or the basidiospores (Zhao and Kwan, 1999). A number of laccase genes have been cloned from white rot basidiomycetes, *e.g.* from *Pycnoporus cinnabarinus* (Eggert *et al.*, 1998; Temp *et al.*, 1999), *Phlebia radiata* (Saloheimo *et al.*, 1991), *Trametes villosa* (Yaver and Golightly, 1996; Yaver *et al.*, 1996), *Trametes versicolor* (Jonsson *et al.*, 1995; Ong *et al.*, 1997) and from mushrooms including *A. bisporus* (Perry *et al.*, 1993), *C. cinereus* (Bottoli *et al.*, 1999; Yaver *et al.*, 1999), *L. edodes* (Zhao and Kwan, 1999) and *P. ostreatus* (Giardina *et al.*, 1995). Laccases are needed for utilizing lignocellulosic substrates [for review see (Whiteford and Thurston, 2000)] and may play in this way a role in fruiting body morphogenesis [for review see (Thurston, 1994)]. In *A. bisporus* (Wood, 1980) and *L. edodes* (Ohga *et al.*, 2000), laccase activity accumulates during vegetative growth in precise parallel with the mycelial mass, but undergoes rapid inactivation shortly after the onset of fruiting. In *A. bisporus*, the level of laccase transcripts was highest in colonized compost prior to the onset of the fruiting, declined during the subsequent fruiting steps and increased again after harvesting and during the second flush of fruiting body production (Ohga *et al.*, 1999). Studies on *C. congregatus* rather suggest the involvement of laccase at the initial light-requiring step. Laccase activities are higher in cultures grown at fruiting conditions than those grown at non-fruiting conditions, *e.g.* in complete darkness or at 30°C. Within the mycelium of cultures, high laccase activity was localized to areas where subsequently primordia differentiated (Ross, 1982b). In *S. commune*, laccase activity increases during fruiting until the mature fruiting bodies are formed, afterwards declines rapidly (Leonard and Phillips, 1973). However, dikaryons of *S. commune* synthesize much more laccase at 30°C in darkness when no fruiting body is formed (De Vries *et al.*, 1986). In *L. edodes*, the highest amounts of transcripts for laccase genes *lac1* and *lac2* were detected in the cap (Zhao and Kwan, 1999). In consistency, very high laccase activity was found in the uppermost and pigmented rind of the pileus and in the stipe of mature fruiting bodies. Increased laccase activity was associated with the rapid growth of nonpigmented aerial mycelium and the formation of pigmented primordia and fruiting bodies (Leatham and Stahmann, 1981). From all these data, the role of laccases in fruiting body development remains obscure, but it is clear that at least some basidiomycetes regulate laccase activity in relation to and during morphogenesis (Wood, 1985).

In most basidiomycetes, fruiting is initiated by light (section 1.4.2). In *C. cinereus* (Uno *et al.*, 1974) and *L. edodes* (Takagi *et al.*, 1988), light induces cAMP production in parallel to fruiting body initiation. In *L. edodes*, intracellular cAMP accumulated in

condensed mycelia just prior to the onset of primordium formation (Takagi *et al.*, 1988). In *C. cinereus*, high amount of cAMP was detected in illuminated mycelia of the monokaryotic fruiting competent mutant strain fis^c compared to the dark mycelia of monokaryotic strains fis^c and fis^- (Uno and Ishikawa, 1982; Uno *et al.*, 1974). High level of cAMP was found in normal dikaryons as well as in self-compatible *Amut Bmut* strains at the point of fruiting, but not in semi-compatible common-*A* and common-*B* heterokaryons (Swamy *et al.*, 1985). These data suggest a correlation between the high level of cAMP and fruiting body initiation. cAMP is therefore thought to be an important messager especially at the light induced transition to secondary hyphal knot formation in *C. cinereus* (Bottoli, 2001). The current hypothesis about how cAMP acts in signalling in *C. cinereus* and other basidiomycetes is mainly based on the cAMP signalling model for pathogenic fungi and the yeast *Saccharomyces cerevisiae* (Borges-Walmsley and Walmsley, 2000): external signals such as light are thought to be perceived by light receptors, which transduce the signals to RAS proteins to form RAS-GTP from RAS-GDP. In *S. cerevisiae*, RAS-GTP activates adenylate cyclase, the enzyme synthesizing cAMP. Binding of cAMP to the regulatory subunit of protein kinase A (PKA) causes the dissociation of the regulatory subunit from the catalytic subunits of PKA. The catalytic subunits can phosphorylate target transcription factors, which in turn regulate the expression of specific genes (Borges-Walmsley and Walmsley, 2000). As the first step in direction to unravel this signalling pathway, *ras* homologs have been isolated in *C. cinereus* (Bottoli *et al.*, 1999; Ishibashi and Shishido, 1993), in *L. edodes* [(Hori *et al.*, 1991) and in *S. commune* (accession number AF268471), *cgpα1* encoding for a G protein α-subunit homolog in *C. congregatus* (Kozak *et al.*, 1995), the adenylate cyclase gene *cac* in *C. cinereus* (Bottoli *et al.*, 1999), gene *cap* encoding a adenylyl-cyclase-associated protein and a gene for protein 14-3-3 that interacts with CAP from *L. edodes* (Zhou *et al.*, 1998; Zhou *et al.*, 2000). *cgpα1* of *C. congregatus* is transcribed in the light-receptive hyphal tip tissues of the dikaryon, supporting its possible role in light-induced signalling transduction pathway (Kozak *et al.*, 1995). Cac activity in *C. cinereus* is light stimulated (Uno *et al.*, 1974), but the gene is constitutively transcribed (Bottoli *et al.*, 1999), suggesting the regulation of Cac activity is at the post-transcriptional level (Bottoli, 2001).

Other candidates acting in signal transduction pathways in control of fruiting body formation include a number of potential protein kinases and transcription factors, whose genes are specifically transcribed during early processes of fruiting body development.

For example, the gene *LeMapK* for the protein kinase homolog in *L. edodes* is mainly transcribed in primordia (Leung *et al.*, 2000) and a number of genes for potential transcription factors (*LeNot1*, *LeJun*, *priA* and *priB*) are all highly transcribed during primordium formation (Endo *et al.*, 1994; Kajiwara *et al.*, 1992; Leung *et al.*, 2000).

AtpD, a homolog of α subunit of mitochondrial ATP synthase from *A. bisporus* (De Groot *et al.*, 1997) and *β-MPP*, a homolog of the β subunit of mitochondrial processing peptidase from *L. edodes* (Leung *et al.*, 2000) are highly transcribed before or during the formation of primordia. The requirement of *β-MPP* for mitochondrial biogenesis suggests the participation of mitochondria in the very energy-demanding fruiting process (Leung *et al.*, 2000). Mutant complementation in *C. cinereus* detected a constitutively transcribed gene *cln2* for a microsomal cytochrome P450 homologs acting in tissue organization of the primordial shaft (Muraguchi and Kamada, 2000). The analog gene *CypA* from *A. bisporus* was also isolated. This gene however is transcribed before primordium formation (De Groot *et al.*, 1997). Another gene with a definitive role in fruiting body development in *C. cinereus* is *ich1* (Muraguchi and Kamada, 1998), whose gene product has a potential S-adenosyl-methionine binding domain, suggesting it to possibly act as a methyltransferase (Kües and Liu, 2000). Mutants with defects in *ich1* lack centrifugally growing hyphal tissues within the pileus. This gene is specifically transcribed in the pileus (Muraguchi and Kamada, 1998).

1.4.1.3 Genes for homokaryotic fruiting

Fruiting body formation in heterothallic basidiomycetes is not always limited to dikaryons. Although rarely occuring in nature, homokaryotic fruiting (Esser, 1979) has been observed in several species of Poriales and Agaricales under laboratory conditions (Stahl and Esser, 1976). Homokaryotic fruiting bypasses plasmogamy, which is a prerequisite for dikaryotic fruiting in most basidiomycetes. Three types of homokaryotic fruiting were observed in basidiomycetes. Type I is the abortive homokaryotic fruiting (AHF), where only fruiting intermediate structures or never sporulating fruiting bodies are formed (Esser *et al.*, 1979). Type II is the true homokaryotic fruiting (THF), in which homokaryotic fruiting bodies contain only two- or four-spore bearing basidia, but all basidiospores have the same mating type as the parental homokaryon (Esser and Meinhardt, 1977; Miyake *et al.*, 1980; Stahl and Esser, 1976; Uno and Ishikawa, 1971). Type III is called the pseudo-homokaryotic fruiting (PHF), in which fruiting bodies are

indistinguishable from the normal dikaryotic fruiting bodies and basidiospores are of different mating types (Labarere and Noel, 1992).

Homokaryotic fruiting may occur spontaneously, under stress conditions such as nutritional deficiency (Gibbins and Lu, 1984) and mechanical injury (Leslie and Leonard, 1979a), and after exposure to fruiting-inducing substances (section 1.4.5). In *S. commune*, spontaneous homokaryotic fruiting, fruiting due to nutritional stress and fruiting due to mechanical injury are under separate polygenetic controls (Leslie and Leonard, 1979b). Being under nutritional stress for long periods, some monokaryons of *C. cinereus* change into a mycelium bearing clamp connections. Fruiting bodies arise from this mycelium and contain basidia in which two nuclei undergo morphologically normal meiosis, leading to tetrad basidiospores of the same mating type as their parental monokaryon (Gibbins and Lu, 1984; Walser, 1997; U. Kües, personal communication).

In the strictest sense of AHF and THF fruiting, hyphae from all developmental stages are uninucleate and clampless (Stahl and Esser, 1976). The control of fruiting by the mating-type genes is apparently bypassed. Several loci operating in sequence have been attributed to these two types of homokaryotic fruiting. In *S. commune*, either the fi_1^+ locus or the fi_2^+ locus is required for the initiation of homokaryotic fruiting, whereas the action of both loci leads to the formation of long stipes without lamella and spores. The third locus fb^+ is responsible for the formation of fully developed homokaryotic fruiting bodies, which differ in shape from dikaryotic fruiting bodies. The expressions of all three morphogenetic loci in homokaryotic fruiting can be inhibited by a fourth locus called st^+ (Esser *et al.*, 1979). In addition, two fruiting initiating alleles ($hap\text{-}5^{spn}$ and $hap\text{-}6^{spn}$) from *S. commune* can bypass the mating-type control and promote spontaneous monokaryotic fruiting (Leslie and Leonard, 1979b). In the tetrapolar *Polyporus ciliatus* (Poriales) (Stahl and Esser, 1976) and the bipolar *Agrocybe aegerita* (Agaricales) (Esser and Meinhardt, 1977), only a single locus fi^+ was found to be responsible for the initiation of homokaryotic fruiting. An additional locus fb^+ is subsequently required for the completion of fruiting body formation. So far, none of these genes has been cloned from *S. commune*, *P. ciliatus* or *A. aegerita*. However, a gene *frt1* of *S. commune* was isolated by chance by transforming a genomic library into non-self monokaryons (endogenous *frt1* allele is different from the transgene). The gene induced THF in these transformants and, in addition, precocious fruiting with an increased spore production in dikaryons of *S. commune* (Horton and Raper, 1991). Gene *frt1* encodes a putative nucleotide-binding protein (Horton and Raper, 1995) and is

dispensable for dikaryotic fruiting. Elevated transcription of the dikaryon-specific hydrophobin gene *sc4* in *Δfrt1* homokaryons suggests that *frt1* act as a negative regulator of dikaryon-expressed genes (Horton *et al.*, 1999).

Alterations within the mating-type loci also can cause true homokaryotic fruiting. Homokaryons such as strain AmutBmut of *C. cinereus* (Swamy *et al.*, 1984) and $MATA^{con}MATB^{con}$ strains of *S. commune* (Koltin and Raper, 1968) are self-compatible due to specific mutations in the *A* and the *B* mating-type loci (see chapter 2) and produce fruiting bodies with identical basidiospores. Spontaneous mating-type switching has been described in *A. aegerita* (Labarere and Noel, 1992) and in *Agaricus bitorquis*, giving rise to PHF with fruiting bodies carrying basidiospores of different mating types (Martinez-Carrera *et al.*, 1995). In *P. ostreatus*, monokaryotic regenerants of protoplasts from dikaryons gave rise to fruiting bodies with spores of different mating types, but it is not known whether mating-type switching was the cause of this case (Takehara *et al.*, 1993).

Mutations outside of mating-type genes but within the mating-type regulatory pathway also can cause spontaneous homokaryotic fruiting as in the case of monokaryons fis^c and 5337 of *C. cinereus* (Murata *et al.*, 1998; Uno and Ishikawa, 1971). A nonsense mutation in gene *pcc1* has been shown to be responsible for the formation of monokaryotic fruiting bodies having few but fertile basidiospores. The Pcc1 protein has a HMG domain and a putative nuclear localization signal, suggesting it to act as a transcription factor. The mutation in *pcc1* promotes also the formation of non-fused clamp cells, suggesting its definite role to reside in the *A*-regulated fruiting pathway (Murata *et al.*, 1998).

The molecular mechanism of most genes involved in homokaryotic fruiting is not known. However, the fact that gene *frt1* (Horton *et al.*, 1999) and fi^+ (Esser and Meinhardt, 1977; Stahl and Esser, 1976) not only can induce homokaryotic fruiting but also enhance the production of dikaryotic fruiting bodies suggests a possible overlap between monokaryotic and dikaryotic fruiting pathways (Wessels, 1993; Wessels, 1994).

1.4.2 Environmental regulation

Fruiting body formation is a specific response of the dikaryotic thallus to environmental stimuli, which include temperature, CO_2 concentration, humidity, moisture content of the medium, pH, presence of microbes and light [for review see

(Kües and Liu, 2000)]. The optimal environmental conditions for vegetative growth and for the subsequent fructification are usually very distinct.

In general, higher temperature stimulates the vegetative growth, but fruiting occurs at lower temperature. In *C. cinereus*, the mycelium grows fastest at 37°C, but only temperatures of 25-28°C permit fruiting (Walser *et al.*, 2001). In *F. velutipes*, 24-25°C is optimal for mycelial growth, but fruiting occurs well only at 10-15° (Kinugawa, 1993). For the cultivation of the wood-rotting fungus *L. edodes*, inoculated logs are kept in greenhouses at 10-20°C. Prior to the time for fruiting, logs are cold-treated by soaking in water to induce primordium formation (Leatham, 1982).

In *A. bisporus*, stipes elongate more rapidly in high CO_2 concentrations, whereas cap and gills expand and spores mature more rapidly when CO_2 is removed (Turner, 1977). Exposure of *F. velutipes* cultures to high CO_2 concentration (6000 ppm for 7 days) during spawn running stimulated vegetative mycelial growth, but exposure during fruiting had an inhibitory effect on primordial formation (Kinugawa, 1993). Low CO_2 concentration might cause the formation of malformed fruiting bodies with elongated stipes and reduced pilei (Kües and Liu, 2000). In *L. edodes*, inadequate aeriation near the time of fruiting maturation results in the failure of cap expansion (Leatham and Stahmann, 1987). Sufficient ventilation improves the yield of fruiting bodies in *A. mellea* (Togashi and Takizawa, 1995).

High relative humidity (95-90%) is generally in favor of fruiting in *L. edodes* (Leatham, 1982). In *L. edodes*, substrate of high and constant moisture content is important for obtaining a good mushroom yield and good quality (Ohga, 1999a). The optimal moisture content for wood substrate is 35-60% and 60-80% for other substrates (Kües and Liu, 2000). *A. mellea* fruits synchronously with high yield on the artificial (yeast extract + malt extract + glucose)-agar medium with 66-75% moisture content (Togashi and Takizawa, 1995).

In *F. velutipes*, vegetative growth occurs within a wider range of pH (ca. 3-11), and a pH value around 6-7 permits fruiting (Kinugawa, 1993). Sawdust-based substrate with pH 4.0 permits fruiting in *L. edodes* (Ohga, 1999b).

The presence of microbes stimulates fruiting in *Agaricus* by eliminating inhibitory compounds from the substrates (De Groot *et al.*, 1998).

Light has been most intensively studied as a modulating factor for fruiting body development. Although *A. bisporus* can complete fruiting body formation independently from light, most other mushrooms need light for fruiting (Eger-Hummel, 1980). The

light requirement regarding dosage and fruiting stage varies widely from species to species, even among different strains within a species. Fruiting body formation in *Favolus arcularius* takes place in continuous light. *Lentinus tigrinus* only needs light for the maturation of primordia (Eger-Hummel, 1980), but *Coprinus stercorarius* (Ellis *et al.*, 1999) and *L. edodes* (Mohamed *et al.*, 1992a) require two separate light signals for initiation and maturation. In *C. stercorarius*, a 2 seconds light exposure is sufficient for primordium formation, whilst a 16 h illumination period is needed for fruiting body maturation (Ellis *et al.*, 1999). In *L. edodes*, 30 minutes of illumination lead to primordium formation (Mohamed *et al.*, 1992a; Mohamed *et al.*, 1992b). Continuous light inhibits the development of mature fruiting bodies in *Coprinus congregatus* (Durand and Furuya, 1985). Light accelerates the formation of *S. commune* hyphal aggregates in stage I-II and differentiation of the hymenium is also light dependent (Perkins, 1969). Homokaryon AmutBmut of *C. cinereus* needs alternating periods of dark and light to develop fully mature fruiting bodies (Kües, 2000; Lu, 2000). However, other *C. cinereus* isolates readily fruit in continuous light (Madelin, 1956a; Morimoto and Oda, 1973). Due to the light sensitivity commonly found in fruiting body development, standard fruiting conditions for *C. cinereus* are either a 12 h light/12 h dark regime (Granado *et al.*, 1997; Muraguchi *et al.*, 1999; Seitz *et al.*, 1996) or a 16 h light/8 h dark regime (Lu, 2000; Pukkila *et al.*, 1984).

The earliest stage of fruiting body development in *C. cinereus*, the formation of primary hyphal knots, needs darkness (continuous light inhibits primary hyphal knot formation) (Boulianne *et al.*, 2000; Kües *et al.*, 1998). If light is absent, the primary hyphal knots develop into aerial sclerotia (Boulianne *et al.*, 2000; Moore, 1981). In contrast, the transition from the primary to the secondary hyphal knots is light dependent (Boulianne *et al.*, 2000; Lu, 1974; Morimoto and Oda, 1973). The effect of light in fruiting body initiation is local in *C. cinereus* (Madelin, 1956a) and in *Melanotus* spp. (Newman, 1968), but not in *C. stercorarius* (Ellis *et al.*, 1999).

In *C. cinereus*, differentiation within the secondary hyphal knots requires also a light signal. In the presence of light, hymenium differentiates and gives rise to gills (Lu, 1974). If light is absent, hymenium differentiation is blocked and only malformed fruiting bodies with undifferentiated caps and elongated stipes (called "etiolated stipes" or "dark stipes") will be formed (Lu, 1974; Morimoto and Oda, 1973; Tsusue, 1969). The same light effect was also observed in *L. edodes*, which only forms such "pseudo fruit bodies" without differentiated cap in the absence of a second light signal

(Mohamed *et al.*, 1992a). In *C. stercorarius*, three days of constant light only gives rise to tall stipes with little or no pileus expansion (Ellis *et al.*, 1999).

After completion of gill formation in *C. cinereus*, light is required for the DNA synthesis at the prekaryogamy stage, but the light intensity is not critical (Morimoto and Oda, 1974; Lu, 2000). However, light intensity determines the duration of the nuclear fusion and the timing of meiotic events. Higher light intensity enhances basidia to enter karyogamy (Lu, 2000), but temperature also plays a role in how reactions occur upon light (Lu, 1972; Lu, 1974).

Following nuclear fusion, meiosis in certain strains of *C. cinereus* never proceeded beyond prophase I at 28°C in the absence of darkness (Kamada *et al.*, 1978). A 3 h dark period is necessary for nuclei to enter the meiotic metaphase, but also the dark requirement is strain, temperature and light intensity dependent. When temperature is below 25°C, even continuous light might not arrest meiosis (Lu, 2000). In *C. congregatus*, when nuclei are arrested in meiosis, stipe elongation will not occur (Robert and Bret, 1987). In *C. cinereus*, the induction of stipe elongation needs a dark-induced diffusible factor from the cap (Kamada and Tsuji, 1979). Once a rapid stipe elongation has started, cap expansion and fruiting body maturation in *C. cinereus* is independent of light (Table 1). A dark dependence in stipe elongation is not seen in *C. stercorarius* (Ellis *et al.*, 1999). However, in *L. edodes*, light promotes the pigmentation of primordia and only such pigmentated primordia are capable to expand later (Leatham and Stahmann, 1987). Moreover, light is required for the development of cap and basidiospores in *F. velutipes*, *S. commune* and *Trametes versicolor* (Croan and Kim, 1997; Perkins, 1969).

Most basidiomycetes, including *C. cinereus* (Kertesz-Chaloupkova *et al.*, 1998; Kües *et al.*, 1998) and *S. commune* (Perkins and Gordon, 1969; Yli-Mattila, 1991), respond to blue light (360-490 nm). Very little light (1 foot-candle of 410-450 nm blue light) is needed for the fruiting body initiation in *C. cinereus* (Lu, 1974). As few as 2-3 s (0.5 μmol m^{-2}) of blue light (440-470 nm) initiate primordium formation in *C. stercorarius* (Ellis *et al.*, 1999). In *P. ostreatus*, hyphal aggregation during the fruiting initial stage is stimulated by light in the near-UV (370 nm) and blue (440-450 nm) region (Richartz and MacLellan, 1987). In *C. congregatus*, a photo fluence of 5 μmol m^{-2} at 260-280 nm is sufficient for fruiting body initiation, and the effectiveness at 280 nm is four times higher than that at 440 nm (Durand, 1987). Other basidiomycetes act on different spectra, *e.g.* blue and red light are effective to *Melanotus* spp. (Newman, 1968) and

Sphaerobolus stellatus (Ingold and Nawaz, 1967). In *Melanotus* spp., primordium formation requires blue or red light. However, mature fruiting bodies are only formed in blue light and fruiting bodies with abnormal pilei are formed in red light (Newman, 1968). In *S. stellatus*, blue light is essential for fruiting body initiation and primordium development, but the later spore discharge is more accelerated by red than by blue light (Ingold and Nawaz, 1967). In *L. edodes*, the stimulatory wavelength in fruiting is dependent on the composition of growth medium. Red light (620-680 nm) stimulates fruiting on media with low calcium content (<40 p.p.m.). Blue light (400-500 nm) inhibits fruiting when the concentration of calcium is low in the medium, but stimulates fruiting on medium with high calcium content (>130 p.p.m.) (Leatham and Stahmann, 1987). Blue light is also responsible for primordium pigmentation in *L. edodes* (Leatham and Stahmann, 1987).

The transduction of a light signal into the interior of cells is thought to be via photoreceptors (Ross, 1985). The photoreceptor responsible for UV/blue perception has been sought for without success for decades. Action spectra (280-460 nm) for different blue light responses suggest them to be flavin-type or carotene-type photoreceptors (Durand, 1987; Elliott, 1994; Yli-Mattila, 1991).

1.4.3 Nutritional regulation

Carbohydrates except lactose and galactose are good carbon sources for the growth of *C. cinereus* (Madelin, 1956b). Glucose and other carbohydrates (acetate, fructose, maltose, mannitol, mannose, xylose, cellulose and starch) can support growth of monokaryons as the sole source of carbon and energy (Moore, 1969). Glucose is widely used in all artificial media for cultivating *C. cinereus* (Bottoli, 2001; Walser, 1997). Similar to the situation in environmental conditions, nutrients that well support vegetative growth do not necessarily support reproduction. For example, the range of glucose concentrations permitting vegetative growth is much broader than the one permitting fruiting, and their optima do not or only little overlap (Bottoli, 2001; Madelin, 1956b). 2% (w/v) glucose inhibits primary hyphal knot formation, but this repression rapidly diminishes as the glucose concentration reduces from 2% to 1% (Bottoli, 2001; Matthews and Niederpruem, 1972). Glucose concentration also negatively affects the development following primary hyphal knot formation. In fact, primordia occur at the time when glucose is depleted (Rao and Niederpruem, 1969). Development is usually not completely blocked but malformed primordia and

malformed fruiting bodies will arise, when the glucose concentration is raised after the step of primary and secondary hyphal knot formation (Bottoli, 2001).

The carbon sources utilized by basidiomycetes are usually lignocelluloses. Many commercial mushrooms such as *A. bisporus*, *F. velutipes, L. edodes, Pleurotus* spp. and *Volvariella* spp. are cultivated on a wide variety of inexpensive substrate/wastes including bagasse, banana leaves, cereal straws, cotton waste, coffee ground, rice bran and sawdust (Chang and Quimio, 1989; Stamets, 1993). It is frequently observed that a high concentration of nutrients encourages vegetative growth and that fruiting may be stimulated by mechanisms causing the stop of vegetative growth (Miles, 1993). Therefore, fruiting body development in *A. bisporus* is often induced by covering the compost, which has been colonized by vegetative mycelium, with a layer of moist peat and chalk having only limited nutrients (Kües and Liu, 2000).

Primordia of *Coprinus* spp. are usually formed in a peripheral zone of the culture (Bottoli, 2001; Ross, 1982a) (see chapter 3), but not all primordia develop into mature fruiting bodies (Bottoli, 2001; Madelin, 1956b). Indeed, fruiting body maturation might not equally occur within a culture. The unequal distribution of mature fruitbodies led to the suggestion that fruiting bodies acted as a sink, which withdrew nutrients from the whole colony (Madelin, 1956b). Glycogen-storage cells were found in submerged mycelia of undifferentiated culture in *C. cinereus*. Upon fruiting, vacuoles appeared in these glycogen-storage cells and their enlargement resulted in the depletion of glycogen from those cells (Madelin, 1960). The quantitative decline of glycogen in the dikaryotic mycelia coincided with the appearance of mature sclerotia (Jirjis and Moore, 1976), implying that glycogen was transported towards sites where sclerotia are formed. Accumulation of glycogen in fruiting body tissues is evident in 1 mm primordia (Matthews and Niederpruem, 1973), initially in the stipe base and subsequently, with the progression of primordium maturation, in the cap (Balyney and Marchant, 1977; Moore *et al.*, 1979). In the cap, glycogen becomes concentrated in gills within the subhymenial tissues (McLaughlin, 1974). Up to 1 mg of glycogen is accumulated in each young fruiting body, all of which disappears as basidiospores are produced (Moore *et al.*, 1979). It appears that glycogen serves as storage polysaccharide and a breakdown of glycogen provides energy and carbon supply for basidiospores formation. No polysaccharide deposits can be detected in specimens bearing mature spores (Moore *et al.*, 1979). All these observations suggest that an organized translocating route (mycelium $\Rightarrow$ stipe $\Rightarrow$ cap) is used to supply the polysaccharide for the development of

fruiting bodies. However, the temporal pattern of glycogen accumulation/degradation was very variable in 23 tested individual fruiting body caps during the period from karyogamy to sporulation, indicating that the glycogen content was not closely associated with any single step from meiosis to spore formation (Ji and Moore, 1993). Glycogen is not the only storage carbohydrate used in basidiomycetes for fruiting: *A. bisporus* degrades trehalose to provide energy and carbon supply for fruiting body development (Kües and Liu, 2000).

Works in *C. cinereus* showed that not only the absolute concentration of carbon is important to decide the initiation of fruiting body development, but also the relative C/N ratio is crucial. A C/N ratio permitting formation of mature fruiting bodies ranges from 10:1 to 5:1. A ratio below 5:1 may sometimes lead to the formation of fruiting bodies with basidiospores, but these basidiospores do not germinate (Bottoli, 2001; Madelin, 1956b). In *A. bisporus*, the optimal C/N ratio in compost (straw, manure and calcium sulphate) for fruiting is between 80:1 and 10:1 (Kües and Liu, 2000).

For *C. cinereus*, the utilizable nitrogen sources include ammonium and organic nitrogen, but not nitrate (Madelin, 1956b). *C. cinereus*, *A. bisporus* and *Volvariella volvacea* can utilize proteins as sole source of carbon, nitrogen or sulphur (Kalisz *et al.*, 1986). They appear to use proteins as effectively as glucose when used as the sole carbon source (Kalisz *et al.*, 1986). In *C. cinereus*, high concentrations of amino nitrogen increase the colony growth rate but block fruiting body initiation and maturation at several distinct steps (primary and secondary hyphal knot formation, primordium formation and maturation up to the stage of postmeiotic rapid stipe elongation) (Bottoli, 2001). The negative effect of a high nitrogen concentration on fruiting body initiation may be a result of the ammonium excreted into the media (Kalisz *et al.*, 1986). However, secretion of ammonia from primary hyphal knots into the medium positively influences fruiting body initiation by inhibiting primary hyphal knots from maturing into sclerotia (Moore and Jirjis, 1976). Addition of ammonia water (NH_3) to 5-day old *C. cinereus* cultures induced primordia formation in the dark at 25°C, while a potato dextrose medium free of ammonia only produced vegetative mycelia in the dark or developed primordia upon light exposure. These data suggest that the effect of light on fruiting body initiation can be replaced by ammonia treatment (Morimoto *et al.*, 1981). Later in development, application of ammonium salts inhibits the differentiation of basidia within explanted gill segments at any time during meiosis and promotes the outgrowth of vegetative hyphal tips from specific regions of the

basidium, which are normally involved in active wall synthesis during basidiospore formation (Chiu and Moore, 1988a). The very early outgrowth of sterigmata from the basidium is the most sensitive stage to the inhibition by ammonium (Moore *et al.*, 1993).

1.4.4 The mycelial competence in fruiting body initiation

Not every hypha from the entire mycelial thallus is uniformly competent to differentiate. Under standard fruiting conditions, primordia are mostly initiated in the peripheral zone in *C. cinereus* cultures (Bottoli, 2001) (see chapter 3). Following a cold-shock at 5°C for 24 hr, 3-day old *C. cinereus* colonies synchronously developed primordia in a ring that is 1-5 mm inside the margin of the colony formed at the time of the cold treatment. This observation suggests that only hyphae of a certain age are responsive to the cold-treatment (Tsusue, 1969). In *C. congregatus*, mycelium at least 3-day old can initiate fruiting in response to light only in the newly growing peripheral zone. The light inducible zone migrates outwards with the peripheral hyphae as the mycelium expands, while previously competent zones become inactive and non-inducible (Ross, 1982a). Likewise, fruiting in *S. commune* can be induced by light only in colonies that are a few days old (Leonard and Dick, 1968; Perkins, 1969). After light induction, fruiting bodies of *S. commune* are formed at the edge of the culture behind the apical region of the hyphae, but the first five hyphal cells in the apical region never respond to light for fruiting (Raudaskoski and Viitanen, 1982). Homokaryotic fruiting bodies in *S. commune* and in homokaryon AmutBmut of *C. cinereus* can locally be induced by mechanically cutting into an established mycelium. This mechanical injury induces new hyphal tip growth at the cuts (Granado *et al.*, 1997; Leslie and Leonard, 1979a).

From all these observations, it seems that only young hyphae on an established thallus are competent for fruiting. Currently, the molecular nature of mycelial competence is not known in any basidiomycete, except that high laccase activity in the fruiting zone of *C. congregatus* was suggested to be responsible for attaining mycelial competence to initiate fruiting body development (Ross, 1982a).

1.4.5 Substances inducing fruiting in basidiomycetes

Substances that can induce or enhance fruiting in basidiomycetes are of different chemical nature and have been isolated from nearly all kingdoms (Table 3). In some

Table 3. Substances inducing fruiting in basidiomycetes

Substance	Source / organism	Tester organism	Fruiting inducing activity	Reference
Fruiting body extract	*A. bisporus*	monokaryon of *S. commune*	homokaryotic fruiting	1
Cell-free extracts	*Hormodendrum cladosporioides*	monokaryon of *S. commune*	homokaryotic fruiting	2
Spent agar beneath colonies	*Phellinus contiguus*	*P. contiguus*	precocious fruiting	3
Liquid culture filtrate	*P. contiguus,* *P. occidentalis* *P. ferruginosus* *P. igniarius* *P. pini* *Trametes versicolor*	*P. contiguus* strain GWR	higher production	4
Oligosaccharide	Carrot	*Lentinus cladopus*	precocious fruiting	5
Cerebrosides (details in Table 4)	*Ganoderma lucidum*	dikaryon of *C. cinereus*	precocious fruiting	6
	S. commune *L. edodes*	dikaryon of *S. commune*		7, 8
	Candida albicans *Candida deformans* *Penicillium funiculosum* *Fusicoccum amygdali*			9, 10, 11
	wheat grain pea seed			12
	Hirudo nipponica *Marphysa sanguinea* *Neanthes diversicolor* *Pheretima asiatica*			13
Cyclic AMP from fruiting body	*Agaricus* spp.	monokaryon fis$^+$ of *C. cinereus*	higher production	14, 15, 16
	C. cinereus	dikaryon of *C. cinereus*	hyphal knots	17

Table 3. - *continued*

Substance	Source / organism	Tester organism	Fruiting inducing activity	Reference
Triterpenoid Saponins	*Quillaja*	*P. ostreatus*	fruiting body initiation	18
Veratryl alcohol	fungal secondary metabolite	*P. ostreatus*	higher production	19
10-oxo-trans-8-decenoic acid	*A. bisporus*	*A. bisporus*	stimulated mycelial growth and stipe elongation	20
Pepsin inhibitor (S-PI)	*Streptomyces*	*L. edodes*	precocious fruiting and higher production	21
Anthranilic acid	Actinomycetes strain TA7	*Favolus arcularius*	stipe formation in the dark	22
Cyclootasulfur	*Streptomyces albulus*			23
Basidifferquinone	*Streptomyces* strain B-412			24
Papulacandin B Aculeacin A Gramicidin S	glucan synthase inhibitors	dikaryon of *S. commune*	precocious fruiting and inhibiting hyphal growth	25
Gramicidin D Tyrocidine Valinomycin	peptide ionophores			
Digitonin	detergent and chitin synthase inhibitor			
Luteinizing hormone-releasing hormone (LH-RH)	linear peptide hormone			

1(Rusmin and Leonard, 1978), 2(Leonard and Dick, 1968), 3(Butler, 1995), 4(Butler and Pearce, 1999), 5(Devi and Swamy, 1993), 6(Mizushina *et al.*, 1998), 7(Kawai and Ikeda, 1982), 8(Kawai, 1989), 9, 10, 11(Kawai *et al.*, 1985; Matsubara *et al.*, 1987; Mineki *et al.*, 1994), 9(Kawai *et al.*, 1985), 10(Matsubara *et al.*, 1987), 11(Mineki *et al.*, 1994), 12(Kawai *et al.*, 1986), 13(Tanaka *et al.*, 1997), 14(Uno and Ishikawa, 1971), 15(Uno and Ishikawa, 1973b), 16(Uno and Ishikawa, 1973a), 17(Rao and Niederpruem, 1969), 18(Magae, 1999), 19 (Suguimoto *et al.*, 2001), 20(Mau *et al.*, 1992), 21(Terashita *et al.*, 1981), 22(Murao *et al.*, 1984), 23(Hayashi *et al.*, 1985), 24(Azuma *et al.*, 1990), 25(Oita and Yanagi, 1993).

cases, the fruiting-inducing activity was found in undefined extracts from fruiting bodies of basidiomycetes (Rusmin and Leonard, 1978), from mycelial pads (Leonard and Dick, 1968), from spent agar beneath the mycelial mat (Butler, 1995) and from the liquid of a mycelial culture (Butler and Pearce, 1999). In other cases, the active compounds were purified and their chemical structures were identified (Table 3).

A group of cerebrosides belongs to such defined active compounds (Table 3 and 4). A cerebroside is a glycolipid containing a ceremide and one sugar residue (Fig. 7). The ceremide is a sphingosine linked to a fatty acid via an amide bridge (Stryer, 1995). A variety of cerebrosides promoted precocious fruiting in dikaryons or induced fruiting in specific monokaryotic strains of *S. commune* and *C. cinereus* (Table 3). Since fruiting normally occurs on dikaryons in these two fungi, it implies that cerebrosides act as fruiting-enhancing and fruiting-inducing substances. Cerebrosides that induce precocious fruiting in *S. commune* are not species specific. Cerebrosides from *Ganoderma lucidum* also induced precocious fruiting in *C. cinereus* (Table 3). In addition, the chemically synthesized cerebrosides SchII (Funaki *et al.*, 1986; Mori and Funaki, 1984; Mori and Funaki, 1985), PenII (Mori and Uenishi, 1996), PenIII (Abe and Mori, 1994) and WheII (Mori and Kinsho, 1991), having the sterochemical structures of their natural counterparts, were found active in a fruiting bioassay with *S. commune*.

The sugar residue of the cerebrosides tested for fruiting induction is a monoglucose or a phophocholoine-glucose. The sphingosine is a C18-sphingadienine where position C-9 is sometimes methylated. In general the fatty acid moiety is saturated and hydroxylated at position C-2, but the chain length varies from 15 to 24 carbons (Table 4). The occurance of bioactive cerebrosides in very different source organisms (fungi, plants, animals) implies that the common structures may be the key factors contributing to their activities. Structural comparison between the active and inactive forms of fungal and plant cerebrosides and their derivatives indicats that the N-acylsphingoid with the methyl group at C-9 is an essential part of active cerebrosides. The double bond at position C-8 of the sphingoid in E (*trans*-) configuration enhances the activity. The 8E double bond and the 9Me can be substituted by an 8Z (*cis*-) double bond without losing the activity. The sugar residue is not necessary, but a sugar moiety with more than two hexose units may interfere negatively with the activity. Fatty acids with a chain length of less than 22 and with a 2-hydroxy group show increased activity. In contrast, the E double bond at position C'-3 of the fatty acid has no influence on the fruiting-inducing

Table 4: Bioactive and inactive forms of different cerebrosides as tested on fruiting body development in *C. cinereus* and *S. commune*

Organism / compound		Cerebroside structure			Fruiting inducing activity	Reference
		Sugar	Ceremide			
			Fatty acid	Sphingoid		
S. commune	Sch I	Glc	h 15:0 h 16:0	d19:2 d18:2	+	1
	Sch II	Glc	h 16:0	d19:2 (4E, 8E, 9Me)	+	
	Sch III	Glc	h 17:0	d19:2	+	
	Sch IV	Glc	h 18:0	d19:2	+	
	Sch V	Glc	h 24:0	d19:2 (4E, 8E, 9Me)	-	
L. edodes	Len I	Glc	h 14:0	d19:2 (4E, 8E, 9Me)	+	2
	Len II	Glc	h 15:0	d19:2 (4E, 8E, 9Me)	+	
	Len III	Glc	h 16:0	d19:2 (4E, 8E, 9Me)	+	
	Len IV	Glc	h 17:0	d19:2 (4E, 8E, 9Me)	+	
	Len V	Glc	h 18:0	d19:2 (4E, 8E, 9Me)	+	
	Len VI	Glc	h 22:0	d19:2 (4E, 8E, 9Me)	+	
	Len VII	Glc	h 23:0	d19:2 (4E, 8E, 9Me)	+	
	Len VIII	Glc	h 24:0	d19:2 (4E, 8E, 9Me)	-	
	Len IX	Glc	h 25:0	d19:2 (4E, 8E, 9Me)	-	
	Len X	Glc	h 26:0	d19:2 (4E, 8E, 9Me)	-	
Ganoderma lucidum		Glc	h 16:0 h 18:0	d19:2 (4E, 8E, 9Me)	+	3
Candida albicans		Glc	h 18:0	d19:2 (4E, 8E, 9Me)	+	4
Candida deformans		Glc	h 16:0	d19:2 (5E, 9Z)	+	5
Penicillium funiculosum	Pen I	Glc	h 16:0 h 17:1	d19:2	+	6
	Pen II	Glc	h 18:1 (3E)	d19:2 (4E, 8E, 9Me)	+	
	Pen III	Glc	h 18:0	d19:2	+	
Triticum aestivum (wheat)	Whe I	Glc	h 16:0	d18:2 (4E, 8Z)	+	7
	Whe II	Glc	h 16:0	d18:1 (8Z)	+	
	Whe III	Glc	h 18:0	d18:2 (4E, 8Z)	+	
	Whe IV	Glc	h 18:0	d18:1 (8Z)	+	
Neanthes diversicolor (Annelida)		CPG	h 16:0	d18:0	-	8
		CPG	h 16:0	d18:1 (8E)	-	
		CPG	h 16:0	d18:2 (4E, 8E)	+	
Marphysa sanguines (Annelida)		CPG	h 16:0	d18:3 (4E, 8E, 10E)	+	
		CPG	h 16:0	d18:3 (4E, 8E, 10E, 9Me)	+	
		CPG	h 20:0	d18:1 (4E)	-	
Hirudo nipponica (Annelida)		CPG	h 18:0	d18:1 (4E)	-	
		CPG	h 22:0	d22:3 (4E, 8Z, 11Z)	+	
		CPG	h 24:0	d22:3 (4E, 8Z, 11Z)	+	
Pheretima asiatica (Annelida)		CPG	h 22:0	d18:1 (4E)	-	
		CPG	h 24:0	d18:1 (4E)	-	

Legend: Glc = glucose; CPG = phosphocholoine-glucose; h = 2-hydroxy fatty acid; d = dihydroxy sphingoid; C : x = carbon number : degree of unsaturation; d19:2 (4E, 8E, 9Me) = (4E,8E)-9-methyl-4,8-sphingadienine; E = *trans*-; Z = *cis*-; + = yes; - = no.

1(Kawai and Ikeda, 1985), 2(Kawai, 1989), 3(Mizushina *et al.*, 1998), 4(Matsubara *et al.*, 1987), 5(Mineki *et al.*, 1994), 6(Kawai *et al.*, 1985), 7(Kawai *et al.*, 1986), 8(Tanaka *et al.*, 1997).

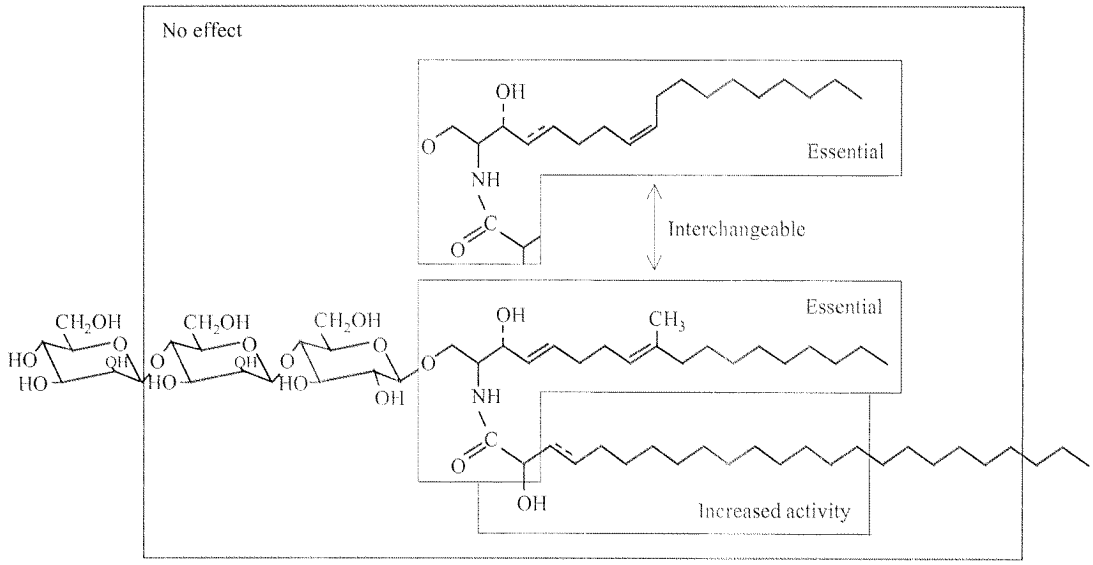

Figure 3. Structure-activity relationship in the fruiting-inducing cerebrosides [from (Kawai, 1989)].

activity (Kawai, 1989). All findings in the structure-bioactivity relationship of cerebrosides are summarized in Figure 3.

Besides cerebrosides, other substances were also isolated for their ability in inducing fruiting in basidiomycetes (Table 3). For example, cyclic AMP from different *Agaricus* spp. and from certain *C. cinereus* strains and synthetic cAMP enhanced fruiting in *C. cinereus* monokaryotic strain fis^{+} (Uno and Ishikawa, 1971; Uno and Ishikawa, 1973a; Uno and Ishikawa, 1973b) and secondary hyphal knot formation in dikaryons of *C. cinereus* (Rao and Niederpruem, 1969).

In *F. arcularius*, anthranic acid (Murao *et al.*, 1984), cyclootasulfur (Hayashi *et al.*, 1985) and basidifferquinone (Azuma *et al.*, 1990), isolated from soil actinomycetes, induced in continuous darkness the formation of incomplete fruiting bodies or long stipes without pileus, resembling the "etiolated stipe" of *C. cinereus* (section 1.4.2). [As in *C. cinereus*, light is required for the normal initiation and development of fruiting in *F. arcularius* (Croan and Kim, 1997)]. In *Lentinus cladopus*, an oligosaccharide from carrot with a polymeric structure containing aromatic residues induced precocious fruiting (Devi and Swamy, 1993). A triterpenoid saponin from the plant *Quillaja* promoted fruiting body initiation by increasing the number of primordia and shortened the period for primordia differentiation in *P. ostreatus* in a dosage-dependent manner (Magae, 1999). 10-oxo-8Z-decenoic acid (ODA), isolated from *A. bisporus*, stimulated mycelial growth and stipe elongation in the same fungus (Mau *et al.*, 1992). ODA has a

structure similar to the "queen substance", the sex pheromone of honey bees, and to traumatin, a wound hormone of plants. In *A. bisporus* it is produced concurrently with the major mushroom aroma component, 1-octen-3-ol, especially when tissues of fruiting bodies or filaments of the mycelium are damaged or disrupted (Mau *et al.*, 1992).

Although many active substances in promoting fruiting in basidiomycetes have been isolated, the mechanisms of their molecular actions still remain unknown or very speculative. Cerebrosides may induce precocious fruiting by suppressing DNA replication or stopping the vegetative growth of the mycelium, since two cerebrosides isolated from *G. lucidum* specifically inhibit activities of replicative DNA polymerases (Mizushina *et al.*, 1998). This assumption may be true, if cerebrosides can penetrate viable mycelium cells and nuclei. However, this has not yet been demonstrated and an increased DNA replication is rather expected, due to the localized proliferation of fruiting body specific cells during fruiting body initiation.

Structural changes of cell wall may be another trigger for fruiting. Evidences supporting this hyphothesis come from inhibitors studies. Glucan and chitin are two major components of fungal cell wall. The addition of glucan synthase inhibitors (papulacandin B and aculeacin A) and chitin synthase inhibitor (digitonin) greatly shortened the time for fruiting body formation in *S. commune* (Oita and Yanagi, 1993). In *C. cinereus*, it is known that the fungal cell wall in secondary hyphal knots differs from that of vegetative hyphal cells (Kamada and Tsuru, 1993).

Membrane modification was speculated as the explanation for the fruiting initiation activity of saponins, also because a saponin from the plant *Gypsophila* interacted with cell membranes and altered cell morphology in colon carcinoma cells (Sung *et al.*, 1995). Peptide ionophores that can increase permeability of membrane to ions, *e.g.* gramicidin S, tyrocidine and valinomycin, enhanced precocious fruiting in *S. commune* (Oita and Yanagi, 1993). However, surfactants like Tween 80, SDS, triton X were not very effective in inducing fruiting. Thus, it is hard to attibute the fruiting inducing ability only to the surfactant activity of saponins (Magae, 1999).

In *F. arcularius*, *F. velutipes*, *L. edodes* and *P. ostreatus*, a *Streptomyces*-pepsin inhibitor (S-PI), a specific inhibitor of carboxyl proteinase, shortened the time required for mature fruiting body formation and increased the number of fruiting bodies per culture (Terashita *et al.*, 1981). When S-PI was added to *L. edodes* cultures, carboxyl proteinase activity in the mycelium decreased, while metal proteinase activity increased. The increased activity of intracellular metal proteinase may be the cause of the

accelerated fruiting, since in *F. velutipes* the highest activity of intracellar metal proteinase was observed at the period of fruiting body formation. Moreover, the addition of metal proteinase inhibitor prohibited fruiting body formation in *F. velutipes* (Terashita *et al.*, 1981).

1.5 Aim of this work

The literature overview on fruiting body development in basidiomycetes showed that the descriptions of cellular processes during fruiting body development are often isolated observations. Environmental signals that act in fruiting body initiation and maturation have been partially described. However, only little is understood on the perception of these signals and on their molecular and genetic regulations. *C. cinereus* appears to be the best basidiomycete to study fruiting body development since the most intensive descriptions of cellular events and environmental and genetic regulations in fruiting body development are from this fungus (Kües, 2000) (chapter 1). In addition, it is one of the two model basidiomycetes where classical and molecular genetics techniques have been well established (Walser *et al.*, 2001). The most fascinating but likely also the most difficult point to study in the fruiting pathway is the fruiting body initiation. The aim of the work presented in this thesis was to isolate and characterize genes involved in fruiting body initiation in the basidiomycete *C. cinereus*. To reach this goal, we needed first to define more precisely the initial cellular steps in fruiting by microscopical observation and to analyze these initial steps with stage specific molecular markers (chapter 3). This knowledge helped to identify a group of mutants specifically defective in fruiting body initiation (chapter 2). From one mutant carrying a single mutation in a recessive allele, an essential gene involved in fruiting body initiation was isolated by using a genomic library with SIB-selection transformation procedure. This gene was sequenced and the defect in the mutant allele was identified (chapter 4).

1.6 References

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Chapter 2

Characterization of mutants defective in fruiting body initiation

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2 Characterization of mutants defective in fruiting body initiation

2.1 Abstract

Homokaryon AmutBmut is a specific strain of the basidiomycete *Coprinus cinereus* that, due to mutations in the mating-type loci, produces fruiting bodies without prior mating to another strain. The homokaryon has therefore been used for creating mutants in fruiting body development. Early stages of fruiting body development include the dark-dependent formation of primary hyphal knots and the light-induced transition from primary hyphal knots to the more compact secondary hyphal knots. 550 mutants of homokaryon AmutBmut with potential defects in these early steps in fruiting were subjected to a screening program. Mutants with phenotypes that can be influenced by available nutritional resources were first excluded. Mutants with a stable phenotype on the artificial YMG/T agar were analyzed for colony growth characteristics, for clamp cell formation (indicator for the function of mating type genes) and for their ability to react to light signals. 50 strains with specific mutations in fruiting body initiation were identified and classified into two groups. A group of 10 mutants was defective in forming primary hyphal knots (*pkn*). The other group of 40 mutants formed primary hyphal knots, but the development was arrested at the transition from primary to secondary hyphal knots (*skn*). Complementation studies between these mutants identified at least 5 and 11 genes involved in the formation of primary and secondary hyphal knots, respectively.

2.2 Introduction

The Basidiomycete *Coprinus cinereus* is a model organism for studying fruiting body development. Fruiting bodies normally occur on the fertile thallus of a dikaryon. In the dark, the vegetative mycelium produces primary hyphal knots by localized intense branching, giving rise to small loose hyphal complexes with numerous short hyphal branches (see chapter 3). A short light signal induces the transformation of these primary hyphal knots into secondary hyphal knots, which are round compact hyphal aggregates of undifferentiated cells. A further light signal stimulates cellular differentiation and regional growth within this structure leading to the formation of specialized cap and stipe tissues. The differentiated structure is called a primordium. In

the cap of a young primordium, karyogamy takes place in basidia upon light stimulation. Karyogamy is immediately followed by meiosis and basidiospore formation. In parallel, the stipe elongates and the cap expands, resulting in the formation of a mature fruiting body. Shortly after maturation, basidiospores are released by cap autolysis [for review see (Kües, 2000)].

Much is known about the environmental and physiological regulation during the fruiting body formation. Light, temperature, mycelial age and nutrition are identified as important factors that influence the onset of fruiting body initiation and maturation. The cellular differentiation within developing and mature fruiting bodies is well dissected by cytological and histological studies (Kües, 2000; Lu, 1991, Moore, 1995; McLaughlin, 1982; Moore, 1998; Moore *et al.*, 1979; Reijnders, 1977). In contrast, genetic studies are far less advanced. Early genetic studies revealed that several developmental steps such as meiosis, basidiospore formation, cap expansion and autolysis could operate independently of each other (Pukkila and Casselton, 1991; Takemaru and Kamada, 1972). In order to isolate genes involved in fruiting body development, attempts were made to generate developmental mutants. Since fruiting bodies normally develop on dikaryons, the earliest developmental mutants were generated from fertile dikaryons (Takemaru and Kamada, 1972). Most mutations identified in these variants were thought to be dominant (Takemaru and Kamada, 1972). Later, Moore (Moore, 1981) pointed out that many of these mutations actually might be recessive, since the parental monokaryons had suboptimal genetic backgrounds for the development of fruiting bodies. However, it is still very difficult to identify recessive mutations in dikaryotic mutants, due to an impractical and work-intensive analysis of F1 generations in case basidiospores are still obtained. If fruiting is severely impaired, meiotic spores are not produced and classic genetics can no longer offer a possibility to unravel whether a mutation is recessive or dominant. Therefore, the generated dikaryotic developmental variants were mostly left aside.

Progress in obtaining genetically accessible mutants in fruiting body development was greatly facilitated by the isolation of *Amut Bmut* homokaryons (Pukkila, 1993; Swamy *et al.*, 1984). As indicated by the name, *Amut Bmut* strains have specific mutations at the *A* and *B* mating-type loci, which enables them to develop fruiting bodies without the need to mate with another strain (Swamy *et al.*, 1984). They produce mature and viable basidiospores, allowing us to perform classic genetic analysis through crosses with normal monokaryons (Liu *et al.*, 1999). *Amut Bmut* homokaryons contain

one type of haploid nuclei. Mutations in such haploid genetic background directly cause mutant phenotypes regardless of their dominance (Pukkila, 1993; Swamy *et al.*, 1984). In addition, *Amut Bmut* homokaryons produce numerous uninucleate mitotic haploid spores (oidia) in the aerial mycelium (Swamy *et al.*, 1984) in a light dependent manner (Kertesz-Chaloupkova *et al.*, 1998), which can be used as starting material for mutagenesis. Collections of developmental variants have been generated by chemical mutagens (Kanda and Ishikawa, 1986), ultraviolet light (UV) (Muraguchi *et al.*, 1999) (J.D. Granado, unpublishes results) and REMI mutagenesis (Cummings *et al.*, 1999; Granado *et al.*, 1997). For classically generated chemical or UV mutants, once a specific phenotype is defined, the mutated DNA sequence can be identified by complementation through transformation with a suitable DNA fragment (Pukkila, 1993) (see chapter 4). In REMI mutants, when the mutation of interest is tagged by an integrated plasmid, the mutated gene can be recovered together with the integrated vector by plasmid rescue strategy (Walser *et al.*, 2001) or alternatively by the recently developed semi-random PCR (Celerin *et al.*, 2000; Cummings *et al.*, 1999).

Homokaryon AmutBmut (*A43mut B43mut pab-1*) was used in our laboratory to produce 9018 mutants by REMI- and UV-mutagenesis. These mutants were first subjected to a standard fruiting test on YMG/T complete medium. In total, 550 clones defective in fruiting body initiation were identified (U. Kües, *et al.*, in prep). Since the morphological changes during fruiting body initiation are influenced by external and internal stimuli (Kües, 2000; Moore *et al.*, 1979; Wessels, 1994), the phenotypes of these mutants can be caused by a subtle interplay between genetic, environmental and physiological factors. In this study, we set up a series of tests on these 550 strains in order to define stable mutants with specific defects in the initial steps of the fruiting body development pathway.

2.3 Materials and methods

2.3.1 Strains

550 different mutants strains of homokaryon AmutBmut (*A43mut B43mut pab1*) (May *et al.*, 1991) were found in a previous screen to be defective in fruiting body initiation (Granado *et al.*, 1997; U. Kües *et al*, in prep.). 66 of these strains had a poor viability and did not grow any more after storage at 4 °C for four weeks or at –70 °C as frozen stocks, leaving 484 strains (267 REMI mutants, 216 UV mutants and 1 mutant

isolated after protoplasting and regenerating oidia) for further study. UV mutants are indicated by a name consisting of only numbers. REMI mutants can be recognized by the initial letter in their names: B stands for restriction enzyme *Bam*HI, E for *Eco*RI and P for *Pst*I that were used in transformation for integration of plasmid pPAB1-2 into the host's genome (Granado *et al.*, 1997). The single mutant obtained after protoplasting and regenerating oidia is called Proto-159.

Since REMI-mutants have integrated plasmid pPAB1-2 containing the wild-type *pab-1*$^{+}$ gene in their genome, they are prototrophic and kept on *Coprinus* minimal medium (Granado *et al.*, 1997). All other mutants are auxotrophic for p-aminobenzoate (PABA) because of the *pab-1* mutation and were therefore cultured on YMG/T complete medium (Granado *et al.*, 1997). Mutants were stored for short periods (up to a month) at 4°C (working cultures) and for years at −70°C as glycerol stock cultures (Walser *et al.*, 2001).

2.3.2 Phenotypic characterization

The ability to fruit was tested by inoculating strains in the middle of YMG/T agar plates, incubating them at 37 °C in the dark for about 5 days until the colonies reached the edge of the Petri dishes (at ca. 1-2 mm distance from the edge) and transferring them into standard fruiting conditions (12 h-light / 12 h-dark regime, 25°C, 90% humidity) (Granado *et al.*, 1997). Alternatively, 40-50 g of fresh horse dung were sterilized in 300-ml transparent glass bottles and were inoculated with ca. 15 pieces of mycelium-containing agar. The horse dung cultures were grown at 37°C in the dark until the mycelium covered the substrate. Subsequently, the bottles were transferred into standard fruiting conditions (Kües *et al.*, 1998). Cold induction of fruiting body initiation was tested by treating YMG/T cultures grown for 5 days in the dark at 5°C for 2 or 8 hours, and subsequently transferring them into standard fruiting conditions for further growth.

For analyzing oidia production at 37 °C in the dark and in the light, mutants were grown on YMG/T medium as previously described (Kertesz-Chaloupkova *et al.*, 1998). The oidia produced by a given mutant were collected in 10 ml of water (Kertesz-Chaloupkova *et al.*, 1998). The optical density of the spore suspension was measured with a photometer at 600 nm (Polak *et al.*, 1997) and compared to that obtained from parallel cultures of the parental homokaryon AmutBmut.

The presence of the hyphal knots and sclerotia was examined in YMG/T cultures grown for 7-8 days at 37 °C in the dark with a stereomicroscope (Zeiss Stemi 2000-C) illuminating the cultures from below. The formation of clamp cells on the hyphal septa and fusion of clamp cells were microscopically analyzed with young hyphae isolated from the growing edge of YMG/T cultures.

For determining the radial growth rate on YMG/T agar at 37°C in the dark, the diameter of a fungal colony was measured at day 2 (D2), day 3 (D3) and day 4 (D4) after inoculation at a 24-hour interval. The radial growth rate (mm/d) was calculated by the formula [(D3-D2)/2 + (D4-D3)/2]/2.

2.3.3 Southern hybridization analysis of the pPAB1-2 ectopic integration in REMI-mutants

REMI-mutants were grown in liquid minimal medium at 37°C in the dark for 4-5 days and their genomic DNAs were isolated from lyophilized mycelium (Zolan and Pukkila, 1986). Genomic DNAs of *Bam*HI-generated mutants were either digested with *Bam*HI alone or double-digested with *Bam*HI + *Sph*I. For *Eco*RI-generated mutants, *Eco*RI and *Eco*RI + *Pst*I were applied to digest genomic DNAs. For *Pst*I-generated mutants, *Pst*I or *Eco*RI + *Pst*I were used. After separation on 1% agarose gels, DNAs were transferred onto Hybond-N nylon membranes (Amersham) and used in Southern hybridization analysis (Sambrook *et al.*, 1989). DNA blots were separately hybridized with two α-^{32}P labelled DNA probes. One probe was a 3.3 kb *Eco*RI-*Pst*I fragment of plasmid pPAB1-2, containing the *C. cinereus pab-1*$^{+}$ gene. Plasmid pTZ19 (Pharmacia), the backbone of plasmid pPAB1-2 (Granado *et al.*, 1997), was the second probe.

2.3.4 Complementation test

Mutants were pair-wise crossed in all possible combinations on YMG/T agar and incubated first at 37°C in the dark. As soon as the colony reached the border of the Petri dish, plates were transferred to standard fruiting conditions (Granado *et al.*, 1997) (see above) for two to three weeks. Size and location of fruiting structures in these cultures were recorded.

2.4 Results

2.4.1 Identification of 137 stable mutants defective in fruiting body initiation

One practical advantage of studying fruiting body development in *C. cinereus* is that this fungus can develop mature fruiting bodies on artificial medium within a short time of two weeks (Moore and Pukkila, 1985). In a first screen, mutants of homokaryon AmutBmut were tested 2-3 times for fruiting on YMG/T agar. 550 strains did not initiate fruiting body development, *i.e.*, they failed to form secondary hyphal knots (U. Kües *et al.*, in preparation). 66 of these strains had a poor viability and did not grow any more after storage at 4 °C for four weeks or at –70 °C as frozen stocks, leaving 484 strains (267 REMI mutants, 216 UV mutants and 1 mutant isolated after protoplasting and regenerating oidia) for further study. According to the nomenclature of Takemaru and Kamada (Takemaru and Kamada, 1972), these mutants were classified as knotless clones (*knt*), since none of them produced fruiting body initials, *i.e.* secondary hyphal knots (see chapter 1 for definition). In the first screen performed on YMG/T medium, sclerotia development was also recorded, enabling to subdivide the *knt* mutants into two subclasses (U. Kües *et al.*, in prep). Mutants forming sclerotia must have previously formed the microscopically small, dark-dependent primary hyphal knots, from which sclerotia develop by further incubation in the dark (Waters *et al.*, 1975) (see chapter 3). These mutants (257 in total) were arbitrarily called *skn* mutants for lack of secondary hyphal knot formation. All others (227 in total) were arbitrarily classified as *pkn* mutants for lack of primary hyphal knot formation, although the definitive microscopical proof was only done later for a small number of selected mutants (see below).

Homokaryon AmutBmut of *C. cinereus* forms ca. 5 cm mature fruiting bodies on YMG/T medium. However, this artificial medium may not be as versatile as the natural substrate (horse dung) in helping the fungus to overcome minor variations in nutritional regulation of fruiting body development. In fact, strain AmutBmut develops ca. 12 cm mature fruiting bodies on horse dung under standard fruiting conditions. The observed size difference of mature fruiting bodies formed on different substrates suggested that the use of natural substrate would enable us to select against mutants that react negatively in development in suboptimal nutritional conditions. Therefore, all 484 viable mutants defective in fruiting body initiation on YMG/T medium were subjected to a fruiting test on horse dung. Various phenotypes were observed and mutants were

grouped according to the maximum developmental stage reached in fruiting body development (Table 1). 43 of the previously defined *pkn* mutants (19%) and 77 of the *skn* mutants (30%) developed mature fruiting bodies on horse dung (Table 1), suggesting that they are only slightly defective in nutritional regulation. Many others [86 of the *pkn* clones (38%), 141 of the *skn* clones (55%)] managed to enter the fruiting pathway, but stopped in development at various stages (Table 1). The fact that a total of 347 *knt* mutants showed a more advanced development on horse dung as compared to the formerly tested YMG/T medium demonstrates the important role of nutrition in regulating fruiting body production. It is interesting to note that more *skn* mutants than *pkn* mutants initiate fruiting body development on horse dung. When further tested for stability of the phenotypes on YMG/T medium (each clone independently for four times), all 137 *knt* (69 *pkn* + 68 *skn*) mutants did not initiate fruiting and were therefore regarded as stable fruiting body initiation mutants.

Table 1. Fruiting behavior of fruiting body initiation mutants of homokaryon AmutBmut on horse dung

Phenotype as defined on YMG/T medium	Number of mutants / phenotype[1] on horse dung													total
	knt		prm	mat	eln	exp	spo	eln exp	eln exp spo	eln spo	exp spo	other	wt	
	pkn[2]	skn[2]												
pkn[2]	60	38	27	50	0	1	2	2	0	0	2	2	43	227
skn[2]	9	30	15	98	2	2	5	9	5	1	3	1	77	257
total	69	68	42	148	2	3	7	11	5	1	5	3	120	484

[1] Phenotypes were defined according to Takemaru and Kamada (1972):
knt = knotless, mutants that did not form secondary hyphal knots
prm = primordiumless, mutants that did not produce primordia
mat = maturationless, mutants in which the maturation of primordia into fruiting bodies did not occur
eln = elongationless, mutants in which stipe elongation was blocked
exp = expansionless, mutants in which the cap did not expand
spo = sporeless, mutants with white caps because of defects in basidiospore formation
other = phenotypes that did not fit any of the other definitions
wt = wild-type

[2] pkn = defective in primary hyphal knot formation and skn = defective in secondary hyphal knot formation. In this table, the classification as pkn and skn was only preliminary based on the ability of knotless mutants defined on YMG/T medium to form (skn) or not to form sclerotia (pkn).

2.4.2 112 *knt* mutants have normal mating-type pathways

Mating-type genes of *C. cinereus* control the establishment and maintenance of a stable dikaryon as well as fruiting body initiation (Kües, 2000; Kües *et al.*, 1998). In *C.*

cinereus there are two unlinked loci containing the *A* and *B* mating-type genes (Casselton and Olesnicky, 1998; Hiscock and Kües, 1999). To form a dikaryon, the two fusing monokaryotic strains need to be different at both loci (A≠B≠). In the dikaryon, fused-clamp cells are found above each hyphal septum as the result of mating-type gene action. The different *A* genes control formation of clamp cells, whereas the different *B* genes cause clamp cells to fuse with their subapical hyphal cells (Casselton and Olesnicky, 1998; Kües, 2000). In *Amut Bmut* homokaryons, the specific mutation in the *A* locus causes expression of clamp cells and the mutation in the *B* locus is responsible for the fusion with the subapical hyphal cell (Swamy *et al.*, 1984). Therefore, clamp cells can be used as an indicator for the action of the mating-type genes, and we examined the mycelial morphology of the 137 stable mutants in fruiting body initiation. Fused clamp cells were found on mycelia of 112 of the mutants. 17 clones only formed non-fused clamp cells and thus should have defects in the *B* mating type pathway (Table 8 in the Appendix). 8 clones did not produce any clamp cell, suggesting defect(s) either in the *A* mating-type pathway or in other genes needed for clamp cell formation (Table 8 in the Appendix).

2.4.3 90 *knt* mutants perceive light normally

The 112 mutants with fused clamp cells were all analyzed for their ability to react to light. Constant incubation under blue or white light represses primary hyphal knot formation (Kües *et al.*, 1998), and a blue light signal is needed for the transition from primary to secondary hyphal knots (Boulianne *et al.*, 2000; Kües, 2000). Light also regulates oidiophore formation and oidia production in dikaryons and homokaryon AmutBmut of *C. cinereus* (Kertesz-Chaloupkova *et al.*, 1998; Polak *et al.*, 1997). In contrast to the complicated light requirements in the proceeding of fruiting body development (Kües, 2000; Lu, 1974; Lu, 2000), only a short light exposure is sufficient to induce oidia production in homokaryon AmutBmut (Kertesz-Chaloupkova *et al.*, 1998). Our present working hypothesis is that fruiting body development and oidiation in homokaryon AmutBmut are under the control of a common light induced signalling pathway. Therefore, light induced oidiation provides us with a very simple detection system to examine light perception in fruiting body initiation mutants, expecially in the *pkn* mutants where we cannot follow light effect on primary hyphal knot formation. In consequence, oidia production was compared in all mutants grown on YMG/T medium at 27°C under constant darkness and under constant light. In 90 mutants, oidia

production was low in the dark, but enhanced by light. In some cases, the light induced oidia production reached normal level. These clones therefore appear to have a normal light perception. 22 other mutants constitutively produced few oidia under both conditions (Table 8 in Appendix), possibly indicating a light blindness in these strains. However, in this last group of mutants, 2 *skn* mutants, strains 7-280 and B-0436, did not produce sclerotia under constant light illumination, indicating a normal light control on sclerotia formation as in the wild-type homokaryon AmutBmut.

2.4.4 63 *knt* mutants have a growth rate similar to the wild-type homokaryon AmutBmut

In the multicellular fungi, the fundamental growth unit is the hypha, which is a highly polarized structure that grows only at the tip. Hyphae regularly branch and give rise to a mycelium that forms a colony (Wessels, 1993). The polarized growth mode requires continuous synthesis and transport of cell wall materials towards the tip region (Moore, 1998). Therefore, a normal growth rate of the fungal colony reflects to a certain extent a well functioning primary metabolism. During the linear extension phase, the colony of the wild-type strain AmutBmut grew 7-8 mm/day on YMG/T agar at 37°C. Of the 90 *knt* mutants with normal light perception, 63 *knt* grew 4.5-8 mm/day, which is similar to the wild type homokaryon AmutBmut. Other mutants showed clearly reduced growth rates, ranging from 0.5 to 4.5 mm/day (Table 8 in the Appendix).

2.4.5 47 *knt* mutants produced abundant aerial mycelia like homokaryon AmutBmut

The multicellular fruiting bodies of *C. cinereus* arise from primary hyphal knots in the aerial mycelium (Matthews and Niederpruem, 1972). Homokaryon AmutBmut produces vigorous mycelium with a fluffy appearance when grown on YMG/T medium at 37°C in the dark (Polak *et al.*, 2000). 47 clones of the 63 well-growing mutants had the same or a very similar fluffy appearance when grown under the same condition. Other strains showed diverse mycelial morphologies, *e.g.* they produced dense, flat, wet or crusty-looking aerial mycelia. Sometimes the shapes of the colonies were irregular, possibly due to irregular hyphal branching and tip growth (Table 8 in the Appendix). A dense morphology may arise from increased hyphal branching and a flat morphology may be caused by the loss or reduction of producing aerial mycelium. Crusty-looking mycelium appears to have an invasive growth into the fungal substrate and/or may

excrete substances to form a hard crusty colony surface. Colonies with a wet morphology could be very interesting, since they may have a defect in producing hydrophobins. Hydrophobins are small cysteine-rich hydrophobic proteins that are secreted from the fungal cell and assemble into a hydrophobic rodlet film covering the aerial hyphae. In consequence, the aerial mycelium is hydrophobic and water repellent. In contrast, the absence of any hydrophobins leads to the easily wettable aerial mycelium (Wessels, 1997).

2.4.6 10 *pkn* and 40 *skn* mutants were defective in primary and secondary hyphal knot formation, respectively.

Originally starting from 484 mutants, so far we have excluded strains with minor defects in nutritional regulation and selected 47 stable *knt* mutants with functional mating-type pathways, normal light perception and normal growth characteristics. We also selected 3 other *knt* mutants (strains 7-280, B-0436 and B-2054) that have very similar characterictics except for a constitutively low oidiation (Table 8 in Appendix). This selection procedure in principle should help us to identify mutations that are closely related to the fruiting pathway. To definitively describe their phenotypes, we examined with a stereomicroscope the presence of any fruiting structures formed in the aerial mycelium of these mutants grown on YMG/T medium at 37°C in the dark or in the light. 10 strains did not form primary hyphal knots, neither in the dark nor in the light (listed in Table 2). Therefore, these 10 knotless clones were classified as *pkn* mutants for lack of primary hyphal knots. The other 40 mutants formed primary hyphal knots only in the dark (listed in Table 3). In all cases, the primary hyphal knots developed into sclerotia by extended incubation in the dark. None of the 40 mutants was able to transform the primary hyphal knots into secondary hyphal knots under standard fruiting conditions. Therefore, these 40 strains were classified as *skn* mutants for lack of secondary hyphal knot formation. In total, 19 of the mutants (3 *pkn* + 16 *skn* mutants) are derived from UV-mutagenesis, 30 of the mutants (7 *pkn* + 23 *skn* mutants) derived from REMI-mutagenesis and 1 *skn* mutant from protoplasting and regenerating oidia of homokaryon AmutBmut. *skn* mutant B-0436 was special, since it was the only strain where fruiting could be induced by a cold treatment at 5°C for 2 or 8 hours. When transferred to the standard fruiting conditions, mutant 7-275 developed at the edge of cultures rockeries, hard aggregated tumor-like structures that were described before in

fruiting body initiation mutants of *C. cinereus* to occur instead of fruiting bodies [Nyunoya, 1979 #532].

2.4.7 Mutant complementation studies

A complementation test, also called *cis-trans* test, is a genetic tool to determine whether two different mutations are in the same functional unit or gene. The test is based on the principle that a wild-type phenotype is produced when two different mutations are combined in a diploid or in a heterokaryotic/dikaryotic cell (Suzuki *et al.*, 1986). In Basidiomycetes, hyphal fusion (plasmogamy) may occur at any time during growth (Kemp, 1975), independently of the mating-type genes present in the fusing hyphae (Buller, 1933). In *Amut Bmut* homokaryons, the specific mutations in the mating-type loci overcome the natural incompatibility between endogenous mating-type gene products (Hiscock and Kües, 1999). Therefore, the formation of dikaryotic-like mycelium that carries two different nuclei in their cells might be expected when crossing two different mutants of homokaryon AmutBmut. In pioneering experiments, fused clamp cells were observed in the contact zone between two clampless mutants of homokaryon AmutBmut, suggesting that both hyphal fusion and nuclear complementation occurred in the intermediary zone (Liu *et al.*, 1999). Therefore, the 50 selected mutants with defects in fruiting body initiation were crossed in a pair-wise order. Since the mating-type pathways function normally in all these mutants, one should expect that a defective gene in fruiting body initiation of one mutant could be functionally complemented in a cross by the wild-type allele present in the other mutant. In consequence, gene complementation should allow the formation of fruiting structures at least in the contact zone between two crossed mutants when incubated in standard fruiting conditions. Fruiting structures may also develop at the outer edge of the mutant colonies, if nuclei are able to bypass the contact zone and migrate through the mycelium of the other strain. This situation is typical when crossing normal monokaryons of different mating-type genes (Casselton and Olesnicky, 1998; Hiscock and Kües, 1999; Kües, 2000).

When crossing the 50 fruiting body initiation mutants in all possible combinations, six different types of interactions between colonies were observed (Fig. 1). In the first three types of interactions, fruiting body development initiated in the contact zone between the two crossed mutants. In type 1, fruiting initiated only in this zone (Fig. 1). In type 2, fruiting body development started also at the outer edge of one of the two

mating partners (Fig. 1). In type 3, fruiting body initiation additionally occurred at the outer edges of both strains (Fig. 1). In the three other types (type 4-6) of interactions, fruiting initiation did not occur in the contact zone (Fig. 1). Type 4 refers to cases where fruiting body development did not occur at all (Fig. 1). Type 5 refers to cases where fruiting initiation was only observed on the outer edge of one colony and type 6 to cases where fruiting initiated on both colonies (Fig. 1). Generally, fruiting body initiation at

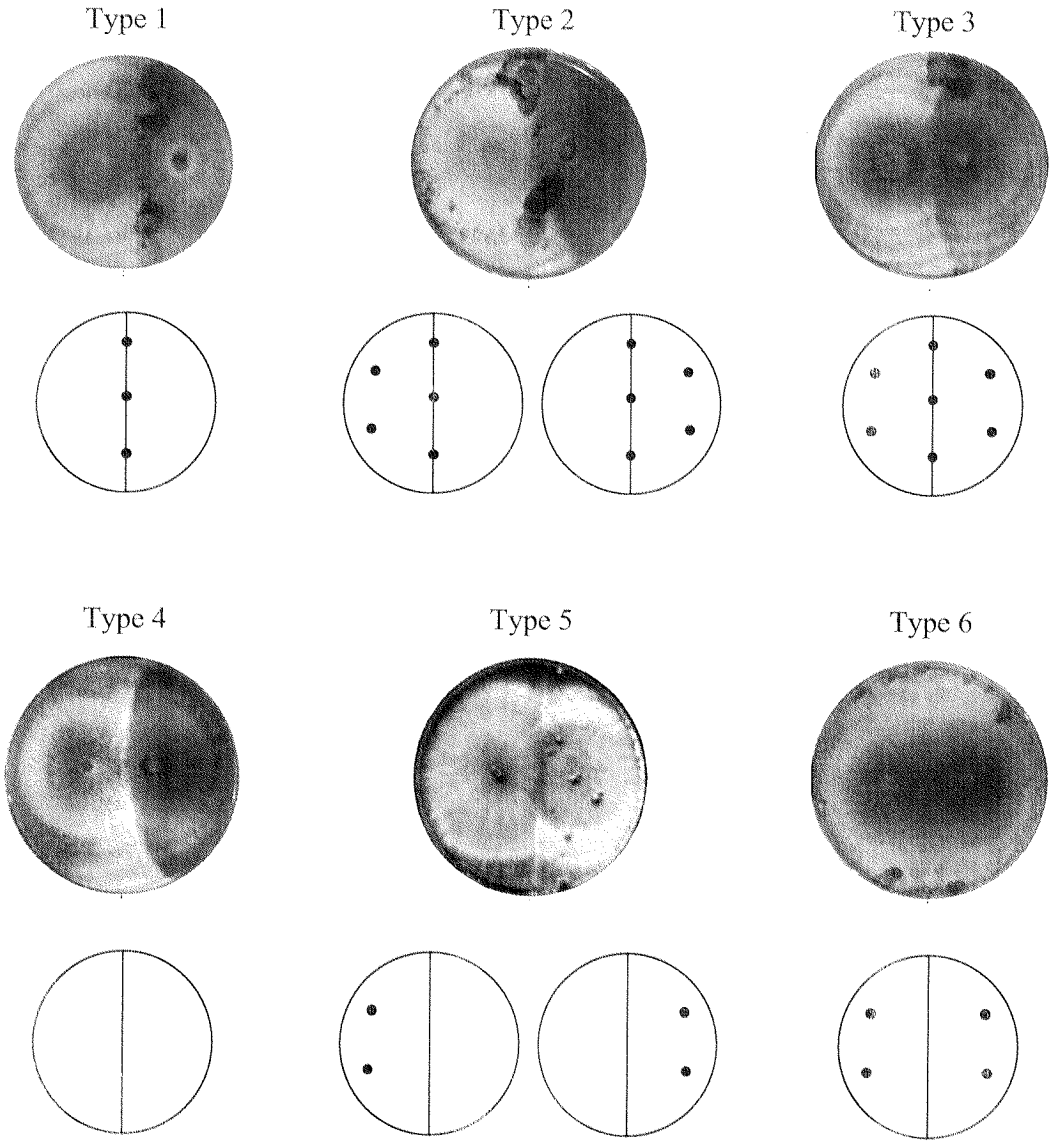

Figure 1. Six different types of interactions were observed in crosses between fruiting body initiation mutants of homokaryon AmutBmut as exemplified with the photos of selected crosses and in schematic diagrams. The vertical line in the diagrams represents the contact zone between mating strains, black dots indicate positions of fruiting structures.

one or more places in a cross did not guarantee that development proceeded to fruiting body maturation. Development could arrest at any stage (s: secondary hyphal knots, p: 1-9 mm sized primordia up to the stage of karyogamy, m: young fruiting bodies in meiosis, f: mature fruiting bodies; see Tables 6 and 7 in the Appendix). It is interesting to note that the type 6 pattern is the usual pattern observed when confronting two colonies of the wild-type homokaryon AmutBmut (not shown).

2.4.8 At least 5 and 11 genes were identified in primary and secondary hyphal knot formation, respectively.

Mating test between 10 *pkn* mutants allowed us to identify 5 different complementation groups in primary hyphal knot formation. These groups are represented by mutants B-0016, 7-K11, B-1533, 3-127/B-2641 and 6-536, respectively. These mutants positively interacted with each other in terms of fruiting body initiation, but did not self-interact (Table 2). In contrast, the *pkn* mutant B-1057 formed primordia in the contact zone when confronted with itself (Table 6 in Appendix). Generally, complementation between *pkn* mutants was achieved in only 61 % of all crosses (Table 2), not allowing any further conclusion on the other *pkn* mutants.

Table 2. Complementation test between *pkn* mutants of homokaryon AmutBmut

Mutant	Light induction of oidiation	Mutant									
		B-0016	7-K11	B-1533	3-127	B-2641	6-536	B-1856	B-1977	B-2054	B-1057
B-0016	yes, low		+	+	+	+	+	+	+	+	+
7-K11	yes, low	+		+	+	+	+	+	+	+	+
B-1533	yes	+	+		+	+	+				+
3-127	yes	+	+	+			+	+			+
B-2641	yes	+	+	+			+		+		+
6-536	yes	+	+	+	+	+					+
B-1856	yes	+	+		+						
B-1977	yes, low	+	+			+					+
B-2054	no, low[1]	+	+								+
B-1057	yes	+	+	+	+	+	+		+	+	+

[1] "no, low" refers to cases of low constitutive oidia production.

Note: Cells with + denote complementation patterns of type 1-3 and 5-6 where fruiting structures were developed by interactions between mutant colonies. The type 4 pattern is indicated by a blank cell. Different complementation groups are separated by thick lines. A thick line also separates the self-interacting mutant. Note also that more detailed results on the position of fruiting body initiation and the maximum stage reached can be found in Tables 6-7 in the Appendix.

11 different complementation groups for secondary hyphal knot formation were identified using the same mating approach. These groups were represented by mutants 6-031/P-1429, B-2018, B-2536, 6-608, B-0436, B-0724, B-0659, B-2521, B-2798, B-1347, B-1331 (Table 3). Self-interaction was observed in the 5 *skn* mutants B-2404, 3-020, 6-500, 4-001 and 7-171 (Table 3). The remaining 23 mutants with poor complementation could not be assigned to a specific group. In total, complementation between *skn* mutants was achieved in 71 % of all crosses (Table 3).

Complementation between the *pkn* and *skn* mutants was also not perfectly efficient (Table 4). Fruiting structures were only observed in 70 % of all crosses between *pkn* and *skn* mutants (Table 4), instead of the expected 100% complementation. Assuming that this lack of complementation was not always due to epistatic effects of mutation, the lack of complementation in crosses between *pkn* mutants and lack of complementation in crosses between *skn* mutants in many cases cannot be based on defects within the same complementation groups.

The self-interactions observed in one *pkn* and five *skn* mutants (Table 2 and 3) might point to the possibility that hyphal contacts induce fruiting or that the phenotypes of these mutants were less stable. Previously, we did not observe the development of any fruiting structures in these six mutants, when single colonies of these mutants were tested on YMG/T medium for fruiting. In the future, it would be worthwhile to perform a parallel fruiting test on these mutants by inoculating a single or two blocks of mycelium onto the YMG/T plates.

Since fruiting structures developed not only in contact zones (type 1-3 in Fig. 1), but also on one or both sides of the mating colonies (type 2-3, 5-6 in Fig. 1), we plotted the fruiting structures developed in the contact zone between two mating mutants in Table 6 (Appendix), and the fruiting structures developed on the side of each crossed mutant on the rows of Table 7 (Appendix). From these two tables, it becomes obvious that complementation in fruiting body development occurred in most cases in the contact zone of two mating mutants. In only 11 % of these cases, mature fruiting bodies developed. In 12% of these cases, young elongating fruiting bodies in meiosis were developed. Most of the time, development stopped at primordia (24%). In 2% of these cases, secondary hyphal knots or hyphal aggregates were formed. It should be noted that these hyphal aggregates appeared to be different from normal secondary hyphal knots. These results suggest that there might be further mutations present in one or both of the mating mutants acting at later stages of fruiting body development. This assumption

Table 3. Complementation test between *skn* mutants of homokaryon AmutBmut (for explanations see Table 2)

Mutant	Light induction of oidiation	Mutant																																							
		6-031	P-1429	B-2018	B-2536	6-608	B-0436	B-0724	B-0659	B-2521	B-2798	B-1347	B-1331	6-207	6-625	E-0782	7-237	P-2020	6-541	E-1593	6-430	E-1775	7-297	5-057	5-021	P-0678	E-2115	E-1686	E-1025	6-529	B-0134	B-1995	E-1574	Proto-159	B-1741	7-280	B-2404	3-020	6-500	4-001	7-171
6-031	yes			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+			+	+	+	+	+
P-1429	yes			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+					+	+	+	+	+
B-2018	yes	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		−	+	+	+	+	+	+	+	+	+	+	+	+
B-2536	yes, low	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6-608	yes	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+		+	+	+	+	+
B-0436	no, low	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			+	+	+	+	+	+	+	+		+	+	+	+	+
B-0724	yes	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+					+			+	+	+	+	+	+
B-0659	yes	+	+	+	+	+	+	+		+	+	+	+	+							+	+	+	+	+	+	+	+		+	+		+	+	+	+	+	+	+	+	+
B-2521	yes	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+						+	+	+		+		+	+	+	+	+			+	+	+	+	+	+
B-2798	yes	+	+	+	+	+	+	+	+	+		+	+	+	+	+									+		+	+	+	+	+	+	+		+	+	+	+	+	+	+
B-1347	yes	+	+	+	+	+	+	+	+	+	+		+		+	+	+	+		+	+			+	+		+	+	+		+	+	+		+	+	+	+	+	+	+
B-1331	yes	+	+	+	+	+	+	+	+	+	+	+			+			+	+	+	+	+	+		+	+							+	+			+	+	+	+	
6-207	yes	+	+	+	+	+	+	+	+	+	+							+		+	+				+			+	+	+							+	+	+	+	
6-625	yes	+	+	+	+	+	+	+		+	+	+	+			+		+	+	+	+		+	+		+	+	+	+		+		+			+	+	+	+	+	+
E-0782	yes, low	+	+	+	+	+	+	+		+	+	+			+		+		+	+			+	+	+		+	+	+	+		+		+			+	+	+	+	+
7-237	yes, low	+	+	+	+	+	+	+		+		+				+			+	+	+	+			+	+	+	+	+	+			+		+	+	+	+	+	+	+
P-2020	yes	+	+	+	+	+	+	+				+	+	+	+				+					+	+	+	+	+	+	+	+	+	+			+	+	+	+	+	
6-541	yes	+	+	+	+	+	+	+					+		+	+	+	+					+	+	+		+	+	+		+	+	+		+	+	+	+	+	+	
E-1593	yes, low	+	+	+	+	+	+	+				+	+	+	+	+	+				+							+			+		+			+	+	+	+	+	+
6-430	yes	+	+	+	+	+	+	+	+			+	+	+	+		+			+				+	+		+	+	+	+	+		+			+	+	+	+	+	
E-1775	yes, low	+	+	+	+	+	+	+	+				+				+						+				+		+		+		+			+	+	+	+	+	
7-297	yes, low	+	+	+	+	+	+	+	+	+			+		+	+			+			+		+	+					+							+	+	+	+	
5-057	yes	+	+	+	+	+	+	+	+	+		+			+	+		+	+		+		+		+					+			+			+	+	+	+	+	+
5-021	yes	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+		+		+	+				+	+	+			+		+			+	+	+	
P-0678	yes, low	+	+	+	+	+		+	+				+		+		+	+										+	+	+	+		+				+	+	+	+	+
E-2115	yes	+	+	+	+	+		+	+	+	+	+			+	+	+	+	+		+	+							+	+	+		+		+	+	+	+	+	+	
E-1686	yes	+	+		+	+	+	+	+		+	+		+	+	+	+	+	+	+	+				+	+			+			+	+	+			+	+	+	+	+
E-1025	yes, low	+	+	+	+	+	+			+	+	+		+	+	+	+	+	+		+	+			+	+	+	+		+	+	+					+	+	+	+	+
6-529	yes	+	+	+	+	+	+		+	+	+			+		+	+	+			+		+	+	+	+	+		+						+		+	+	+	+	
B-0134	yes, low	+	+	+	+	+	+		+	+	+	+			+			+	+	+	+	+				+	+		+								+	+	+	+	+
B-1995	yes	+	+	+	+	+	+			+	+	+				+		+	+									+	+								+	+	+	+	
E-1574	yes, low	+		+	+	+	+	+	+	+	+	+	+		+		+	+	+	+	+	+		+	+	+	+	+								+	+	+	+	+	+
Proto-159	yes	+		+	+		+		+				+			+												+									+	+	+	+	
B-1741	yes			+	+	+	+		+		+	+					+		+						+		+			+							+	+	+	+	
7-280	no, low			+	+			+	+	+	+	+			+		+	+	+	+	+	+		+			+						+				+	+	+	+	+
B-2404	yes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3-020	yes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6-500	yes, low	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4-001	yes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7-171	yes	+	+	+	+	+	+	+	+	+	+	+			+	+	+			+				+		+		+	+		+		+			+	+	+	+	+	+

was confirmed for mutant 6-031 that obviously carries an additional defect in primordium maturation (see chapter 4). However, for our analysis on complementation groups in primary and in secondary hyphal knot formation, the formation of mature fruiting bodies is not relevant.

Interestingly, when looking at fruiting body initiation at the outer edges of mating colonies, a very regular mating behaviour was found for six mutants (pkn mutant B-0016 and skn mutants B-2536, B-2402, 3-20, 6-500, 4-001, see shaded rows in Table 7 in the Appendix). These mutants in nearly every case initiated fruiting body development at their outer edges of the colonies, unlike most of their mating partners. Such a mating pattern might be the result of nuclei belonging to their mating partners bypassing the contact zone and migrating through the mycelium of these mutants. Alternatively, some kind of product(s) contributing to fruiting body initiation from the mating partners might diffuse through the mycelia of these six special mutants. In any case, these mutants appear to react as recipients, whereas other mutants behave like donors. However, it should be noted that four of these six mutants (strains B-2402, 3-20, 6-500, 4-001) initiated fruiting body development at the outer colony edges at both sides also in self-matings. In these four cases, it is not clear whether the self-reaction is due to a phenotypic instability or due to a special interaction caused by mycelial contact.

2.4.9 The integration patterns of pPAB1-2 in REMI mutants

For later recovery of mutated genes, it is important to know whether the mutations in REMI mutants of interest are linked to a plasmid integration and how often the plasmid is inserted in the respective mutants (Celerin *et al.*, 2000; Cummings *et al.*, 1999; Liu *et al.*, 1999; Muraguchi and Kamada, 2000). Therefore, we analyzed the pPAB1-2 integration pattern in the 7 *pkn* and 23 *skn* stable REMI mutants. 7 of the mutants had perfect REMI integration events. They contain only one copy of pPAB1-2 that was integrated into their genomes via a restriction site (*Bam*HI, *Eco*RI or *Pst*I) (Fig. 2, Table 5) and can be released by digesting the genomic DNAs of the mutants with the same restriction enzyme used for integration. 4 other mutants had pPAB1-2 integrated only once but in an undefined way (Fig. 2, Table 5). The most frequent pattern was found in 12 mutants, where pPAB1-2 was integrated twice with one case of one perfect REMI event (Table 5). The last 7 mutants showed multiple pPAB1-2 integrations (2 or more) with very complicated integration patterns (Table 5). In some of the cases of multiple

Table 4. Complementation between *pkn* and *skn* mutants of homokaryon AmutBmut (for explanations see Table 2)

skn mutant	Oidia light induction	*pkn* mutant									
		B-0016	7-K11	B-1533	3-127	B-2541	6-536	B-1856	B-1977	B-2054	B-1057
6-031	yes	+	+	+	+	+	+	+	+	+	+
P-1429	yes	+	+	+	+		+	+			+
B-2018	yes	+	+	+	+	+	+		+	+	+
B-2536	yes, low	+	+	+	+	+	+	+	+	+	+
6-608	yes	+	+	+	+	+	+	+		+	+
B-0436	no, low	+	+	+	+	+	+	+	+		+
B-0724	yes	+	+	+	+	+	+		+		+
B-0659	yes	+	+	+	+	+		+			+
B-2521	yes	+	+		+	+	+				+
B-2798	yes	+	+	+	+	+	+	+		+	+
B-1347	yes	+	+	+	+	+	+		+		+
B-1331	yes	+	+			+					
6-207	yes	+	+	+		+			+		+
6-625	yes	+	+		+		+				+
E-0782	yes, low	+	+		+		+			+	+
7-237	yes, low	+	+	+	+					+	+
P-2020	yes	+		+	+	+	+				+
6-541	yes	+			+	+		+			+
E-1593	yes, low	+	+		+	+	+				+
6-430	yes	+	+	+		+	+				+
E-1775	yes, low	+	+		+	+					+
7-297	yes, low	+	+		+		+				+
5-057	yes	+	+		+	+		+	+		
5-021	yes	+	+	+	+	+	+	+			+
P-0678	yes, low	+	+	+		+		+			+
E-2115	yes	+	+	+	+	+	+	+			+
E-1686	yes	+	+	+	+		+	+			+
E-1025	yes, low	+	+	+	+	+	+				
6-529	yes	+	+	+	+	+	+	+			
B-0134	yes, low	+	+	+	+	+	+				+
B-1995	yes	+	+		+				+		
E-1574	yes, low	+	+	+	+	+	+		+	+	+
Proto-159	yes	+	+								+
B-1741	yes	+	+						+		+
7-280	no, low	+	+	+	+		+				
B-2404	yes	+	+	+	+	+	+	+	+	+	+
3-020	yes	+	+	+	+	+	+	+	+	+	+
6-500	yes, low	+	+	+	+	+	+	+	+	+	+
4-001	yes	+	+	+	+	+	+	+	+	+	+
7-171	yes	+	+	+		+	+	+			+

integrations, a complete pPAB1-2 copy was recovered in digestion but the analysis did not allow us to distinguish perfect-REMI integrations from a tandem integration of two pPAB1-2 copies (Table 5). In general, the pPAB1-2 integration pattern in fruiting body initiation mutants occurred in a similar frequency as that observed before for randomly analyzed *C. cinereus* REMI transformants (Granado *et al.*, 1997). In addition, the hybridization patterns of the different *pkn* and *skn* mutants distinguished all from each other, indicating random ectopic integrations of the plasmid.

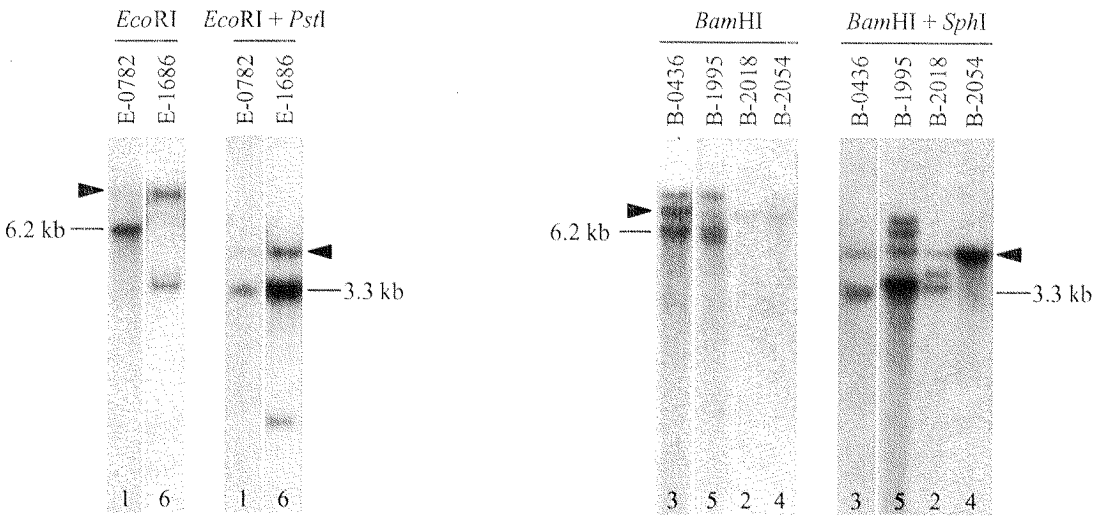

Figure 2. Integration pattern of plasmid pPAB1-2 in REMI mutants of homokaryon AmutBmut being defective in fruiting body initiation. Genomic DNAs of mutants were digested either with a single restriction enzyme or with two enzymes as indicated. Digested DNAs were separated on 1% agarose gels and transferred to nylon membranes for Southern hybridization analysis. A 3.3 kb *Eco*RI-*Pst*I fragment of PAB1-2 with the *C. cinereus pab-1*+ gene was used as a probe for hybridization. The examples given in the figure show a pefect REMI integration of pPAB1-2 (1), a single integration of pPAB1-2 in an undefined way (2), a case where pPAB1-2 integrated twice into the genome: once possibly as a perfect REMI integration event and once in an undefined way (3), two undefined integrations with pPAB1-2 (4), multiple integrations of pPAB1-2 with a possible perfect REMI integration or a tandem integration (5), and a case of undefined multiple integrations (6). Arrows mark the positions of the endogenous *pab1* sequence, 6.2 kb points to the positions of a full-length pPAB1-2 fragment and 3.3 kb to the positions of the *pab-1*+ insert of pPAB1-2 released by *Eco*RI + *Pst*I and *Bam*HI + *Sph*I, respectively.

Table 5. The integration pattern of pPAB1-2 in 30 different REMI mutants

Integration patterns		*pkn* mutants	*skn* mutants
1 insertion	perfect REMI insertion	B-1856	B-1741, B-2521, B-2798, E-0782, P-1429, P-2020
	undefined	B-1533	B-2018, B-2536, P-0678
2 insertions	1 possible perfect REMI insertion and 1 undefined		B-0436
	both undefined	B-0016, B-0659, B-1057, B-1977, B-2054, B-2641	B-0134, B-0724, E-1025, E-1574, E-1775
≥2 insertions	1 perfect REMI insertion or tandem integration and 1 or more undefined		B-1995, B-2404, E-1593
	all undefined		B-1331, E-1686, E-2115, B-1347

2.5 Discussion

In this study, we identified 10 stable *pkn* mutants and 40 stable *skn* mutants of *C. cinereus* homokaryon AmutBmut, being defective in fruiting body development at the stages of primary and secondary hyphal knot formation, respectively. These mutants can now be used to clone genes acting specifically in the early steps of fruiting body development. The first gene cloned from one of these mutants is described in chapter 4.

Different approaches can be taken to clone the genes of interest from our specific fruiting body initiation mutants. In REMI mutants, if a mutation is tagged by the insertion of the pPAB1-2 plasmid, we can recover in *E. coli* the plasmid DNA including flanking chromosomal DNA regions of the *Coprinus* mutant. These flanking regions will be used to identify the corresponding wild-type locus in a genomic cosmid library made from homokaryon AmutBmut (Bottoli *et al.*, 1999). Such an approach has successfully been employed to isolate genes from REMI mutants of the basidiomycete *Ustilago maydis* (Bolker *et al.*, 1995), but not yet of *C. cinereus*. Multiple insertions and/or plasmid rearrangements hindered the DNA recovery from a few *C. cinereus* REMI mutant that so far have been looked at (Celerin *et al.*, 2000; Cummings *et al.*,

1999) (J.D. Granado, personal communication). However, a semi-random PCR technique allowed at least in one case the targeted recovery of tagged mutated sequences from *C. cinereus* (Celerin *et al.*, 2000; Cummings *et al.*, 1999). In other cases, plasmid insertions have been shown by genetic analysis to be unlinked to a mutation of interest (Muraguchi and Kamada, 2000; Polak, 1999). Such untagged mutations can be isolated by crossing the paba-prototrophic *Amut Bmut* mutants with suitable monokaryons. Once present in an auxotrophic strain useful for transformation, such untagged mutations can be complemented with a genomic library (Bottoli *et al.*, 1999) by a SIB-selection transformation procedure [*i.e.*, repeated subdivision of a genomic library and progressively enriching for the transforming gene (Akins and Lambowitz, 1985)]. The same SIB-selection transformation strategy can be employed to identify mutated genes in paba-auxotrophic UV mutants of homokaryon AmutBmut (see chapter 4).

An important question not yet fully answered in our genetic analysis is how many different genes are mutated in the identified 10 *pkn* mutants and 40 *skn* mutants. The complementation tests between mutants identified at least 5 complementation groups for primary hyphal knot formation (Table 2) and 11 for secondary hyphal knot formation (Table 4). However, when no complementation was observed, we cannot exclude that hyphal fusion between crossing partners was inefficient. In the crosses between different *pkn* and *skn* mutants, we would have expected 100% complementation, but in reality, only 70% of the crosses produced fruiting structures. This result strongly suggests that the number of different genes identified in this study from the 10 *pkn* and 40 *skn* mutants by complementation is smaller than the actual number of genes affected in these mutants.

Hyphal fusion in basidiomycetes generally occurs between confronted genetically identical homokaryons and dikaryons (Aylmore and Todd, 1984; Kües, 2000; Todd and Aylmore, 1985). However, a nuclear replacement reaction usually follows the fusion between two different unrelated monokaryotic hyphae, a monokaryotic and an unrelated dikaryotic hypha, or even between genetically different dikaryotic hyphae (Aylmore and Todd, 1984; Kües, 2000; Todd and Aylmore, 1985). In consequence, heterokaryotic (dikaryotic) cells with two different nuclei are created and genetic defects in these nuclei might be complemented through the presence of the other nucleus. The situation differs when the two hyphae identical in one or both mating-type loci meet. They still fuse and the situation with two different nuclei is generated in the fusion cell, but this

status is very unstable. Usually, there is no or only very poor outgrowth of heterokaryotic hyphae (Kües, 2000). Since mating-type controlled self-incompatibility is overcome in *Amut Bmut* homokaryons (Casselton and Olesnicky, 1998; Hiscock and Kües, 1999), outgrowth of more stable heterokaryotic hyphae with two different mutant nuclei might be expected upon crossing two different mutants of homokaryon AmutBmut. Our current experiences from mutants of homokaryon AmutBmut with defects in clamp cell formation (Liu *et al.*, 1999) and from mutants defective in fruiting body initiation (this study) suggest that this is sometimes but not always the case. Complementation between *Amut Bmut* homokaryons appears to be a subtle event. When few of the crosses presented here were repeated, different complementation patterns were sometimes observed, *e.g.* in some crosses complementation was only achieved in this study, and in some crosses complementation occurred in the second time despite a negative result in this study (G. Ruprich-Robert, personal communication). Therefore, careful examination should be made before a mutant is selected for further studies.

Besides complementation reactions in the contact zone of two mutants, in particular with six of the mutants, fruiting structures were also observed at the outer edge of one or both mating partners. It is possible that the latter is caused by invasion of nuclei from one mating partner into the foreign mycelium of the other partner. However, if *Amut Bmut* homokaryons behave in mating events similarly to a wild-type dikaryon that only can donate but can not accept nuclei (Buller phenomenon) (Kües, 2000; Quintanilha, 1937), fruiting body formation on the outer edges of colonies is unlikely the result of a direct nuclear interaction but rather a result of gene products or secondary metabolites diffusing through the mycelial network.

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2.6 References

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Appendix

Table 6. Fruiting structures developed in the contact zone between two mutant colonies of homokaryon AmutBmut.
(s: secondary hyphal knots, p: primordia up to the stage of karyogamy, m: young elongating fruiting bodies in meiosis, f: mature fruiting bodies)

Mutant	Phenotype	Mutant	B-0016	7-K11	B-1533	3-127	B-2641	B-536	B-1856	B-1977	B-2054	B-1057	6-031	P-1429	B-2018	B-2535	6-609	B-5426	B-0724	B-0659	B-2521	B-2798	B-1347	B-1331	6-207	6-625	E-0782	7-237	P-2020	6-541	E-1593	6-430	E-1775	7-297	5-057	5-021	P-0678	E-2115	E-1638	E-1025	6-529	B-0134	B-1095	E-1574	Proto-159	B-1741	7-280	B-2404	3-020	6-500	4-001	7-171
B-0016	*pkn*			p	p	f	f	p	m				f	p	f	m																																					
7-K11	*pkn*		p		p	m	m	p	p	s			m	m	f		m	p	m	m	f	m	p	m	p																															
B-1533	*pkn*		p	p		p		p				p				p	p	m																																				
3-127	*pkn*		m	m	p		p	m					p	m	f	m	p		m	p	p																																	
B-2641	*pkn*		p	m		p			p				p	p	m	f	p	p																																				
B-536	*pkn*		f	p	p	p							p	m		f	m	f	p	p	m		m	f																														
B-1856	*pkn*		p	p		m							p				p																																					
B-1977	*pkn*			s			p						s																																									
B-2054	*pkn*													p																																								
B-1057	*pkn*		f	m	p	p	p	p					m	p	m	p	p	m	p	f	f	p	p																															
6-031	*skn*		p	m	p	m	p	s	p	m				f	m	p	m	f	f	f	f	f																															
P-1429	*skn*		f	m	p	f									f	f	p	m	f	f	f	f			p	p																										
B-2018	*skn*		m	f		m	p		p				m			f	p	m	p	f	f	f																														
B-2535	*skn*				p	p	m	p					m	f	p		m	p	f	m	f	f																														
6-609	*skn*		f	f	p	m	p	m	p				m	p	p	f	p	k	m	p	p	p																														
B-5426	*skn*		f	m	m	f	f	m	m				f	m	f	m	k		p	f	f	m																														
B-0724	*skn*			f	m	l	p	m					p	f	p	m	p	p	f	p	p	p																														
B-0659	*skn*		m	p	p	p							f	f	p	p	f	p	f	f	p	p																														
B-2521	*skn*		p		m	f							f	f	f	f	f	f	p	f	f	p																														
B-2798	*skn*		m			m	p		p	p			f	m	p		m	p																																			
B-1347	*skn*		f		f		f	p					f	m	f	m	m		p	f	f	p																														
B-1331	*skn*		f	m										p	p	p		p	f	p	p	p																														
6-207	*skn*				p	p			s		f			p	p	p	m	p	p	p	s																														
6-625	*skn*		m	p		p		p			p			p	p	p	m	p	s	p	f																														
E-0782	*skn*		p	m					p		p		m		f	m		p	p	f	p																														
7-237	*skn*		m			p				p			m	p	p		p	p	p	f	p																														
P-2020	*skn*			p	p	f	p		p					p	f																																					
6-541	*skn*		p			p	p	p		p			m	f	f	p	m	p	p																																
E-1593	*skn*			m		m	p	m					m		f	f	p	m	p																																
6-430	*skn*		f	p	p			f					p	f	f		p	f																																
E-1775	*skn*			p									m	f	s		p	m	p	s																														
7-297	*skn*		p	p		p		m		p			p	p	f		p	p																																
5-057	*skn*															p																																		
5-021	*skn*		p	m		m	p	p	s	p			m	f	m	f	m	m	f	p	p																														
P-0678	*skn*		s	p	s		m	f	s				m	p		p	m		p																																
E-2115	*skn*		m	m	p	m	p	p		f			m	f	p	p	f	m		m	p	p	f	f																										
E-1638	*skn*		p	p	m		s	p					m	f		m	p	p																																
E-1025	*skn*		m	p	p	p	p						m	p	m	p	p		m	f	f																													
6-529	*skn*		p	f				p					p	p	a	m	p																																	
B-0134	*skn*		f		p	s	p		p				p	s	m	p			s	f	s	f																												
B-1095	*skn*			f					p	p	p	p	p	p	p	p	p			f	f	f																												
E-1574	*skn*		m	p		s	p		p	p	m		p	p	p	p	p	k		p																													
Proto-159	*skn*		p											p																																			
B-1741	*skn*			p			p																																										
7-280	*skn*		f	p													m																																
B-2404	*skn*		p	m	f		p			m				f	m																																		
3-020	*skn*		m		f		p		p	p				p	p	f	m		f	p	f	m																												
6-500	*skn*			m		p									f																																		
4-001	*skn*		f		p	p	m	p	m				m	p	p	p	p	f	m																															
7-171	*skn*		m	p	p	p	s	p		f			m	m	p	p	p	p	p	f	p	p																												

Table 7: Fruiting structures developed on the side(s) of crossed mutant colonies of homokaryon AmutBmut. Data in each row describe the maximal fruiting stages (symbols as in Table 6) observed on the colony outer edge of a given mutant (indicated on the row) when crossed with other mutants (on the column).

Mutant	Phenotype	Mutant: B-0016	7-K11	B-1533	3-127	B-2641	6-536	B-1856	B-1977	B-2054	B-1057	6-031	P-1429	B-2018	B-2536	6-608	B-0436	B-0724	B-0659	B-2521	B-2798	B-1347	B-1331	6-207	6-625	E-0782	7-237	P-2020	6-541	E-1593	6-430	E-1775	7-297	5-057	5-021	P-0678	E-2115	E-1686	E-1025	6-529	B-0134	B-1995	E-1574	Proto-159	B-1741	7-280	B-2404	3-020	6-500	4-001	7-171
B-0016	*pkn*		p		p		p	p	p		p	p	p	f		p	f	p	p		f	f	p	s	f	p	p	p		p	p	p	p	p	p	f															p	
7-K11	*pkn*	p				p						s	p																						m				p					s									
B-1533	*pkn*	p				p						p		p	p		p				m						p								p			p			p					p						
3-127	*pkn*																				p				p	s									m					p	p				m				p	p		
B-2641	*pkn*						s							p		p	p		s	s		p	s	s					s					p	s				s										s		
6-536	*pkn*																								p																			s			s			s		s		
B-1856	*pkn*														m	s			s																s			s														
B-1977	*pkn*																																																				
B-2054	*pkn*		s								s																																									
B-1057	*pkn*	p				p	p		p					s					s								p										p		p				s	s		s					s	
6-031	*skn*														p	p										s		p							s								s	s	s	s						
P-1429	*skn*						p	p																		s		p						p	p																
B-2018	*skn*	p	p	p	p		p		m	p	p				p		s				p	p			p	p	p		p		p	p	p	p			p				m	s		s	p	p			p	p
B-2536	*skn*	s	p		p	p	p	p	m	p	p	p	p	s	p		p	p	p	p	p	p			p	p	s	p	p	p	p	p	p	p	p	p	p	p	p	p	p		p	p	p	p		s	p	
6-608	*skn*	s	p			p	p	p		p	p		p	p	p			p							s	p	s				m			s	s			p	p			p	p		m		p	p	p	p	
B-0436	*skn*			p	p		p		p		p			s	s					p						p												p			p						p	f		p	p
B-0724	*skn*						m		p																										p											p						
B-0659	*skn*	s									s																								p											p						s
B-2521	*skn*	p			p																														m																	p	
B-2798	*skn*											f	f			f		p							m		p												m				p			p		p			m	
B-1347	*skn*	p		p					p			p	f		f		f				p					p	p			p	f				f						m					f					
B-1331	*skn*					s									s				s	s						s					s			s									p	s							
6-207	*skn*																																																				
6-625	*skn*	s																																																			
E-0782	*skn*		s				p			p			p			p		p								s		p		s	p			p	p			p	p	s			s					p	p	p
7-237	*skn*														p												p																										
P-2020	*skn*	p														p		s								f												p										p				
6-541	*skn*											p	p		f													s					p	m					p										p		p		
E-1593	*skn*											m																		p										p										p		s	
6-430	*skn*						p						f				f														f				f											f						
E-1775	*skn*				p	m							p				p																																					
7-297	*skn*																																																				
5-057	*skn*																p																																				
5-021	*skn*																									p								p											p						
P-0678	*skn*																																																			
E-2115	*skn*																																																	p			
E-1686	*skn*	s															s		p																																	p	p
E-1025	*skn*																									p																										
6-529	*skn*			p		p	p	p							p	p			p	p	p			p			p							p	p	p		p					p				p	p		
B-0134	*skn*										s					p																																			p			
B-1995	*skn*								s																	p																										
E-1574	*skn*														p												p																										
Proto-159	*skn*																p																																				
B-1741	*skn*																s												s																								
7-280	*skn*																			m																																	
B-2404	*skn*	f	m	p	f	m	f	f	p	m	p	f	f	p	p	p	f	f	m	f	p		p	m	f	m	f	p	f	f	f	f	f	f		f	f	f	f	f	m	f	f	f	p	p	f	p	f	f	p
3-020	*skn*	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p
6-500	*skn*	p	p	p		p	p	p	p	p	p	p	p	p	p	p	p		p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p	
4-001	*skn*	f		p	p	s	p	p	s	s	s	s	p	s	s	p	p	p	s	s	p	p	p		p	p		p	p		s		s	p	p	p	p	s	s	p	p	p	p	p	s	p	p		s	p	p	s
7-171	*skn*	p		s							s	p		s													p			s							p						s								s

Table 8. Characteristics of fruiting body initiation mutants generated from *C. cinereus* homokaryon AmutBmut

Clamp cells at hyphal septa	Oidia production	Growth rate (mm/day)	Aerial mycelium morphology*	Names of mutants
Fused clamps	Light induced	4.5-8	Similar	3-020, 3-127, 4-001, 5-021, 5-057, 6-031, 6-207, 6-430, 6-500, 6-529, 6-536, 6-541, 6-608, 6-625, 7-171, 7-237, 7-297, 7-K11, B-0016, B-0134, B-0659, B-0724, B-1057, B-1331, B-1347, B-1533, B-1741, B-1856, B-1977, B-1995, B-2018, B-2404, B-2521, B-2536, B-2641, B-2798, E-0782, E-1025, E-1574, E-1593, E-1686, E-1775, E-2115, P-0678, P-1429, P-2020, Proto-159
			Different	0-050, 6-523, 7-275, B-0381, B-0565, B-0965, B-1578, B-1969, E-0143, E-0335, E-1071, E-1809, E-2081, P-0301, P-0572, P-1789
		0.5-4.5	Similar	6-533, 6-K09, 6-K18, B-0805, B-0966, B-2800
			Different	6-611, 6-620, 6-632, 6-K03, 6-K14, 6-K19, 7-216, 7-258, 7-301, 7-302, B-0121, B-1149s, B-1245, B-2253, B-2795, E-0008, E-0963, E-1200, E-1328, P-0775, P1759
	Constitutively low	4.5-8	Similar	7-280, B-0436, B-2054
			Different	4-077, 6-587, B-0873, B-1191, B-2216, P-0276
		0.5-4.5	Similar	6-K21, 6-K22, 6-K30
			Different	6-605, 6-K05, 6-K25, 6-K31, 6-K46, 7-278, B-0877, B-0888, B1400, E-1136s
Nonfused clamps	Light induced	4.5-8	Similar	6-577, 7-252, B-0617, E-0601
		0.5-4.5	Different	6-594, 7-295, B-0116, P-1614
	Constitutively low	4.5-8	Similar	B-1375
			Different	6-543
		0.5-4.5	Similar	6-580
			Different	6-K26, 6-K45, 7-K09, B-0513, B-2863
	Constitutively high	4.5-8	Different	6-K08
No clamps	Light induced	0.5-4.5	Different	E-0003
	Constitutively low	4.5-8	Different	E-0978
		0.5-4.5	Different	6-K16, 7-173, 7-K16, B-0281, E-0301
	Constitutively high	4.5-8	Similar	E-2095

* When grown on YMG/T medium at 37°C in the dark, the aerial mycelium morphology of mutants listed here is either similar to the wild-type homokaryon AmutBmut or different.

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Chapter 3

Microscopic observations on primary hyphal knot formation and the correlation of galectin expression to fruiting body initiation

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3 Microscopic observations on primary hyphal knot formation and the correlation of galectin expression to fruiting body initiation

3.1 Abstract

The formation of primary hyphal knots initiates fruiting body development in the higher basidiomycete *Coprinus cinereus*. The microscopic three-dimensional structures are formed in the dark and derived from localized intense hyphal branching in the aerial mycelium. A primary hyphal knot can arise from a single vegetative hypha, or from cooperative interactions between two or more neighboring vegetative hyphae. Two fungal galectin genes (*cgl1* and *cgl2*) were differentially expressed at both the transcriptional and the translational levels during early fruiting body development. Proteins of these genes were absent in pure vegetative mycelium. Expression of Cgl2 protein coincided with the emergence of primary hyphal knots, expression of Cgl1 with the light-induced secondary hyphal knot formation. The secondary hyphal knot is a compact hyphal aggregate without any internal differentiation. Cgl2 expression increased with the proceeding of primordium development, whereas the expression of Cgl1 was induced at the stage of the light-dependent hymenium differentiation in the primordium. Studies with mutants defective in primary hyphal knot formation (*pkn*) showed that Cgl2 can be expressed in the absence of primary hyphal knots and *vice versa*, suggesting that Cgl2 expression and primary hyphal knot formation are independent from each other, although light and temperature regulate both events in the same way. In two mutants defective in secondary hyphal knot formation (*skn*), strong Cgl1 expression occurred in the absence of secondary hyphal knot formation, showing that also these two events occur independently from each other. Several other *pkn* and *skn* mutants were found to have an altered light and/or temperature regulation on one or both of the galectin genes.

3.2 Introduction

A lectin is a sugar-binding protein or glycoprotein of non-immune origin that agglutinates cells and/or precipitates glycoconjugates. The sugar-binding site(s) enables a lectin to recognize specific monosaccharides or oligosaccharides (Goldstein *et al.*, 1980). Lectins are widely distributed in living organisms including bacteria, viruses, animals, plants and fungi. Fungal lectins, isolated from thirteen mushroom species,

differ largely in molecular characteristics such as molecular weight, number of subunits, carbohydrate content and carbohydrate binding specificity (Wang *et al.*, 1998). Galectins, or S-type lectins, specifically bind β-galactoside sugars in a calcium-independent manner and share sequence homology within the carbohydrate-recognition domain (CRD) (Barondes *et al.*, 1994). Currently, ten mammalian galectins (Cooper and Barondes, 1999) and two fungal galectins (Cooper *et al.*, 1997) have been discovered, each containing one or two highly conserved CRDs made up of about 135 amino acid residues (Hughes, 1999). The identification of galectins from these two kingdoms implies that galectins perform very basic biological functions (Cooper *et al.*, 1997), since animals and fungi shared a common ancestor approximately 1 billion years ago (Doolittle *et al.*, 1996).

Members of the mammalian galectin family have been shown to mediate cell adhesion, to regulate cell proliferation, to trigger or inhibit apoptosis and to be required for pre-mRNA splicing (Perillo *et al.*, 1998). In animals, galectins are found on the cell surface and within the extracellular matrix, as well as in the cytoplasm and the nucleus (Perillo *et al.*, 1998). In fungi, two galectins, Cgl 1 and Cgl 2 (each containing one CRD), were isolated from the heterothallic basidiomycete *Coprinus cinereus*. These galectins are localized in the extracellular matrix, in cell walls as well as in cytoplasmic bodies and are specific to the fruiting bodies (Boulianne *et al.*, 2000; Cooper *et al.*, 1997). Fruiting body development in *C. cinereus* is a process involving various hyphal-hyphal interactions (Kües, 2000; Moore, 1998). The localization of galectins in the extracellular matrix suggests their involvement in cellular hyphal interactions, which might be mediated by their ability of binding specific carbohydrates.

The early stages during fruiting body development in *C. cinereus* are classified by three emergent structures: the primary hyphal knot, the secondary hyphal knot and the primordium (Kües, 2000) (see chapter 1). The primary hyphal knot derives from localized intense hyphal branching that happens in the dark (Boulianne *et al.*, 2000; Kües *et al.*, 1998). A light signal stimulates the transformation of the primary hyphal knot into the secondary hyphal knot, the latter being the first fruiting specific structure. Stimulated by further light signals, regional growth and cellular organization within the secondary hyphal knot lead to the formation of the primordium, in which cap and stipe tissues are differentiated. In the cap of a young primordium, light induces karyogamy within the basidia. Karyogamy is followed by meiosis and basidiospore formation. In parallel, the stipe elongates and the cap expands, giving rise to a mature fruiting body

(Kües, 2000). In this study, we followed galectin expression by Western blot analysis through the developmental pathway of fruiting body formation. The early stages in fruiting body development were more precisely defined and galectin expression was correlated to these early events.

3.3 Materials and methods

3.3.1 Fungal strains

Strain AmutBmut is a self-compatible homokaryon with mutations in both mating-type loci that fruits without prior mating to another strain (Swamy *et al.*, 1984). Strains B-0016, B-1057, B-1533, B-1856, B-1977, B-2054 and B-2641 are REMI-mutants and strains 3-127, 6-536 and 7-K11 are UV-mutants of homokaryon AmutBmut with defects in primary hyphal knot formation (*pkn*) (U. Kües *et al.*, in prep). Strains B-0134, B-0436, B-0659, B-0724, B-1331, B-1347, B-1741, B-1995, B-2018, B-2404, B-2521, B-2536, B-2798, E-0782, E-1025, E-1574, E-1593, E-1686, E-1775, E-2115, P-0678, P-1429 and P-2020 are REMI-mutants and strains 3-020, 3-149, 4-001, 5-021, 5-057, 6-031, 6-207, 6-430, 6-500, 6-529, 6-541, 6-608, 6-625, 7-171, 7-237, 7-279, 7-280 and 7-297 are UV-mutants of homokaryon AmutBmut defective in secondary hyphal knot formation (*skn*) (U. Kües *et al.*, in prep). Strain Proto-159 is a *skn* mutant derived from protoplasting of AmutBmut oidia and subsequent regeneration (Granado *et al.*, 1997; U. Kües, *et al*, in prep) (see chapter 2). Mutant cultures were stored at –70°C in 15% glycerol (Walser *et al.*, 2001). For regrowth, frozen cultures were thawed and transferred onto YMG/T agar in case of paba-auxotrophic UV-mutants and strain Proto-159, or onto *Coprinus* minimal medium in case of paba-prototrophic REMI-mutants (Granado *et al.*, 1997). These cultures were used to inoculate YMG/T agar for studying galectin expression. All mutants were also stored for three years at 4°C. The 4°C cultures were refreshed by regrowing on YMG/T or minimal media and inoculated onto YMG/T medium to check the stability of their fruiting phenotypes (defects in primary or secondary hyphal knot formation).

3.3.2 Microscopic observation of primary hyphal knot formation

After homokaryon AmutBmut was grown on YMG/T agar at 37°C in a light-proof ventilated box for 5 days, a block of agar (1 x 1 cm) was cut out to create an observation window. Cultures were returned into the light-proof box and incubated for two more

days. Processes occurring in primary hyphal knot formation in strain AmutBmut were observed in one- or twenty-hour intervals and at random time points with a inverse microscope Axiovert 25 (Zeiss). Mycelium with primary hyphal knots was photographed with a color chilled 3CCD Camera (Hamamatsu C5810). Digital images were processed with the computer program Adobe Photoshop 5.5.

3.3.3 Detection of galectin expression under different growth conditions

Homokaryon AmutBmut was inoculated on YMG/T agar in the center of Petri dishes (day 0) and incubated under various environmental conditions (Table 1). Aerial mycelium with or without fruiting structures (primary hyphal knots, secondary hyphal knots, primordia in the size of 1 or 8 mm) was collected and lyophilized, as well as cap and stipe tissues from the postmeiotic (ca. 2.4 cm) and mature fruiting bodies. Saline-soluble proteins were extracted from these lyophilized materials and analyzed by SDS-PAGE and Western blotting (Boulianne *et al.*, 2000). Blots were incubated with galectin-specific rabbit antiserum (α-Cgl, primary antibody) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit (secondary) antibody and detected according to the supplier's instructions (Bio-Rad) (Charlton *et al.*, 1992). Alternatively, they were incubated with α-Cgl and protein A coupled-HRP, detected by ECL chemiluminescence (Amersham Life Science) (Boulianne *et al.*, 2000).

Table 1. Growth conditions used to study galectin expression in homokaryon AmutBmut

	Condition
I	37°C continuous darkness for 5 days, followed by 8 days of incubation in a 12 h light/12 h dark regime[a] (L/D) at 25°C (the standard fruiting conditions, (Granado *et al.*, 1997))
II	25°C 12 h light/12 h dark regime[a] (L/D) for 7 to 16 days
III	37°C in continuous darkness (D) for 5 to 12 days
IV	25°C continuous darkness for (D) 7 to 15 days
V	37°C continuous light [b] (L) for 5 to 12 days
VI	25°C continuous light regime[b] (L) for 7 to 14 days
VII	37°C continuous darkness (D) for 5 days, followed by 7 days of exposure in continuous light (L) at 37°C[b]

[a]light source: white fluorescent tubes (Osram L40/25 Universal-Weiß, emission spectrum 295-780 nm), light intensity: 35-70 $\mu E\ s^{-1}m^{-2}$

[b]light source: as in [a], light intensity: 20 $\mu E\ s^{-1}\ m^{-2}$

To analyze galectin expression in *pkn* and *skn* mutants, the aerial mycelium of whole cultures grown either at conditions I or III for 7 days, or at condition II for 10 days (5

days at 37°C and 5 days at 25°C) were collected and analyzed by SDS-PAGE and Western blotting with α-Cgl and protein A coupled-HRP by ECL chemiluminescence (Boulianne *et al.*, 2000).

3.3.4 Northern hybridization analysis

Total RNAs was extracted with a guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987) from powdered lyophilized *C. cinereus* AmutBmut mycelium or tissues of different fruiting stages. 10 µg of total RNAs per sample were used in Northern hybridization analysis (Ausubel *et al.*, 2000). Hybridization signals were produced with a 1 kb *Bgl*II-*Nhe*I DNA fragment that was isolated from plasmid pCGLI-6R, containing the *cgl1* coding region inserted in pBluescript KS(-), and labeled with [α-^{32}P]dCTP (specific probe activity: 3 x 10^9 cpm/µg DNA) by random primed DNA labeling (Boehringer Mannheim).

3.4 Results and discussion

3.4.1 Primary hyphal knots are derived from localized intense hyphal branching

In the literature, the formation of primary hyphal knots is poorly defined (see chapter 1). Therefore, we tried to observe this process by following individual hyphae in dark-grown cultures of homokaryon AmutBmut under a microscope at one-hour intervals. At the beginning, we observed the formation of a regular hyphal network (Fig. 1a) on the surface of the plastic Petri dish, where an observation window was created (see Materials and methods). Branches appeared at large distances in more or less regular angles (this study; Polak *et al.*, 2000; Polak *et al.*, 1997) on some but not all cells of a given hypha ("main hypha"). These side branches tended to elongate in one direction and occasionally gave rise to new branches (Fig. 1a). After 10 hours, several short hyphal branches emerged in a confined area between two main hyphae (Fig. 1b and 1c). Unfortunately, the strong white light source of the microscope (about 12 mE s^{-1} m^{-2}) led to an arrest in development of those short hyphae. In the next 5 hours, no further development in the confined area shown in Fig. 1b or 1c was encountered (not shown). Therefore, we were confronted with the problem that blue light represses primary hyphal knot formation in vegetative mycelium (Kües *et al.*, 1998). In addition, blue light was shown to induce oidiophore formation and asexual spore (oidia) production

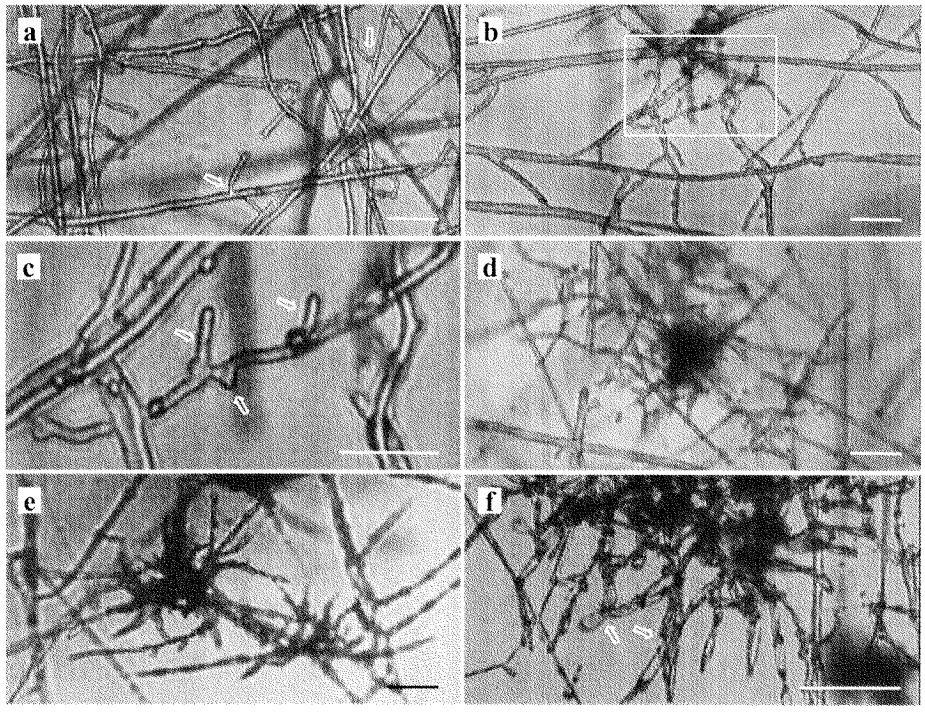

Figure 1. Primary hyphal knots are derived from localized intense branching. Primary hyphal knot formation was observed in cultures of homokaryon AmutBmut as described in Materials and methods: 48 hours after the creation of an observation window, hyphae colonized the agar-free Petri dish surface by regular formation of side branches (a). Ten hours later, many short branches appeared in a confined area between two main hyphae (b, see the inset enlarged in c). More than one short branch may develop on one hyphal cell (c). Arrow heads in a and c point to branching positions. In other independent experiments, immature (d) and mature (e, f) primary hyphal knots were detected three days after the creation of the observation window. Note the short hyphae in immature and mature primary hyphal knots. These short hyphal cells have irregular shape and partially twist around each other (f). Size bar in all pictures: 0.1 mm.

(Kertesz-Chaloupkova *et al.*, 1998; Kües *et al.*, 1998; Polak *et al.*, 1997). Consistently, in another experiment, using twenty-hour intervals for observation, further advanced hyphal complexes were seen (Fig. 2a and 2b), but primary hyphal knot development was also aborted by the microscopic light emission. Instead, light induced oidia production on the hyphal complex (Fig. 2b-2f).

The addition of a yellow filter (E520LP, Chroma, Zeiss) to the inverse microscope eliminated light in the UV-blue range and improved our observations. Primary hyphal knot development continued in the cultures well after microscopic inspection (not shown). However, because of fast water condensation in the observation window due to temperature difference between the growth condition (37°C) and the lab (ca. 20°C), it was still difficult to obtain consecutive photos of the same developing hyphal complex. Therefore, we present only steady-state pictures on the formation of primary hyphal knots.

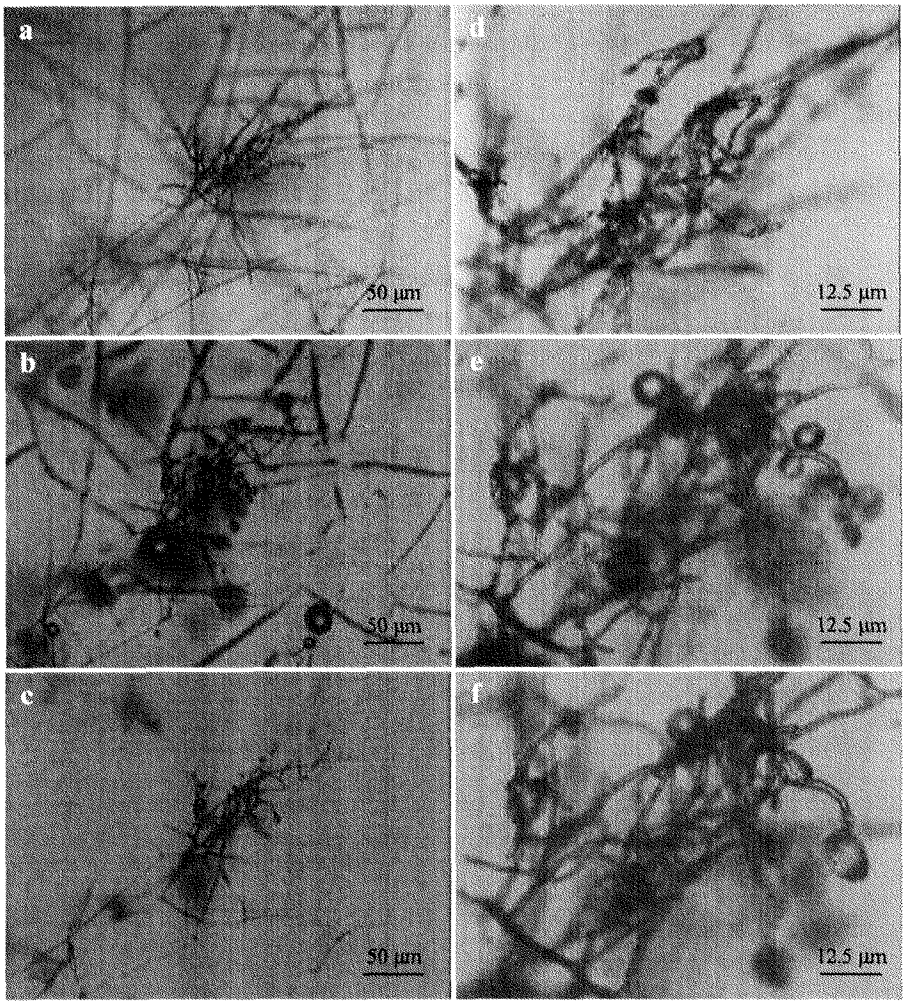

Figure 2. Light illumination inhibits primary hyphal knot formation in the aerial mycelium of a dark-grown culture and induces oidia formation. A young primary hyphal knot consisting of numerous short hyphal branches was detected in the aerial mycelium of homokaryon AmutBmut (a and d) and followed over the time (b and e, twenty hours later; c and f, twenty five hours later). Due to a light emission from the microscope, primary hyphal knot development arrested and numerous oidia droplets developed in the hyphal complex 20 hours later (b and e). After additional 5 hours, oidia droplets fused into larger spore mass and the surrounding aerial mycelium suffered from desiccation (c and f).

Examples of our observations when using the yellow filter at random time points are shown in Fig. 3. Nineteen hours after creating the small observation window, vegetative hyphae formed a hyphal network by regular branching (Fig. 3a). In some confined areas, we detected unusual hyphae with abundant short side branches of limited tip growth (Fig. 3b). 9-12 h later such localized intense branching led to the formation of hyphal complexes that arose either from just a single hypha (Fig. 3c) or from more than one neighboring hyphae (Fig. 3d). After other 14 h, inflated hyphae were found within the hyphal complex (Fig. 3e), which increased three-dimensionally. At this stage the

dimensions of the complex made it difficult to obtain pictures with good resolution (Fig. 3f-3g). After further 10 h, primary hyphal knot formation was basically finished (Fig. 3h).

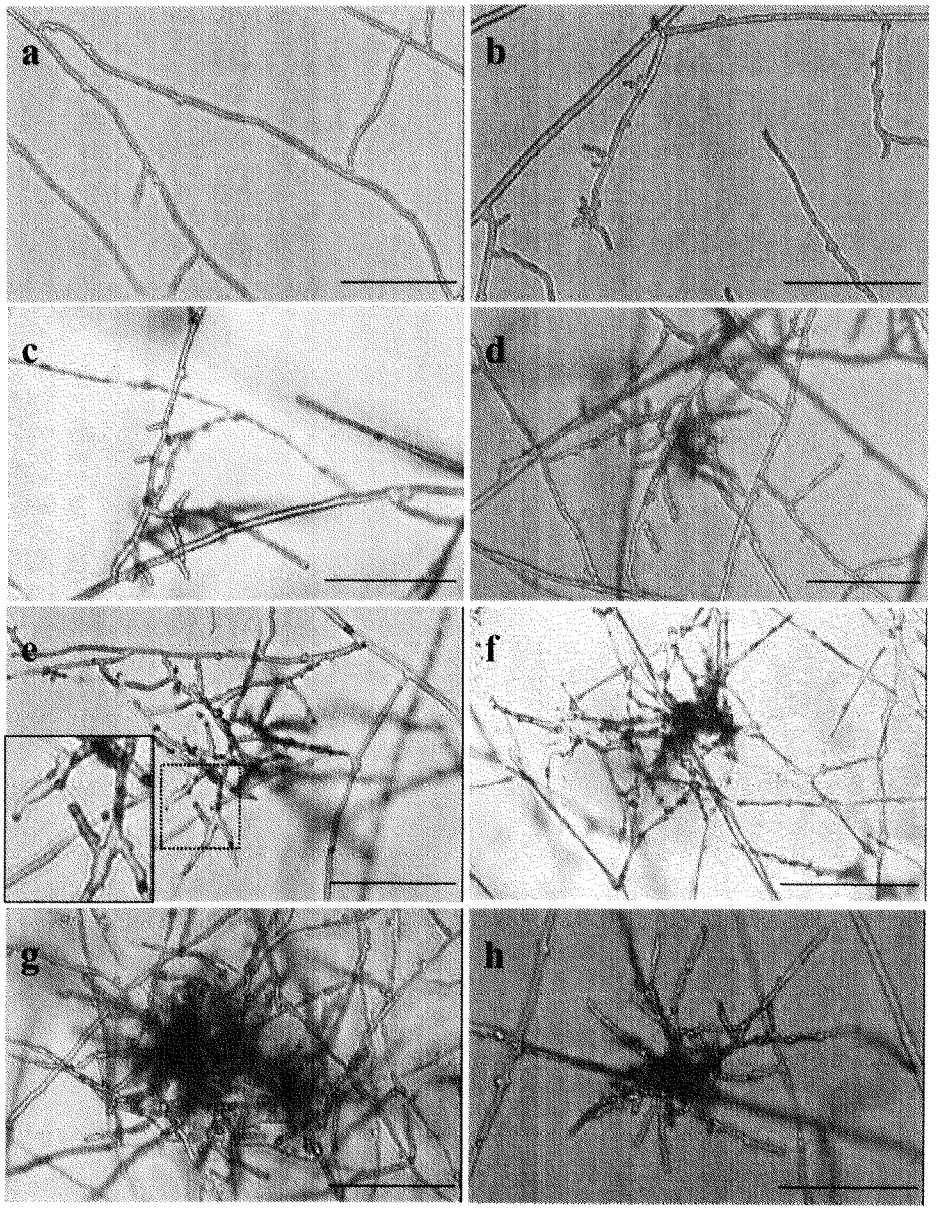

Figure 3. Morphological changes during primary hyphal knot formation in homokaryon AmutBmut. 19 h after creating an observation window, a vegetative hyphal network formed by regular branching (a). At this time point, many short branches appeared on specific individual hyphae (b). 9-12 h later, hyphal complexes were generated by intense localized branching that originated either from a single hypha (c) or from two or more neighboring hyphae (d). After another 14 h, the hyphal complex grew three-dimensionally (e-g). The dashed box in picture e is twice enlarged in the inset and presents examples of inflated hyphal cells. After further 10 h, primary hyphal knots were fully matured (h). Size bar in all pictures: 15 µm.

3.4.2 More observations and aspects related to primary hyphal knots

Based on observations made at 37°C on dark-grown cultures of homokaryon AmutBmut of different age and with different structures, under the microscope with or without the yellow filter, we deduce the following:

1) Following observations of Brefeld, Buller claimed that the basidiocarp of *C. stercorarius* arose from a single cell (Buller, 1933). Other authors (Clémençon, 1997; Reijnders, 1963), however, found that primordia (and thus ultimately primary hyphal knots) in a number of *Coprinus* spp., including *C. cinereus*, originated either from one hypha (monocentric) or from two or more hyphae (polycentric). In cultures of *C. cinereus* homokaryon AmutBmut, we also observed monocentric primary hyphal knot formation (Fig. 3b-3c), but polycentric primary hyphal knot formation (Fig. 1d-1e and 3d-3e) was more frequently observed.

2) Within the aerial mycelium, primary hyphal knots appear to develop in more or less regular pattern (Fig. 4a). Primary hyphal knots in homokaryon AmutBmut formed at distance of 388 ± 151 μm (calculated from 237 primary hyphal knots).

3) It takes 1-2 days from the first sign of development (Fig. 1b) to obtain a fully matured primary hyphal knot (Fig. 3). At the onset of primary hyphal knot formation, a hypha switches from unidirectional growth into localized ramification, which leads to the production of many short branches with restricted hyphal tip growth (Fig. 1b-1c and 3b). There might be one or more of such short branches per hyphal cell (Fig. 1c). Ramification is confined to an active center, resulting in the formation of a hyphal complex that expands three-dimensionally in size by repeated branching (Fig. 1d-1f, 2a, 2d and 3b-3g). At the end, a fully developed primary hyphal knot emerges with a size of approximately 30 μm in diameter (Fig. 1e and 3h). Numerous short hyphal branches within such a primary hyphal knot are characterized by cells of irregular shape (Fig. 1f and 3e). Hyphal branches within the primary hyphal knot partially twist around each other (Fig. 1f). Hyphae anchoring and connecting primary hyphal knots in the aerial mycelia network can also twist (Fig. 4b-4c), possibly to strengthen the anchoring forces.

4) Developing primary hyphal knots are neither committed to the fruiting program nor committed to sclerotium formation (see point 5 below). When exposed to a strong light source (microscopic bulb), oidium droplets clearly developed on branches of the hyphal complex (Fig. 2b-2e), indicating that light determines which developmental program is entered. Primary hyphal knot formation needs darkness (Kües *et al.*, 1998; this study). The induction of secondary hyphal knot formation needs low light energy (1

foot-candle of blue light, 410-450 nm) (Elliott, 1994; Lu, 1974), whereas strong light represses fruiting body initiation but leads to oidia production (Kertesz-Chaloupkova *et al.*, 1998; Kües *et al.*, 1998; this study).

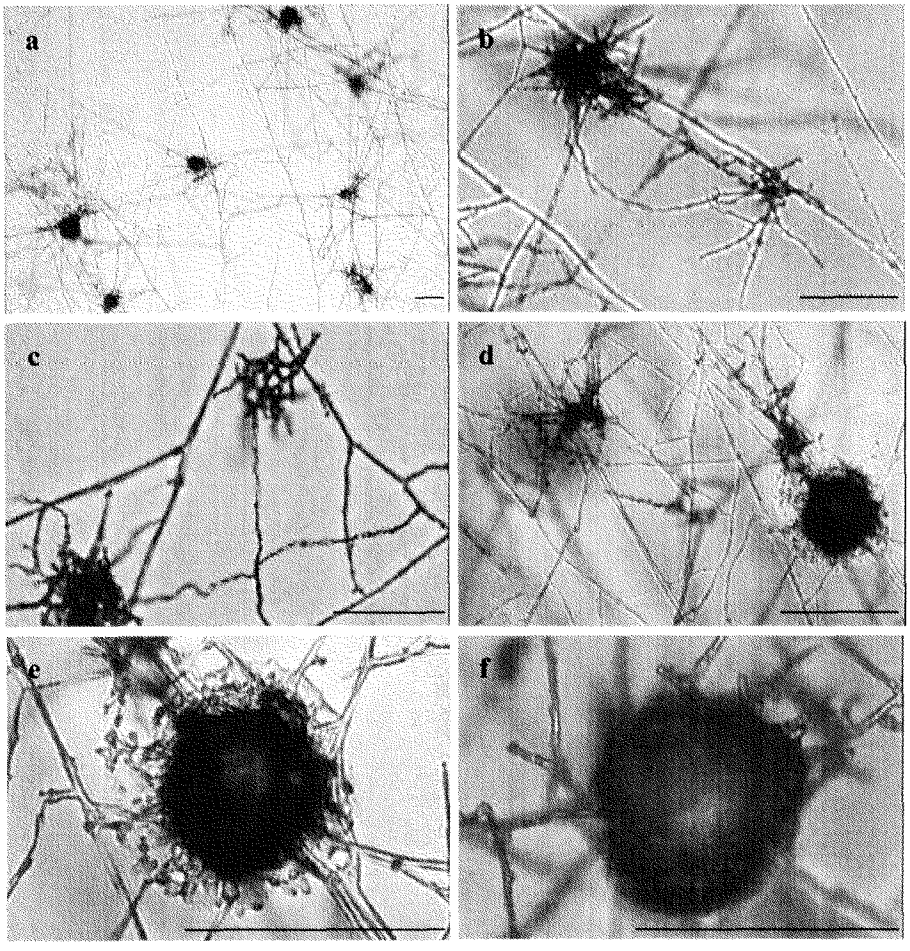

Figure 4. Mature primary hyphal knots in the aerial mycelium of homokaryon AmutBmut eventually develop into sclerotia when cultures are kept in the dark. The overview of aerial mycelium shows that primary hyphal knot formation initiates at regular distances (a). Neighboring primary hyphal knots are sometimes connected by twisted hyphae (b). Such twisted hyphae also anchor primary hyphal knots in the mycelial network (c). After two-day incubation at 37°C in the dark, primary hyphal knots and sclerotia (compact brown spherical structures) coappear in the aerial mycelium (d). The periphery of a young sclerotium consists of many short hyphae (e), which disappear over the time (f). Size bar in all pictures: 30 μm

5) When using the microscope with yellow filter to observe primary hyphal knot formation in the aerial mycelium of homokaryon AmutBmut, we noticed that after 2 days the central region of some primary hyphal knots turned into a brown compact mass (ca. 30 μm in diameter), whereas the peripheral region was still composed of short loosely arranged branches (Fig. 4d). Over the next four days, many of such brown

structures appeared in the aerial mycelium (Fig. 4e). During this time, the brown colour became darker and most of the peripheral short branches disappeared (Fig. 4f). These brown structures are known as aerial sclerotia and their formation from aerial hyphal complexes has been described before (Waters *et al.*, 1975). Genetic data have shown that sclerotia and fruiting body initiation share the same origin (Moore, 1981). It needs darkness to turn a primary hyphal knot into a sclerotium (Kües *et al.*, 1998), whereas a light signal is believed to turn a primary into a secondary hyphal knot having more compact and aggregated structure (Boulianne *et al.*, 2000; Kües, 2000) (see chapter 1). The transition from a primary into a secondary hyphal knot has not yet been described.

3.4.3 Differential expression of galectins (Cgl1 and Cgl2) during fruiting body development at standard fruiting conditions

Two galectins (Cgl1 and Cgl2) with 83% aa sequence identity were cloned and defined by their specific binding ability to β-galactoside sugars (Boulianne *et al.*, 2000; Cooper *et al.*, 1997). These two proteins can be separated by SDS-PAGE and both Cgls can be recognized by α-Cgl antiserum (Fig. 5b, lane 1) (Boulianne *et al.*, 2000). Northern hybridization and Western blot analysis showed that the *cgl* genes expressed only at a very low level in mycelium ready to initiate fruiting, but mainly transcribed in fruiting bodies. In particular, galectins were produced in great quantity during early meiotic stages and decreased to a low level at the end of meiosis (Charlton *et al.*, 1992). This differential expression pattern during fruiting body formation and meiosis prompted us to thoroughly examine the expression of galectins during the entire fruiting body developmental pathway.

When grown in the dark at 37°C for 5 days, colonies of homokaryon AmutBmut nearly reached the edge of Petri dishes. At this time point, cultures only contained undifferentiated hyphae (Fig. 5a) and no galectin was detected (Fig. 5b, lane 2). To induce fruiting body formation, 5-day old dark-grown cultures were transferred to a 12 hour-light/12 h-dark alternating regime at 25°C for further growth (Growth condition I, Table 1), corresponding to the standard fruiting conditions used in our laboratory (Granado *et al.*, 1997). At day 6 (one day after the transfer), primary hyphal knots emerged around the inoculum in the center of the plates and at the edge of Petri dishes. At day 7 (two days after transfer), numerous primary hyphal knots appeared all over the

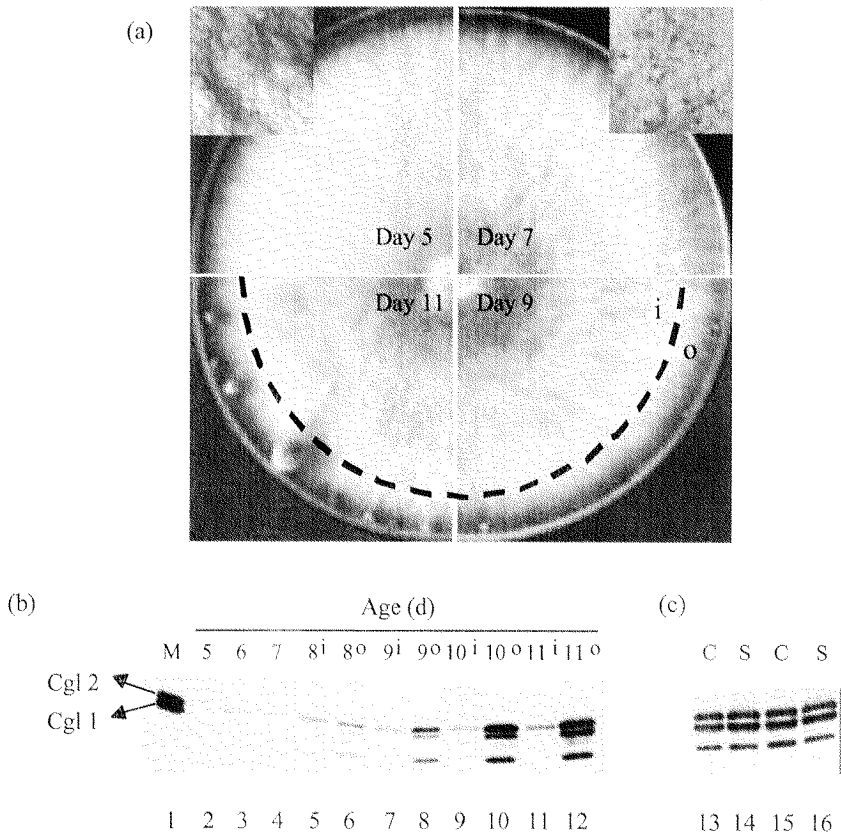

Figure 5. Western blot analysis with rabbit a-Cgl antiserum indicates that the galectin genes *cgl1* and *cgl2* are expressed during fruiting body development. (a) Homokaryon AmutBmut was grown on solid YMG/T medium at 37°C in constant dark for 5 days. Cultures were then transferred to 25°C in a 12 h alternating light/dark regime. At day 5 (top left), the mycelium did not contain any differentiated structures, whereas at day 6 and 7, primary hyphal knots were present (top right). At day 8 and 9 (bottom right), secondary hyphal knots (initials) and small primordia were seen that matured up to day 11 (bottom left) to the stage where karyogamy can be induced. Fruiting occurred primarily in an outer fruiting zone (indicated by a dashed line), whereas hyphal knot formation was observed over the whole mycelium. The insets illustrate the mycelial structure of the culture, photographed through a stereomicroscope. (b) Analysis of galectin expression. The aerial mycelium and tissues were harvested, protein extracted and separated by SDS-PAGE, transferred to nitrocellulose and probed with a-Cgl antibodies as described by Boulianne *et al.* (2000). The age of the culture is given above each lane of the Western blot and the positions of Cgl1 and Cgl2 are indicated. The total mycelium was harvested from cultures at day 5-7 (lane 2-4). For older cultures, the mycelium was separated into an inner non-fruiting zone (i, lanes 5, 7, 9, 11) and an outer fruiting zone (o, lanes 6, 8, 10, 12). Lane 1: purified galectins were applied as a marker (M). (c) Large amounts of galectins were detected in isolated cap (C) and stipe (S) tissues of maturing (lane 13, 14) and mature fruiting bodies (lane 15, 16).
A smaller protein of ca. 12 kDa was also detected in protein samples extracted from mycelium containing primordia (lane 6, 8, 10, 12) and from isolated cap and stipe tissues (lane 13-16). This small protein reacts with the horseradish-peroxidase-coupled protein A, as well as goat IgG antibody. It binds to a peroxidase-coupled Sepharose column but not a lactosyl-coupled Sepharose column, suggesting that it might be another carbohydrate-binding lectin, but not a galectin (P. J. Walser and R. C. Bertossa, personal communication).

fungal colonies (Fig. 5a), but the number of primary hyphal knots increased towards the edge of the plates and towards the inoculum in the center (not shown). Cgl2 was

detected on both day 6 and day 7 in the aerial mycelium of homokaryon AmutBmut that contained primary hyphal knots (Fig. 5b, lane 3 and 4). On day 8 and day 9, primary hyphal knots differentiated into secondary hyphal knots and small primordia (ca. 1 mm). They were exclusively found at the edge of the plates. The uneven distribution of primordia made it possible to separate the colony into an inner non-fruiting zone and an outer fruiting zone (Fig. 5a). Cgl2 was detected in the non-fruiting area. In the outer fruiting zone, not only Cgl2 but also traces of Cgl1 were found (Fig. 5b). Up to day 11, primordia mature to the stage (ca. 5 mm) when karyogamy can be induced by light (not shown). During primordia maturation, both Cgls increased their expression with proceeding time and development in the fruiting zone, whereas the amount of Cgl2 in the non-fruiting area remained low (Fig. 5b, lane 6, 8, 10, 12). On day 12, meiosis completed in the cap of primordia (now ca. 8 mm in size), resulting in the formation of young fruiting bodies with basidiospores (I, Fig. 6). Within the next 24 hours (day 13), mature fruiting bodies were formed by rapid stipe elongation and cap expansion (I, Fig. 6). When we separately examined the presence of galectins in isolated cap and stipe tissues of postmeiotic elongating young fruiting bodies (1-1.5 cm) and of mature fruiting bodies (ca. 4.5 cm), strong signals for both galectins with equal Cgl2/Cgl1 ratios were detected in all samples by Western blot analysis (Fig. 5c, lane 13-16).

Earlier Northern hybridization analysis using a wild-type dikaryon (Charlton *et al.*, 1992) showed that the transcription of *cgl* genes decreased drastically at the second meiotic division and disappeared at the end of meiosis. These data were confirmed by our Northern hybridization analysis using total RNAs of homokaryon AmutBmut (Fig. 7). *Cgl* transcripts were not detected in vegetative mycelia, not even after saturating the exposure of the hybridized blot to X-ray films (Fig. 7, lane 1). When primary hyphal knots were formed in the aerial mycelium (7 days after inoculation), a very weak *cgl* signal was obtained after over-exposure (Fig. 7, lane 2 in the lower panel). Strong signals were detected in total RNAs isolated from 1 mm-sized primordia (Fig. 7, lane 7). Higher amounts of *cgl* transcripts were detected in 5 mm-sized primordia (prekaryogamy, Fig. 7, lane 8) and the *cgl* transcripts were most abundant in primordia of 8 mm in size (meiosis, Fig. 7, lane 9). The *cgl* transcripts became undetectable in maturing fruiting bodies of 1-1.5 cm in length, corresponding to the end of meiosis (Fig. 7, lane 10). Likewise, *cgl* transcripts were not present in mature fruiting bodies (Fig. 7, lane 11). In addition, *cgl* transcripts were not detected in senescent mycelium containing sclerotia (Fig. 7, lane 3), nor in mycelium exposed constantly to light during and after

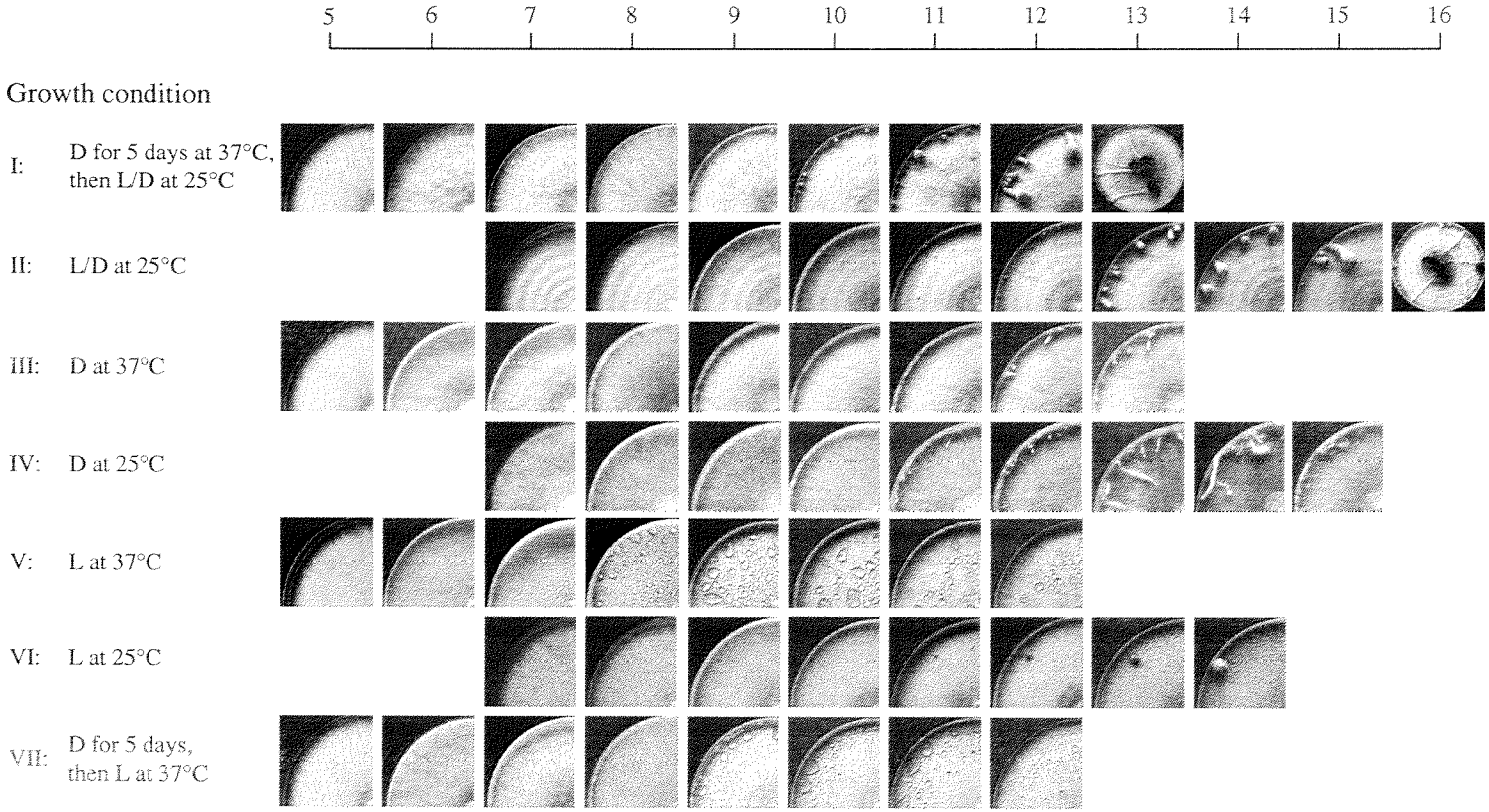

Figure 6. Morphological differences and developmental processes of homokaryon AmutBmut when grown at different conditions (I to VII, see Table 1). Numbers on the top lane indicate days after inoculation. Under growth conditions I, II and VI, normal primordia developed, but only in growth conditions I and II they matured into fully developed fruiting bodies. In contrast, under growth condition III and IV, "etiolated stipes" with underdeveloped caps were formed. Such "etiolated stipes" enlarged in length over the time by proliferation of stipe cells. No fruiting body development occurred under growth conditions V and VII.

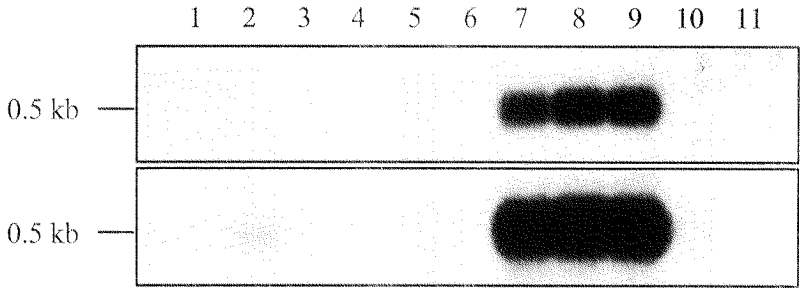

Figure 7. Transcription of *cgl* genes under different growth conditions and in different developmental stages. Total RNAs were extracted from 1) purely vegetative mycelium of homokaryon AmutBmut grown at 37°C in the dark for 4 days; 2) mycelium grown at 37°C in the dark for 7 days that contained primary hyphal knots; 3) mycelium grown at 37°C in the dark for 10 days containing sclerotia; 4) mycelium grown at 37°C in constant light illumination for 7 days without any differentiated structure; 5) vegetative mycelium grown at 37°C in the dark for 5 days, then exposed to light at 37°C for another day; 6) vegetative mycelium grown at 37°C in the dark for 5 days, then exposed to light at 37°C for another 2 days; 7) isolated primordia sized of 1 mm; 8) isolated primordia sized of 5 mm (in the prekaryogamy stage); 9) isolated primordia sized of 8 mm (in meiosis); 10) isolated young postmeiotic fruiting bodies of 1-1.5 cm in length and 11) isolated mature fruiting bodies of ca. 4.5 cm in length. Total RNAs were separated on a 1.2% formaldehyde-agarose gel, transferred to Hybond-N nylon membrane (Amersham life science) and hybridized with a ^{32}P-labeled 1 kb *Bgl*II-*Nhe*I DNA fragment of plasmid pCGLI-6R (containing gene *cgl1*). To visualize the *cgl* transcripts, the blot was exposed to X-ray films for 2 hours (upper panel) and for 13 hours (lower panel).

the vegetative growth phase (Fig. 7, lane 4-6). This transcription profile enforces once again the relationship between galectin expression and early stages of fruiting body development.

Taken all results together, we found that the onset of *cgl2* and *cgl1* expression on both transcription and protein level occurred synchronously to the formation of primary and secondary hyphal knots, respectively. Both Cgls were expressed in young fruiting structures and their expression increased with the progression of fruiting body development up to the stage of meiosis. The transcription of *cgl* genes stopped at the end of meiosis. However, because of a high protein stability, large amounts of Cgl1 and Cgl2 were still present in postmeiotic and mature fruiting bodies. The abundant amount of galectins in these later structures suggests that these proteins are generally very stable.

3.4.4 Effects of light and temperature on galectin expression in relation to fruiting body formation

The development of fruiting bodies in *C. cinereus* is regulated by light and temperature (Kües, 2000). The formation of primary hyphal knots takes place in the

dark at both 37°C and 25°C, but it is repressed by light at 37°C (Kües *et al.*, 1998). Following primary hyphal knot formation, the light-induced development of mature fruiting bodies was only reported to occur at lower temperature such as 25°C. Light is essential for the formation of secondary hyphal knots and the differentiation of cap and stipe tissues within the primordia, especially for the differentiation of the hymenium within the cap (Lu, 1974; Morimoto and Oda, 1973). The absence of light at this stages leads to the formation of so called "etiolated stipes" or "dark stipes" (Tsusue, 1969), which have a small undifferentiated cap and an elongated stipe through cell proliferation (Lu, 1974; Morimoto and Oda, 1973; Muraguchi *et al.*, 1999) (J. D. Granado, personal communication). Moreover, following the formation of basidia in the hymenial trama, light is essential for the meiotic S-phase (64–36 h before karyogamy, prekaryogamy) and higher light intensity enhances the speed of basidia entering karyogamy (Lu, 2000). The coordination between light and temperature controls karyogamy. The normal proceeding of karyogamy can be arrested by high temperature, *e.g.* 35°C. The first two hours at the beginning of the prekaryogamy stage is the light sensitive period (Lu, 1974). This arrest, however, can be overcome by either a 10 h-dark incubation or by a temperature downshift to 25° (Lu, 1972). In contrast to karyogamy, a dark phase is required for the completion of meiosis (Lu, 1974; Lu, 2000). Some *C. cinereus* strains at 28°C never proceed beyond prophase I when darkness is absent (Kamada *et al.*, 1978). When temperature is below 27°C (room temperature), even continuous light does not arrest meiosis in some strains (Lu, 2000). The later developmental processes including basidiospore formation, cap expansion, stipe elongation and cap autolysis are light independent (Kües, 2000). Since light and temperature so critically control fruiting body development, and since galectin expression was shown to be fruiting body specific, we asked how variations of light and temperature conditions during cultivation could influence galectin expression.

Varying temperature conditions: as in growth condition I (Fig. 5 and 6), homokaryon AmutBmut readily developed fruiting bodies in growth condition II (alternating 12 h L/D cycle at 25°C) (Fig. 6). The difference between these two fruiting permissive conditions lies in the temperature, at which vegetative growth takes place. Vegetative growth was slower at 25°C. In consequence, it took 7 days for the fungal colonies to reach the edge of Petri dishes. At this time point, the colony had a ripple-like appearance that was visible even after the formation of mature fruiting bodies (II, Fig.

6). This colony appearance resembles that described in *C. congregatus* under the same condition (Ross, 1982). In growth condition II, primordia developed at the edge of the plates as in growth condition I, but primordia also developed in areas between the edge and the inoculum. However, mature fruiting bodies appeared only at the edge (Fig. 6). When mycelium of different age was examined by Western blot analysis using α-Cgl antiserum, galectin expression followed exactly the same pattern in relation to the developmental stages reached as that seen under growth condition I (Fig. 5). Cgl2 was detected first when primary hyphal knots appeared, Cgl1 with secondary hyphal knot formation. The expression of Cgl2 and Cgl1 increased with the progress in secondary hyphal knot and primordium development (Fig. 5 and 7). The results indicate that the temperature in the range of 25°C-37°C, at which the vegetative mycelium proliferates, has no decisive influence on the later galectin expression during fruiting body formation.

To further test the influence of temperature on galectin expression, homokaryon AmutBmut was grown in the dark (light-proof ventilated boxes) either at 37°C (growth condition III) or at 25°C (growth condition IV). The difference between these two growth conditions was only the temperature and this affected the growth rate of fungal colonies. Major differences in respect of colony morphology and general fungal development including galectin expression were not observed (Fig. 6). Primary hyphal knots emerged first in the aerial mycelium after 5 and 7 days in growth condition III and IV, respectively. Like under fruiting permissive conditions (I and II), low amounts of Cgl2 were synchronously detected at these time points of primary hyphal knot formation. In the following 2-5 days, additional primary hyphal knots were formed and Cgl2 expression increased (Fig. 8). Possibly due to a minor leakage of light - a pinhole in the boxes is sufficient to induce (Lu, 1974) - "etiolated stipes" developed at the edge of Petri dishes in the last few days under both growth conditions (III and IV, Fig. 6). Strong Cgl2 and weak Cgl1 signals were detected at both temperatures in mycelium samples containing "etiolated stipes" from outer edges, as wells as in samples collected from the inner parts of the plates containing primary hyphal knots but being free of "etiolated stipes" (Fig. 8). The Cgl2/Cgl1 ratio detected at the stage of "etiolated stipes" formation was similar to that obtained in 1 mm-sized primordia formed under fruiting permissive conditions (I and II, Fig. 5 and Fig. 8). Such ratio remained constant even when the development of "etiolated stipes" proceeded (III and IV, Fig. 8), indicating

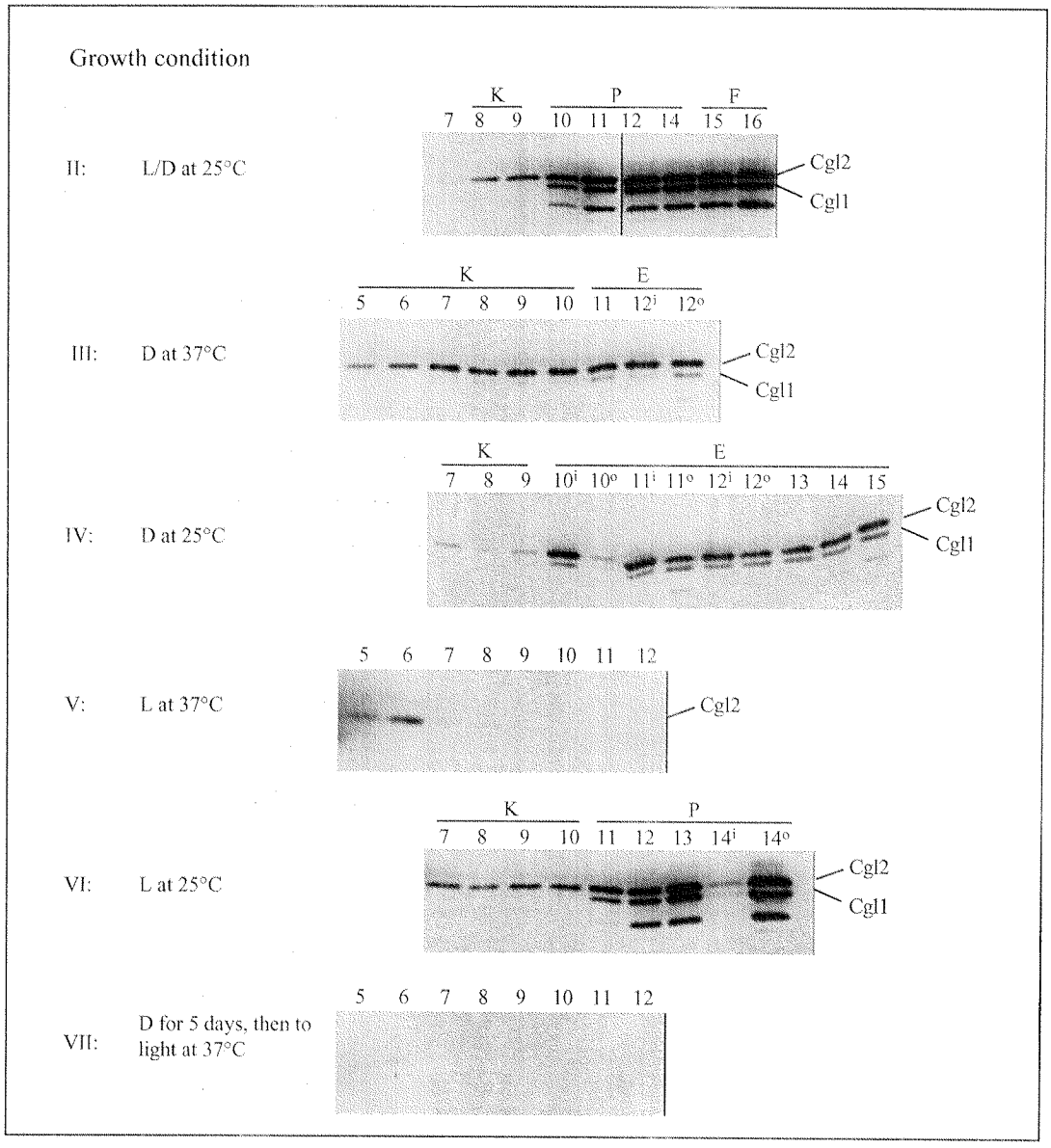

Figure 8. Galectin expression profile of homokaryon AmutBmut under different growth conditions (see Table 1). The appearance of cultures at different growth conditions and different days is shown in Figure 5 (for comparison, galectin expression in growth condition I can be found in Fig. 5). Numbers above each lane denote the day on which the vegetative mycelium or mycelium containing primary hyphal knots (K), secondary hyphal knots and primordia (P), maturing or mature fruiting bodies (F) and "etiolated stipes" (E) were collected. When fruiting structures (P,F,E) occurred at the edge of a Petri dish, the fungal colony was separated into an inner non-fruiting zone (i) and an outer fruiting zone (o). Proteins were extracted and galectins were detected by Western blot analysis. The third nonlabeled band detected in some of the samples does not represent a galectin (for further explanations see the legend of Figure 4). Note that growth condition II, III, IV and VI were exposed to X-ray films for 1-5 seconds, whereas the exposure time for blots of conditions V and VII was 2 minutes to detect any trace of Cgl proteins. The same protein marker as in Fig. 5 was applied on all SDS-polyacrylamide gels for comparison between different blots (not shown).

that the increase in Cgl1 expression in normal fruiting body development (II, Fig. 8) links to the time point at which hymenium differentiates.

Immuno-localization studies showed that galectins are only poorly expressed in the hymenium, but mainly in the outer stipe and veil tissues (Boulianne *et al.*, 2000). Hymenium differentiation runs in parallel to the enlargement of primordia, which is mostly due to an increase in sizes of already existing cells (Kües, 2000; Lu, 1974; Moore, 1998). During the rapid stipe elongation and cap expansion at later stages of fruiting body development cell volumes increase up to 100 folds, which in turn generates tension stress on the stipe-gill conjunction. To maintain the compact aggregated structure during primordium maturation and to avoid any rupture within the structure, the outer stipe and cap tissues especially have to endure such strong forces (Boulianne *et al.*, 2000; Kamada, 1994; Moore, 1998). Based on their functions in cell-cell interaction in animal systems (Perillo *et al.*, 1998) and their synchronous expressions to the early stages of fruiting body development (Fig. 5 and 7), it is likely that the fungal galectins also act in cell aggregation (Boulianne *et al.*, 2000). The changing of the Cgl2/Cgl1 expression ratios suggests that at the beginning of primordium development, gene *cgl2* is sufficient to provide enough protein for aggregation and maintenance of the structural integrity of the very young primordia. However, at later stages of fruiting body formation additional expression of gene *cgl1* may become necessary to quickly react on keeping the rapidly extending cell surfaces of the cap and stipe attached to each other and to resist rupture forces generated during the enlargement processes.

Varying light conditions: light influence on galectin expression was tested with cultures of homokaryon AmutBmut grown in constant light from the moment of inoculation either at 37°C (growth condition V) or at 25°C (growth condition VI). Also in the light, we observed an influence of temperature on the growth rate of fungal colonies (Fig. 6). In contrast to dark-grown cultures, primary hyphal knots were not formed at 37°C in the light. Instead, at day 7 after inoculation, liquid droplets appeared at the surface of the fungal colonies. More droplets were produced over the time (V, Fig. 6). Cgl1 expression was not observed, but traces of Cgl2 were only occasionally detected (on day 5 and day 6 after 2-minute exposure of the labelled Western blots) (V, Fig. 8). This result confirmed earlier observations by Kües *et al.* (Kües *et al.*, 1998) and Boulianne *et al.* (Boulianne *et al.*, 2000) that light represses primary hyphal knot formation and galectin expression, but it also showed that the light repression is not always 100% absolute.

Interestingly, development of homokaryon AmutBmut in constant light at 25°C (growth condition VI) was different from that in constant light at 37°C (growth condition V). In condition VI, primordia sporadically developed randomly on the plates. These primordia did not develop into mature fruiting bodies (Fig. 6). Following the development pattern until the stage of primordium formation, the galectin expression profile in condition VI was identical to that in the fruiting permissive growth conditions I and II (Fig. 5 and 7). The occurrence of fruiting initiation and galectin expression in constant light at 25°C but not at 37°C indicates that light is less effective on both events at 25°C. It should be noted that Lu previously reported fruiting body development on a wild-type dikaryon of *C. cinereus* in constant light at 25°C but not at 37°C (Lu, 1974).

In the last set of experiments (growth condition VII), homokaryon AmutBmut was first grown in the dark at 37°C for 5 days until the mycelium covered the entire plates, then transferred to constant light at 37°C. At the point of transfer, these cultures consisted of vegetative fluffy mycelium but without any primary hyphal knot (note that primary hyphal knot formation usually starts at the end of day 5 or the beginning of day 6, when homokaryon AmutBmut is grown on YMG/T plates at 37°C in the dark). Later, when kept in light, their morphology resembled that of cultures incubated in the constant light at 37°C directly after inoculation (V, Fig. 6). Liquid droplets were produced on the surface of the colonies on day 9 under growth condition VII, but to less extent than that produced under condition V (Fig. 6). Neither primary hyphal knots nor galectin expression was detected even after over exposure (VII, Fig. 8), indicating that light repression is effective on already established vegetative mycelium.

3.4.5 Mutant analysis shows that galectin (Cgl2 and Cgl1) expression is independent of primary and secondary hyphal knot formation

The initial processes of fruiting body development, such as the formation of primary hyphal knots, secondary hyphal knots and primordia, are interactions between hyphae. The coinciding occurrence of these early structures and the differential expression of the two galectins (Cgl1 and Cgl2) implies the involvement of galectins during fruiting body initiation and primordium maturation, possibly in hyphal-hyphal interaction (Boulianne *et al.*, 2000) (see above). To further emphasize the relationships between galectin expression and cellular events in early fruiting body development, we examined the galectin expression in 50 different mutants of homokaryon AmutBmut. These mutants were defective in fruiting initiation either at the stage of primary hyphal knot formation

(*pkn*, 10 strains, see chapter 2) or at the transition to secondary hyphal knot formation (*skn*, 40 strains, see chapter 2). All mutants were subjected to three different growth conditions: i) constant dark at 37°C for 7 days, ii) constant light at 37°C for 7 days or iii) 12 h-light/12 h-dark rhythm at 25°C for 13 days.

In a general overview, mutants were very variable in respect to galectin expression at these growth conditions (see Table 2 and Appendix for detailed results). The expression patterns of Cgl1 were more difficult to analyze than those of Cgl2, in accordance to the normal expression of gene *cgl1* at later stages in fruiting body development (Fig. 5 and 9), which none of the mutants was able to reach. 27 of the mutants (8 *pkn* and 19 *skn* mutants, see Table 2 and Appendix) never produced Cgl1 in all tested conditions. Notably, in two *skn* mutants (strains 3-020 and 6-529) Cgl1 was well expressed at 37°C in the dark but not in the light (see below). Moreover, in two *pkn* mutant (strains 6-536 and B-2641) and 5 *skn* mutants (strains 6-031, B-0659, B-0724, B-1995 and B-2798), reasonable amounts of Cgl1 were detected when grown at 37°C in the dark and/or in the light, indicating that neither primary nor secondary hyphal knot formation is prerequisite for Cgl1 expression. Some of the remaining mutants expressed Cgl1 in traces at 37°C in the dark, like homokaryon AmutBmut, while others produced traces of Cgl1 in the light (see Appendix).

Like the expression of Cgl1, production of Cgl2 can occur independently from primary hyphal knot formation. When grown at 37°C in the dark, 6 *pkn* mutants (strains 6-536, B-0016, B-1057, B-1856, B-2054 and B-2641) had a galectin level comparable to that of homokaryon AmutBmut at the stage of primary hyphal knot formation (see Appendix). In contrast, 4 *pkn* mutants (strains 3-127, 7-K11, B-1533 and B-1977) and, most interestingly, 2 *skn* mutants (strains B-0724 and E-1025) did not produce Cgl2 at 37°C in the dark (see below), unlike the parental homokaryon AmutBmut (Fig. 9) and other mutants (Table 2). Therefore, primary hyphal knot formation can proceed without Cgl2 production or without any other galectin, since neither mutant B-0724 nor mutant E-1025 produced Cgl1 at 37°C in the dark.

In total, 44 mutants (6 *pkn* and 38 *skn*) produced Cgl2 at 37°C in the dark. 22 of the *skn* mutants had a comparable, 14 strains an increased and 2 strains a reduced Cgl2 expression, when compared to the wild-type homokaryon AmutBmut (see Appendix). In constant light at 37°C, all mutants but one (strain B-2018, see below) produced less Cgl2 than in the dark at 37°C (Table 2), showing that light repression was still functional, although it was not always as stringent as in the unmutated parental strain

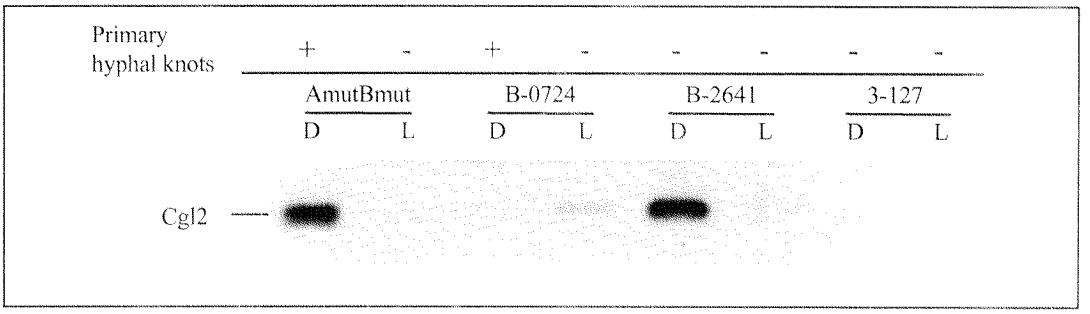

Figure 9. Cgl2 expression is independent of primary hyphal knot formation. In homokaryon AmutBmut, primary hyphal knots formed in the dark (D) and primary hyphal knot formation was repressed by light (L). Cgl2 was detected in the dark-grown cultures of wild-type homokaryon AmutBmut by Western blot analysis, but not in the light-grown cultures. Like the wild-type strain, the *skn* mutant B-0724 formed primary hyphal knots in the dark. However, Cgl2 was detected in the light-grown but not in the dark-grown cultures of this mutant. In the two *pkn* mutants B-2641 and 3-127, primary hyphal knots formed neither in dark-grown nor in light-grown cultures. Nevertheless, a strong Cgl2 signal was detected in the dark-grown culture of mutant B-2641, but not in the dark-grown cultures of mutant 3-127. In all cases, strains were grown at 37°C for 7 days before mycelium was harvested for Western blot analysis.

(see Appendix). Since in the wild-type homokaryon AmutBmut Cgl2 expression and primary hyphal knot formation are tightly linked and both are controlled by the same environmental (light, temperature and nutrition) and genetic regulators (*A* and *B* mating-type genes) (Boulianne *et al.*, 2000; Bottoli, 2001; U. Kües and M.J. Klaus, unpublished results), most of these *skn* mutants might have a defect that is rather specific to fruiting initiation than in one or more of the regulatory pathways - unless other developmental processes such as oidiation are also affected, as for example in mutants 7-280, B-0436 and B-2054 (see below). This view that genes specific to the fruiting pathway will be affected in the group of *skn* mutants with normal Cgl2 expression at 37°C in the dark and light finds support from molecular analysis of mutant 6-031. This mutant was shown to harbor a defect in the structural gene for a potential cyclopropane fatty acid synthase (see chapter 4).

Amongst mutants with an altered Cgl2 and/or Cgl1 expression pattern, we expect to identify genes that most likely act in regulatory pathways of fungal differentiation. The most interesting of the mutants are discussed in the following. Here, we also include our observations on galectin expression at 25°C in a 12 h-light/12 h-dark regime and on light control in oidiation [Note: For Cgl2, we found several cases where the protein level appeared to be increased at either 37°C in the dark and/or at 25°C in a 12 h-light/12 h-dark regime when compared to the stage of secondary hyphal knot formation in the parental homokaryon AmutBmut (marked as "yes, high" in the Appendix). Since

Table 2. Galectin expression in mutants defective in fruiting body initiation at 37°C in the dark and light

Mutant class	Number	Cgl2 production	Number	Cgl1 production*
pkn	10	Light repressed	1	Light repressed
			1	Light induced
			4	No Cgl1
		Light induced	1	No Cgl1
		No Cgl2	3	No Cgl1
skn	40	Light repressed	14	Light repressed
			2	Constitutive
			17	No Cgl1
		Light induced	1	No Cgl1
		Constitutive	3	Light repressed
			1	Light induced
			1	No Cgl1
		No Cgl2	1	Light induced

* Most mutants only produced traces of Cgl1 protein at 37°C in the dark and/or light.

the analysis of galectin expression is already very complex, these in principle interesting cases were left aside].

1. 3 *skn* mutants (strains 3-020, 6-500 and 6-529) were found to constitutively express Cgl2 at 37°C and 25°C, whereas *skn* mutant 6-608 expressed Cgl2 well at 37°C independently of the presence of light and to a less extent at 25°C in the 12 h-light/12 h-dark regime. All these four strains obviously lost the light control in galectin expression. However, their defects are unlikely situated directly in the light signalling pathway, since primary hyphal knots of these mutants formed in the dark but not in the light. In addition, all mutants still produced oidia in a light-dependent manner, like the wild-type homokaryon AmutBmut (Kertesz-Chaloupkova *et al.*, 1998). Two of the four mutants, 3-020 and 6-529, were also special in terms of Cgl1 expression due to an unusual Cgl1 production at 37°C in the dark. From data obtained from the wild-type homokaryon AmutBmut (Fig. 5 and 7), we expect Cgl1 to be highly expressed in the light at lower temperature (*e.g.* 25°C) and at a specific stage (hymenium differentiation) in fruiting body development (see above). With a deregulated expression of both Cgl2 and Cgl1, we probably hit (a) regulatory gene(s) acting on both galectin genes.

2. *skn* mutant B-2018 produced similar amounts of Cgl2 at 37°C in the dark and in the light, but not at 25°C in a 12 h-light/12 h-dark regime, indicating a defect in the temperature regulation pathway. As discussed about the four mutants in point 1, mutant

B-2018 unlikely has a defect directly in the light signalling pathway, since oidiation is still under light control.

3. Other candidates for defects in temperature control of Cgl2 expression are *pkn* mutant B-2054 and *skn* mutants 4-001 and 6-430. They produced Cgl2 well in the dark at 37°C but not at 25°C in the 12 h-light/12 h-dark regime. In contrast, *pkn* mutants 3-127 and B-1977 did not produce any Cgl2 at 37°C in the dark or light, but were able to do so at 25°C under fruiting conditions. With the exception of *pkn* mutant B-2054 having a constitutively low oidia production, light control in oidiation in other four mutants were normal.

4. Surprisingly, *pkn* mutant B-1533 did not produce Cgl2 at 37°C in the dark, but produced considerable amounts of Cgl2 in constant light and at 25°C under fruiting conditions. Thus, the effect of light on Cgl2 expression seems to be reversed in this mutant. Despite of forming primary hyphal knots, *skn* mutant E-1025 also did not produce Cgl2 at 37°C in the dark but expressed traces of Cgl2 at in the light and at 25°C under fruiting conditions. Therefore, these two strains might belong to the same class of mutants. Light control on oidiation was normal in both strains.

5. *skn* mutant B-0724 did not produce Cgl2 in all conditions, suggesting that the *cgl2* gene is not expressed. It cannot be excluded that this strain has a defect in the *cgl2* structural gene, since Cgl1 is expressed, albeit in a deregulated way. Low amounts of Cgl1 were detected at 37°C in the light and at 25°C under fruiting conditions.

6. *pkn* mutant 7-K11 produced neither Cgl1 nor Cgl2 under all growth conditions, implying that both *cgl* genes are not expressed. Unless this strain carries a large deletion on a DNA fragment carrying both *cgl* genes [they are arranged in tandem in the genome of *C. cinereus* (Boulianne *et al.*, 2000)], this mutant is a potential candidate for identifying a central regulator of galectin and primary hyphal knot formation.

7. *pkn* mutant 6-536 and *skn* mutants 7-280 and B-2798 constitutively expressed Cgl1 under all growth conditions. In strain 6-536 and B-2798, Cgl2 expression and oidiation were still under normal light control. In contrast, mutant 7-280 had a low constitutive oidia production and Cgl2 expression was only slightly repressed by light at 37°C, suggesting that this mutant might have a defect in light regulation of developmental processes.

3.4.6 General conclusion: galectins can serve as molecular markers for studying fruiting body initiation

The initial steps in fruiting body development in *C. cinereus* are poorly understood, despite the earlier histological and morphological studies on primary and secondary hyphal knot formation conducted by Matthews and Niederpruem (Matthews and Niederpruem, 1972; Matthews and Niederpruem, 1973). Primary and secondary hyphal knots, dark and light respectively dependent structures as defined in this work (see chapter 1), were formerly referred to as hyphal knots and initials (Kües, 2000). In this study, we observed both monocentric and polycentric modes of primary hyphal knot formation in confined areas within the aerial mycelium. Formation of short hyphae by intense localized branching is the main principle in primary hyphal knot formation. Two genes, *cgl2* and *cgl1*, were transcribed and translated synchronously to the formation of primary and secondary hyphal knots, respectively. An increasing gene expression coincided with the development of primordia up to the premeiotic stage. Vegetative mycelium was devoid of any *cgl* gene expression (this study) (Boulianne *et al.*, 2000; Charlton *et al.*, 1992). The temporal and spatial expression patterns not only strongly suggest the involvement of galectins in fruiting body development, but, as stated already in the work of Boulianne *et al.* (Boulianne *et al.*, 2000), they also provide us with molecular markers to follow the developmental processes during the initial steps of fruiting, Cgl2 for primary hyphal knot formation and Cgl1 for secondary hyphal knot development, respectively.

Cellular processes during fruiting body initiation are controlled by various environmental factors (Kües, 2000). Of these, temperature and light were found most important (Elliott, 1994; Lu, 1974). Concluding from various tested growth conditions, the results obtained in this study reinforced that fruiting body development is under control of a complicated interplay between light and temperature (Elliott, 1994; Lu, 1974). The formation of primary hyphal knots is repressed by light at 37°C (Kües *et al.*, 1998) (this study), as well as Cgl2 expression (Boulianne *et al.*, 2000) (this study). Lower temperature such as 25°C can override the light repression on Cgl2 expression and on fruiting initiation (Lu, 1974) (this study). Hymenium differentiation is light induced (Lu, 1974). Since little Cgl1 protein was found in the dark-grown etiolated stipes having no differentiated hymenium, it is likely that also the Cgl1 expression is light induced. With the two galectins as a read-out system, it will be possible to access the different signaling pathways employed by environmental factors to control fruiting

body initiation at a molecular level. As a first step in this direction, galectins were used to characterize defects in mutants blocked in fruiting body development either at the stage of primary or at the stage of secondary hyphal knot development.

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3.1 Appendix

Table 3. Galectin expression in fruiting body initiation mutants of *C. cinereus* homokaryon AmutBmut[1]

Strain	Genotype[2]	Cgl2			Cgl1			Light induced oidiation[3]
		37°C D	37°C L	25°C D/L	37°C D	37°C L	25°C D/L	
Cgl2 expressed in the dark and repressed by light at 37°C, highly expressed during primordium development at 25°C D/L Cgl1 expressed in traces in the dark and repressed by light at 37°C, highly expressed during primordium development at 25°C D/L								
AmutBmut	wt	yes	no (trace[4])	yes, high	trace	no	yes, high	yes
Cgl2 expressed in the dark and under fruiting conditions, repressed by light Cgl1 (poorly) expressed in the dark, repressed by light								
5-057	*skn*	yes	no	yes	trace	no	no	yes
B-0134	*skn*	yes, high	trace	yes	trace	no	no	yes, low
E-1686	*skn*	yes, high	trace	yes	trace	no	no	yes
B-1995	*skn*	yes, high	trace	yes	yes, low	no	no	yes
B-2641	*pkn*	yes	trace	yes, low	yes, low	no	trace	yes
6-541	*skn*	yes	no	yes, low	trace	no	no	yes
B-2404	*skn*	yes	no	yes, low	trace	no	no	yes
Cgl2 expressed in the dark and under fruiting conditions, repressed by light Cgl1 expressed in all conditions at a low level								
B-2798	*skn*	yes, high	trace	yes, low	yes, low	yes, low	yes, low	yes

Cgl2 expressed in the dark but not or only in traces in the light and under fruiting conditions
Cgl1 expressed in traces in the dark but not in the light and under fruiting conditions

4-001	*skn*	yes	trace	trace	trace	no	no	yes
6-430	*skn*	yes, high	no	no	trace	no	no	yes

Cgl2 expressed in all conditions, slightly repressed by light
Cgl1 (poorly) expressed in the dark, repressed by light

6-207	*skn*	yes	yes, low	yes	trace	no	trace	yes
6-625	*skn*	yes	yes, low	yes	trace	no	no	yes
B-0659	*skn*	yes, high	yes, low	yes	yes, low	trace	no	yes
6-031	*skn*	yes	yes, low	yes, low	yes, low	trace	no	yes
B-1741	*skn*	yes, high	yes, low	yes, low	trace	no	no	yes
E-0782	*skn*	yes, high	yes, low	yes, low	trace	no	no	yes, low

Cgl2 expressed in all conditions, slightly repressed by light
Cgl1 (poorly) expressed in all conditions

6-536	*pkn*	yes	yes, low	yes	trace	yes, low	trace	yes
7-280	*skn*	yes	yes, low	yes, high	trace	trace	trace	no, low

Cgl2 expressed in all conditions
Cgl1 well expressed in the dark and repressed by light

3-020	*skn*	yes, high	yes	yes, high	yes	no	no	yes
6-529	*skn*	yes, high	yes	yes	yes	no	no	yes

Cgl2 expressed in all conditions
Cgl1 poorly expressed in the dark, repressed by light

6-608	*skn*	yes, high	yes	yes, low	trace	no	no	yes

Cgl2 expressed in all conditions
Cgl1 expressed in traces in the light

6-500	*skn*	yes	yes	yes	no	trace	no	yes, low

Cgl2 not expressed in all conditions
Cgl1 expressed in the light

B-0724	*skn*	no	no	no	no	yes, low	yes, low	yes

Cgl2 expressed in the dark and under fruiting conditions, repressed by light
Cgl1 not expressed in all conditions

B-0016	*pkn*	yes	trace	yes, high	no	no	no	yes, low
5-021	*skn*	yes	no	yes	no	no	no	yes
7-297	*skn*	yes, high	trace	yes, high	no	no	no	yes, low
B-2521	*skn*	yes	trace	yes	no	no	no	yes
B-2536	*skn*	yes	trace	yes	no	no	no	yes, low
E-1593	*skn*	yes	no	yes	no	no	no	yes, low
E-2115	*skn*	yes, high	no	yes	no	no	no	yes
P-2020	*skn*	yes	trace	yes	no	no	no	yes
B-1057	*pkn*	yes	no	yes, low	no	no	no	yes
B-1856	*pkn*	yes	trace	yes, low	no	no	no	yes
B-0436	*skn*	yes	no	yes, low	no	no	no	no, low

E-1574	*skn*	yes	no	yes, low	no	no	no	yes, low
Proto-159	*skn*	yes	no	yes, low	no	no	no	yes

Cgl2 expressed in the dark and repressed by light
Cgl1 not expressed in all conditions

B-2054	*pkn*	yes	no	trace	no	no	no	no, low
7-171	*skn*	yes	trace	trace	no	no	no	yes
B-1347	*skn*	yes, low	trace	trace	no	no	no	yes
P-1429	*skn*	yes	no	trace	no	no	no	yes
E-1775	*skn*	yes	no	no	no	no	no	yes, low

Cgl2 expressed in all conditions, slightly repressed by light
Cgl1 not expressed in all conditions

7-237	*skn*	yes	yes, low	yes	no	no	no	yes, low
B-1331	*skn*	yes	yes, low	yes	no	no	no	yes
P-0678	*skn*	yes, high	yes, low	yes	no	no	no	yes, low

Cgl2 expressed at 37°C in the dark and light, but not at 25°C
Cgl1 not expressed in all conditions

B-2018	*skn*	yes, low	yes, low	no	no	no	no	yes

Cgl2 not expressed in the dark, but (poorly) in the light
Cgl1 not expressed in all conditions

B-1533	*pkn*	no	yes	yes, low	no	no	no	yes
E-1025	*skn*	no	trace	trace	no	no	no	yes, low

Cgl2 only expressed in the fruiting conditions
Cgl1 not expressed in all conditions

3-127	*pkn*	no	no	yes, low	no	no	no	yes
B-1977	*pkn*	no	no	yes, low	no	no	no	yes, low

Cgl2 not expressed in all conditions
Cgl1 not expressed in all conditions

7-K11	*pkn*	no	no	no	no	no	no	yes, low

[1] Mutants discussed in detail in chapter 3 are marked by grey shading.
[2] wt = wild-type, *pkn* = defective in primary hyphal knot formation (shaded in dark grey), *skn* = defective in secondary hyphal knot formation (mutants discussed in the text are shaded in light grey).
[3] The phenotype in oidiation is given to enable comparison of the effect of light on galectin expression and oidia production.
[4] Traces of Cgl2 were occasionally detected in the light-grown mycelium of homokaryon AmutBmut.

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Chapter 4

An essential gene for fruiting body initiation in the basidiomycete *Coprinus cinereus* is homologous to bacterial cyclopropane fatty acid synthase genes

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In preparation

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4 An essential gene for fruiting body initiation in the basidiomycete *Coprinus cinereus* is homologous to bacterial cyclopropane fatty acid synthase genes

4.1 Abstract

Homokaryon AmutBmut is a specific strain of the basidiomycete *Coprinus cinereus* that, due to mutations in the mating-type loci, produces fruiting bodies without prior mating to another strain. The homokaryon has therefore been used for creating mutants in fruiting body development. Early stages of fruiting body development include the dark-dependent formation of primary hyphal knots and the light-induced transition from primary hyphal knots to the more compact secondary hyphal knots. UV mutant 6-031 forms primary hyphal knots, but development arrests at the transition state. Genetic analysis indicates that this phenotype is caused by a single recessive defective allele (*skn1*). Using a SIB-selection transformation procedure, a cosmid that complemented the defect was isolated from a genomic DNA library. The responsible wild-type gene (referred to as *cfs1*) on this cosmid encodes a protein highly similar to cyclopropane fatty acid synthases, a class of enzymes so far characterized only in prokaryotes. The *cfs1* allele of mutant 6-031 carries a T to G transversion, leading to an amino acid substitution (Y441D) in a domain suggested to be involved in the catalytic function of the protein. The mutant allele of *cfs1* was unable to complement the fruiting deficiency in strain 6-031, indicating that this gene is essential for fruiting body initiation in *C. cinereus*.

4.2 Introduction

The heterothallic fungus *Coprinus cinereus* serves as a model organism to study fruiting body development in higher basidiomycetes. Fruiting bodies normally form on the dikaryon (Kües, 2000). However, the presence of two genetically distinct nuclei in the dikaryotic mycelium is a major drawback to perform genetic analysis on fruiting body development. The self-compatible homokaryon AmutBmut, having specific mutations in both mating-type loci, gives rise to fruiting bodies without the need to mate with another strain (Swamy *et al.*, 1984). This special feature provides us an easy accessible genetic system. A series of developmental mutants have been generated from

strain AmutBmut by UV- and REMI-mutagenesis (Granado *et al.*, 1997; U.Kües, *et al.*, in preparation), from which genes can now be isolated.

The development of fruiting bodies is a highly organized process, which requires the coordination between genetic, environmental and physiological factors. In the dark, upon nutritional depletion, hyphae locally undergo intense branching to form microscopic primary hyphal knots. Following a light signal, radial growth of primary hyphal knots and hyphal interaction lead to the formation of compact hyphal aggregates, secondary hyphal knots, which are specific fruiting body initials. Cellular differentiation within the secondary hyphal knot results in the formation of cap and stipe tissues. Such differentiated structures are termed primordia (Boulianne *et al.*, 2000; Kües, 2000; Matthews and Niederpruem, 1972). In the primordium cap, induced by a further light signal, karyogamy occurs in specialized cells (basidia). Karyogamy is directly followed by meiosis. In parallel, the stipe elongates and the cap expands, giving rise to a fully developed fruiting body (Kües, 2000; Lu, 1974; Moore *et al.*, 1979).

So far, little is known about the genetic determinants that act in fruiting body initiation and formation. Induction of primary hyphal knots and the morphological transition from primary hyphal knots into secondary hyphal knots were shown to be regulated by the *A* mating-type genes (Kües *et al.*, 1998). A gene *pcc1*, encoding an HMG-box transcription factor, likely acts downstream of the *A* mating-type gene products and appears to negatively regulate fruiting body initiation (Murata *et al.*, 1998). Onset of expression of two genes encoding fruiting body specific galectins (β-galactoside sugar binding lectins) correlates with the formation of primary hyphal knots and fruiting body initials (Boulianne *et al.*, 2000). In later stages of fruiting body development, two genes involved in cap and stipe tissue formation (Muraguchi and Kamada, 1998; Muraguchi and Kamada, 2000) and five genes acting in meiosis (Celerin *et al.*, 2000; Gerecke and Zolan, 2000; Nara *et al.*, 1999; Seitz *et al.*, 1996; Stassen *et al.*, 1997) have been identified.

Within our mutant collection derived from homokaryon AmutBmut, we identified two groups of mutants whose defects link to fruiting body initiation. Members of one group are termed *pkn* (= primary knotless) mutants, because they do not form any primary hyphal knot in the dark. The other group of mutants is arrested at the transition from primary hyphal knots to secondary hyphal knots. Therefore, they are called *skn* (= secondary knotless) mutants (U.Kües *et al.*, in preparation). In this study, we isolated a gene that complemented the defect of fruiting body initiation in the *skn* UV-mutant 6-

031. The predicted gene product is highly homologous to cyclopropane fatty acid synthases, a class of enzymes so far only characterized in bacteria.

4.3 Materials and methods

4.3.1 Fungal strains, culture conditions and transformation

Strain 6-031 (*A43mut, B43mut, pab1, skn1*) is a fruiting body initiation mutant generated from homokaryon AmutBmut (*A43mut, B43mut, pab1*) (May *et al.*, 1991) by UV-mutagenesis (U.Kües *et al.*, in preparation). Monokaryon 5401 [*A1(m), B1(m)*] has the same genetic background as homokaryon AmutBmut (Maida *et al.*, 1997). Monokaryon JV6 (*A42, B42*) is a wild-type strain unrelated to homokaryon AmutBmut (Binninger *et al.*, 1987). Strains were standardly grown at 37°C on YMG/T complete medium and minimal medium (Granado *et al.*, 1997) supplemented with p-aminobenzoate (PABA, 5 mg/l) when required. For oidia induction of mutant 6-031 and other *A43mut B43mut* strains, dark grown cultures were exposed to light for two days (Kertesz-Chaloupkova *et al.*, 1998). The number of oidia per plate was determined by a spectrophotometer as previously described (Polak, 1999). Matings were performed on YMG/T plates by placing two mycelial blocks of inoculum 5 mm apart. For growth and induction of fruiting bodies, mating plates were incubated in standard fruiting conditions (Granado *et al.*, 1997). Randomly isolated basidiospores were germinated on YMG/T medium at 37°C (Walser *et al.*, 2000). Progenies of cross 6-031 x 5401 were analyzed on minimal media for pab-auxotrophy. Presence of unfused and fused clamp cells, indicators of activated *A* and *B* mating-type pathways, respectively (Kües, 2000), was determined under a Zeiss Axiophot microscope. Frequencies of phenotypic distributions in progenies were tested by a Chi-square method. The F1 progeny of cross 6-031 x JV6 was analyzed for fruiting ability by individually inoculating clones on YMG/T agar, growing them for 4 days at 37°C in the dark and subsequently transferring them to standard fruiting conditions. Dikaryons were identified by light inducing oidia production, germinating the spores on YMG/T agar and analyzing pab-auxotrophy on minimal medium.

For DNA transformation, oidia were protoplasted and transformed as previously described (Granado *et al.*, 1997). When necessary for selecting pab-prototrophic transformants, 1 µg of plasmid pPAB1-2 (Granado *et al.*, 1997) was added to perform cotransformation. Upon germination on regeneration agar (Granado *et al.*, 1997),

transformants were individually transferred onto minimal medium for further growth. Subsequently, four individual transformants were inoculated on YMG/T agar per single Petri dish and grown in the dark at 37°C for 2 days to a colony size of 3-3.5 cm in diameter. To induce fruiting, plates were then moved to standard fruiting conditions for 2 weeks. The number and size of primordia per transformant were scored and photographed with a color chilled 3CCD Camera (Hamamatsu C5810). Digital images were processed with Adobe Photoshop 5.5. A small piece of gill tissue from primordia developed upon transformation with cosmid 40-5A was spread and stained with hematoxylin (Lu and Raju, 1970). Basidia in the stained gill tissue were examined under a Zeiss Axiophot microscope and photographed as above.

4.3.2 DNA and RNA techniques

An indexed genomic library derived from homokaryon AmutBmut (Bottoli *et al.*, 1999) was transformed into mutant 6-031 and screened for cosmids that were able to restore fruiting ability in this strain, following a SIB-selection procedure (Akins and Lambowitz, 1985). The *pab1*$^{+}$ wild-type gene of *C. cinereus* present in the cosmid backbone (Bottoli *et al.*, 1999) was used as a selection marker. Cosmid DNAs from 60 pools of each 96 microtiter dish-arranged *E. coli* clones, from subpools of each 8 clones following the rows of microtiter dish 40, and from the 8 individual clones of the fifth row in microtiter dish 40 were isolated by the method of Little (Little, 1987).

Cloning was performed by standard methods (Sambrook *et al.*, 1989). Plasmids were propagated in *E. coli* strain XL1-Blue (Stratagene). Derivative pSphA of cosmid 40-5A is a ligation product between a 16 kb *Sph*I fragment (13 kb genomic DNA + 3 kb cosmid backbone) and a 7.5 kb *Sph*I fragment (2 kb genomic DNA + 5.5 kb cosmid backbone) in their natural order. *Not*I fragments of cosmid 40-5A were cloned into the *Not*I site of pBC SK (+) (Stratagene) and tested for transformation activity in mutant 6-031. The positive plasmids pNotB5 and pNotB7 contained the same DNA insert but in opposite orientation. The insert in pNotB5 was sequenced on both strands by primer walking (Microsynth, Switzerland). Sequences were assembled with program DNASTAR and analyzed with OMIGA 2.0, BLAST (NCBI). The whole sequence of 10526 bp was submitted to GeneBank (accession number AF338438). Note that 32 bp of the *Not*I fragment originated from the linker of the cosmid backbone and therefore was not included in this sequence. The general codon usage of *C. cinereus* was

determined from 70 complete protein coding genes comprising 31276 codons (Codon Usage Database at Kazusa DNA Research Institute, Japan).

pNotB5 and pNotB7 were used to construct the following subclones in pBC SK (+): p5SmaCS and p5BamCS carry gene *arf1* on a 1.4 kb *Not*I-*Sma*I and a 3.5 kb *Not*I-*Bam*HI fragment, respectively. p5EcoCS and p5XbaCS contain *arf1* and a truncated *cfs1* gene on a 3.8 kb *Not*I-*Eco*RI and a 5.5 *Not*I-*Xba*I fragment, respectively. p5SpeCS includes both *arf1* and *cfs1* on a 7 kb *Spe*I fragment. p7XbaCS carries truncated *cfs1* and *kin1* copies on a 5 kb *Xba*I-*Not*I fragment. p7SpeCS contains a truncated *kin1* on a 3.5 kb *Spe*I-*Not*I fragment. Subclones constructed in pBluescript KS (-) (Stratagene) were as follows: pPvu8.5 contains a 8.5 kb *Pvu*II fragment covering the complete *cfs1* gene and the 3' end of *kin1*. pBam3.5 and pSmaSpe5.5 carry *cfs1* on a 3.5 kb *Bam*HI and a 5.5 kb *Sma*I-*Spe*I insert, respectively. pEco4.4 contains truncated *cfs1* and *kin1* copies on a 4.4 kb *Eco*RI fragment. Furthermore, the 8.5 kb *Pvu*II fragment, 3.5 kb *Bam*HI fragment and 4.4 kb *Eco*RI fragment were also cloned into pPAB1-2 containing the *C. cinereus pab1*$^{+}$ gene, resulting in pPvu8.5-pab, pBam3.5-pab and pEco4.4-pab, respectively.

A cut-and-shut strategy using either *Nco*I or *Bst*EII and plasmid pNotB5 resulted in p5NcoCS with a deletion in *cfs1* (Δbp 5296-5476) and p5BstCS with a deletion in *kin1* (Δbp 8502-8619), respectively. An *Aat*II deletion (Δbp 6567-6803) in p5SpeCS yielded p5SpeAatCS. A *Nru*I deletion in *arf1* (Δbp 676-972) in p5SmaCS gave rise to p5SmaCSΔ*arf*, from which the *Sma*I insert was cloned into pSamSpe5.5 to generate p5SpeCSΔ*arf*. The insertion of a 3.5 kb *Spe*I fragment from pNotB7 at the *Spe*I site in p5SpeCSΔ*arf* resulted in pNotB5Δ*arf*. The T to G transversion found in the *cfs1* allele of mutant 6-031 was introduced into plasmid p5SpeCS by exchanging a 1 kb PCR amplified *Stu*I–*Nde*I fragment with the wild-type sequence, giving rise to p5SpeCS/6-031. Plasmid pNotB5/6-031 distinguishes from pNotB5 by the same T to G transversion.

Genomic DNA of *C. cinereus* strains was isolated from powdered lyophilized mycelium (Zolan and Pukkila, 1986). Two overlapping fragments containing the *cfs1* allele of mutant 6-031 were six times independently amplified from genomic DNA with specific primers (a 3.1 kb fragment using primers 5'TCAAGTCGGGTCGGTAGAAG3' and 5'TTTGTTTCGGAGCTTGACTG3' and a 1.1 kb fragment using primers 5'GGACGCTTCAAGATTAGATC3' and 5'CTCTGAAGGAATCGCTCTTG3') and sequenced using a ABI PRISM DNA

Sequencing Kit and a Model 373A DNA sequencer (Perkin-Elmer). Sequences of PCR products separately amplified with the same primer set were identical.

Southern blot analysis was performed with 10 µg of genomic DNA per sample following basic protocols (Sambrook *et al.*, 1989). Total RNA of strain AmutBmut was extracted with a guanidinium isothiocyanate procedure (Chomczynski and Sacchi, 1987) from powdered lyophilized *C. cinereus* mycelia or tissues of different fruiting stages. poly(A)$^+$ RNA was isolated with the Oligotex mRNA Midi kit (Qiagen). Per sample, 10 µg of total RNA or 2.5 µg of poly(A)$^+$ RNA were used for Northern blot analysis (Ausubel *et al.*, 2000). Hybridization signals in Southern and Northern blot analysis were produced with DNA fragments labeled with [α-^{32}P]dCTP (specific probe activity: 10^8 cpm/µg DNA) by random primed DNA labeling (Boehringer Mannheim).

The 5' and 3' cDNA ends of the *cfs1* gene were determined with the 5'/3' RACE Kit of Roche Molecular Biochemicals following the instructions of the manufacturer. poly(A)$^+$ RNA isolated from 5 mm-sized primordia was used for cDNA synthesis. In the 5' RACE, a *cfs1* specific primer sp1 (5'ACAATGCACAGGAGTACATC3') was employed to synthesize the first strand cDNA. Two *cfs1* specific primers, sp2 (5'GCAATGGCATTGAGTCGAG3') and sp3 (5'TAGACGATAGGGTCATCTCC3'), were applied in subsequent PCR reactions. In the 3' RACE, two *cfs1* specific primers, sp4 (5'GATTTTGCCCTCAAGCCAC3') and sp5 (5'CAATTCGAGCCTGCCCAG3') were used. RACE products were cloned into pBluescript KS (-) by T/A cloning (Marchuk *et al.*, 1991) and sequenced as above with a Model 373A DNA sequencer.

4.3.3 Computer analysis of protein sequences

Proteomics tools provided by ExPaSy Molecular Biology Server (Swiss Institute of Bioinformatics, Geneva) were used to perform protein pattern and profile searches (InterPro), transmembrane region detection (TMpred and TMHMM) and secondary structure predication (PSA and PSIpred). Hydrophilicity profile was calculated with Goldman/Engelman/Steitz parameters in OMIGA 2.0.

4.4 Results

4.4.1 Mutant 6-031 carries a single recessive defective allele in secondary hyphal knot formation

UV-mutant 6-031 has a growth rate (8 mm/day on YMG/T agar at 37°C) and a mycelial morphology indistinguishable from its progenitor strain AmutBmut. Like homokaryon AmutBmut (Kertesz-Chaloupkova *et al.*, 1998; Swamy *et al.*, 1984), the mutant forms fused clamp cells at the hyphal septa and produces ca. 10^9 oidia/plate in a light-dependent manner, indicating that mating-type genes in mutant 6-031 are not affected. Mutant 6-031 forms primary hyphal knots in the dark that mature into sclerotia when cultures are further kept in the dark. However, primary hyphal knots do not develop into secondary hyphal knots in a 12 h light/12 h dark regime at 25°C with 90% humidity (standard fruiting conditions), suggesting that mutant 6-031 has a specific defect in fruiting body initiation (*skn1*).

A cross between strain 6-031 and a closely related wild-type monokaryon 5401 gave rise to mature fruiting bodies, indicating that mutant 6-031 carries a recessive allele defective in fruiting body initiation. 97 descendants of cross 6-031 x 5401 with fused clamp cells (*A43mut B43mut* strains) were subjected to a fruiting test. 34 clones (35%) initiated fruiting body development (Liu *et al.*, 1999), but only 14 clones (14% in total and 41% of those that initiated fruiting) developed mature fruiting bodies. In the control cross AmutBmut x 5401, 66 out of 90 descendants with fused clamp cells (73%) initiated fruiting body development and 50 clones (56% in total, 76% of those that initiated fruiting) gave rise to mature fruiting bodies. The reduction by half ($p > 0.9$) of clones from cross 6-031 x 5401 initiating fruiting suggests that mutant 6-031 contains a single defect in fruiting body initiation. A more extensive analysis using the whole viable progeny of cross 6-031 x 5401 came to the same result (Liu *et al.*, 1999).

For the study presented here, it is also important to note the drastic reduction in clones from cross 6-031 x 5401 that completed fruiting body development, when compared to those from cross AmutBmut x 5401. This result points to the presence of additional defects in mutant 6-031, affecting later stages of fruiting body development. Our aim was to investigate genetic factors involved in *C. cinereus* fruiting body initiation. Since, as in the progeny of cross AmutBmut x 5401, parts of the progeny from cross 6-031 x 5401, for unknown reasons, were not viable (Liu *et al.*, 1999) and

since defects in later stages of fruiting body development are not of importance for studying fruiting body initiation, these defects were not further analyzed.

4.4.2 Identification of a cosmid able to restore fruiting body initiation in mutant 6-031

A SIB-selection transformation strategy (Akins and Lambowitz, 1985) was employed to isolate from a genomic library of *C. cinereus* monokaryon AmutBmut (Bottoli *et al.*, 1999) DNA sequences that can restore the fruiting ability of mutant 6-031. In the first round of transformations using pools of 96 different cosmids, one transformant in a total number of 7948 (equivalent to the analysis of ca. 45% of the entire library) formed primordia up to a size of 5-8 mm (Fig. 1). Pool 40 that gave rise to the positive transformant was divided into 12 subpools. In subsequent transformations, 12 out of 208 transformants of subpool 40-5 developed 5-8 mm sized primordia. In the final round of transformation using individual cosmids, 27 out of 45 tested transformants of cosmid 40-5A generated such primordia. Basidia within these primordia had either two distinct nuclei at the prekaryogamy stage (Fig. 1) or no nucleus (data not shown).

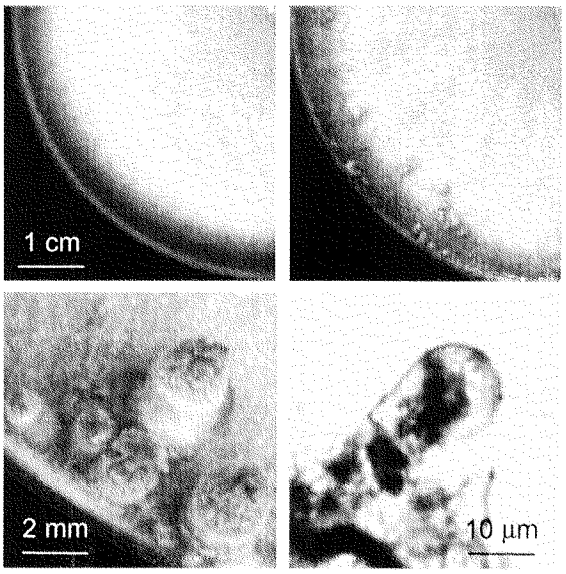
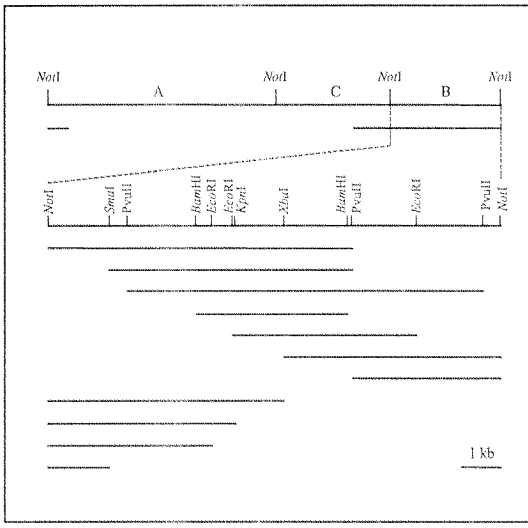


Figure 1. Cosmid 40-5A restores the defect in fruiting initiation in the *Coprinus cinereus* mutant 6-031 upon transformation. Mycelial morphology of mutant 6-031 before transformation (top left) and primordium formation after transformation (top right). Primordia of transformants are shown enlarged at the bottom left. Their basidia (bottom right) are in a stage of prekaryogamy, as indicated by the presence of the two nuclei and their positions within the basidia (Kües, 2000).

The genetic analysis of cross 6-031 x 5401 suggested that mutant 6-031 harbors defects in both early and later stages of the fruiting pathway. Therefore, it is possible that cosmid 40-5A restores only the defect in fruiting body initiation and that primordium development arrests at a prekaryogamy stage due to an additional defect in mutant 6-031 at this stage. To test this assumption, descendants from cross 6-031 x 5401 with the genotype *A43mut B43mut pab1 skn1* were identified by presence of fused clamp cells, pab-auxotrophy and inability to initiate fruiting body formation. When crossed with the parental strains, 18 out of 82 clones formed mature fruiting bodies with monokaryon 5401, but did not initiate fruiting body development with mutant 6-031. 16 of these clones were transformed with cosmid 40-5A. In all cases, transformants developed primordia, but in no case they were bigger than 5-8 mm. This result might indicate that the defect of fruiting body initiation in mutant 6-031 is closely linked to the postulated defect blocking development at the prekaryogamy stage. Alternatively, presence of a suppressor gene on cosmid 40-5A acting in initiation but not at later steps in fruiting would explain the observations.

4.4.3 Transformation activities of subclones derived from cosmid 40-5A

Cosmid 40-5A with a 40 kb sized insert of *C. cinereus* genomic DNA was digested with various restriction enzymes, and digestion mixtures were transformed into mutant 6-031. Digestions with *Bam*HI, *Not*I, *Pvu*II or *Sph*I still allowed initiation of fruiting body development in part of the transformants, unlike those with *Eco*RI, *Eco*RV, *Kpn*I, *Pst*I or *Xho*I. *Not*I divides cosmid 40-5A into three *C. cinereus* genomic DNA fragments (20, 10.6 and 8.9 kb, Fig. 2) plus an extra fragment representing the cosmid backbone (8.9 kb). *Not*I was chosen to construct pBC SK(+) subclones, which were cotransformed with plasmid pPAB1-2 into mutant 6-031. Plasmids pNotB5 and pNotB7 containing the same 10.6 kb insert fragment (*Not*I-B), restored fruiting body initiation (Fig. 2 and 3). Some subclones of this *C. cinereus* genomic fragment (p5SpeCS, pSmaSpe5.5, pPvu8.5 and pBam3.5) were also active in fruiting body initiation. However, we noticed quantitative and qualitative variations in transformation activities, related to the length of transformed DNA fragments (Fig. 2). The most effective constructs were cosmid 40-5A and its deletion derivative pSphA carrying the *Not*I-B fragment together with flanking DNA regions. 20-30 % transformants of these cosmids initiated fruitbody development and developed primordia up to 5-8 mm in size. Three plasmids (pNotB5, p5SpeCS and pSmaSpe5.5, containing a common 5.5 kb sequence)



Constructs	Positive/total transformants I	Positive/total transformants II	Primordium size
cosmid 40-5A	177 / 693	14 / 55	5-8 mm
pSphA	135 / 549	73 / 318	5-8 mm
pNotB5	80 / 832	43 / 442	3-5 mm
pNotB7	21 / 302	-	3-5 mm
p5SpeCS	7 / 201	65 / 364	2-3 mm
pSmaSpe5.5	6 / 93	29 / 248	3-5 mm
pPvu8.5	4 / 342	3 / 138	2-3 mm (deformed)
pBam3.5	8 / 721	1 / 428	1 mm
pEco4.4	1 / 332	0 / 166	3-5 mm
p7XbaCS	0 / 302	0 / 79	
p7SpeCS	0 / 129	-	
p5XbaCS	0 / 430	0 / 154	
p5KpnCS	0 / 158	-	
p5EcoCS	0 / 91	-	
p5SmaCS	0 / 120	-	

Figure 2. Identification of DNA fragments that restore fruiting body initiation in *C. cinereus* mutant 6-031. The three *C. cinereus* genomic *Not*I fragments (A,B,C) present in cosmid 40-5A are shown, as well as the length and position of subfragments present in the cosmid 40-5A derivative pSphA. The *Not*I-B fragment and subfragments were inserted into either pBC SK(+) or pBluescript KS(-). For transformation we used either 1µg of cosmid DNA or 1µg DNA of pBC SK(+) or pBluescript KS(-) based plasmids plus 1µg of pPAB1-2 for cotransformation (experiment setup I). To equalize the absolute number of DNA molecules possibly acting in fruiting body initiation, either 7 µg of cosmid 40-5A, 3.3 µg of pSphA or 1 µg/7 kb DNA of pBC SK(+) or pBluescript KS(-) based plasmids plus 1 µg of pPAB1-2 were applied (experiment setup II). Per single experiment, between 32 to 248 transformants were obtained. Since percentages of transformants initiating fruiting were comparable between different transformations of the same DNA construct (not shown), transformants of different experiments were added up. Transformation with 1 µg of pPAB1-2 and transformation with 1 µg of pPAB1-2 plus 1 µg of pBluescript KS(-) or pBC SK(+) served as negative controls. From these control transformations, a total number of 1909, 215 and 132 transformants were respectively obtained and none of them initiated fruiting body formation. -, not performed.

induced fruiting body initiation in 5-10 % of the transformants, but the primordia formed were of a maximal size of only 3-4 mm (Fig. 2 and 3). Normal cap and stipe differentiation were observed in these primordia (Fig. 3). The reduction in percentage of transformants initiating fruiting might relate to the fact that cosmids 40-5A and pSphA carry the *pab1*$^+$ selection marker, whereas the pBC SK(+) and pBluescript KS(-) constructs need to be cotransformed with the *pab1*$^+$ containing plasmid pPAB1-2. In contrast, this difference in the transformation procedure cannot account for the less developed primordia obtained when transforming with plasmids pNotB5, p5SpeCS and pSmaSpe5.5. Moreover, upon cotransformation of plasmids pPAB1-2 and pPvu8.5 (having a 5.2 kb *C. cinereus* sequence in common with pNotB5, p5SpeCS and pSmaSpe5.5), only 1% of transformants developed primordia, which were malformed and just 1-2 mm in size (Fig. 2 and 3). In these primordia, we observed gill tissue but the internal pileus trama tissue was missing (Fig. 3). Plasmid pBam3.5 with a 3.5 kb *Bam*HI fragment was the smallest construct regularly active in cotransformation, with

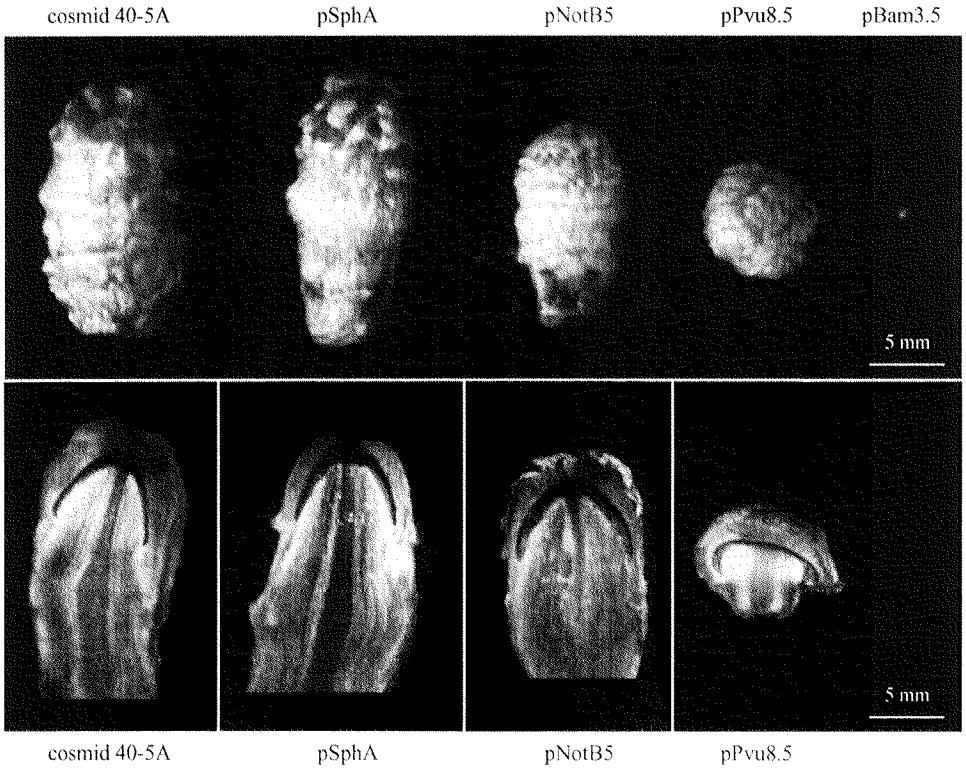


Figure 3. The morphological progress in primordia development declines when reducing the length of DNA fragments in transformation. The phenomenon is indicated by the isolated primordia formed by the cosmid 40-5A, pSphA, pNotB5, pPvu8.5 and pBam3.5 transformants (top panel). Differentiation of cap and stipe tissues are normal in primordia induced by cosmid 40-5A, pSphA and pNotB5 (lower panel). In contrast, the section through a primordium induced by plasmid pPvu8.5 shows that the internal pileus trama is missing (lower panel).

1% of transformants initiating fruiting but development arrested shortly after secondary hyphal knot formation (Fig. 2 and 3). When solely transforming constructs pPvu8.5-pab and pBam3.5-pab, containing the *pab1*$^{+}$ selection marker in addition to the 8.5 kb *Pvu*II fragment or the 3.5 kb *Bam*HI fragment, neither the transformation efficiency increased nor the primordia development improved in positive transformants obtained (not shown). The data suggest that the observed differences in transformation efficiency and degree of primordia maturation obtained with different *C. cinereus* fragments are not simply a result of variations in the transformation procedure.

The 3.5 kb *Bam*HI fragment present in pBam3.5 originated from the central region of the 10.6 kb *Not*I-B fragment (Fig. 2). In a total of 498 clones obtained from cotransformation of pPAB1-2 and pEco4.4, a partial overlapping 4.4 kb *Eco*RI fragment only gave rise to 1 transformant (0.2%) able to initiate fruiting. Two more transformants with primordia out of 200 tested clones were obtained from transforming mutant 6-031

with plasmid pEco4.4-pab, containing both the 4.4 kb *Eco*RI fragment and the *C. cinereus pab1*$^{+}$ gene. Interestingly, primordia of these three transformants developed to a size and shape comparable to that of pNotB5 (not shown). Other plasmids carrying *C. cinereus* inserts either from the flanking regions of the 3.5 kb *Bam*HI fragment or inserts splitting this fragment in half were all negative in transformation (Fig. 2).

4.4.4 The 3.5 kb *Bam*HI fragment is linked to the *skn1* mutation in strain 6-031

To understand the genetic relationship between the positive 3.5 kb *Bam*HI fragment and the fruiting initiation defect in mutant 6-031, the strain was crossed to the *C. cinereus* wild-type monokaryon JV6. These two strains have a distinct *Bgl*II restriction fragment length polymorphism (RFLP) in the DNA region covered by the 3.5 kb *Bam*HI fragment (Fig. 4), which provides a read-out system to follow inheritance of the DNA region in the progeny of cross 6-031 x JV6. 46 out of 588 randomly isolated descendants of this cross initiated fruiting body development on YMG/T medium. 41 of these clones had the RFLP pattern of monokaryon JV6 (Fig. 4). Both parental patterns were detected in the remaining 5 clones, but analysis of a mating-type linked *pab1* allele (May *et al.*, 1991) in their oidia identified them as dikaryons (data not shown). Either the JV6 or the 6-031 RFLP pattern was found in 30 randomly isolated non-fruiting clones (Fig. 4). The data suggest that the 3.5 kb *Bam*HI fragment is linked to the fruiting initiation defect (*skn1*) in mutant 6-031 and that it does not function as a suppressor but actually complements the defect *skn1*.

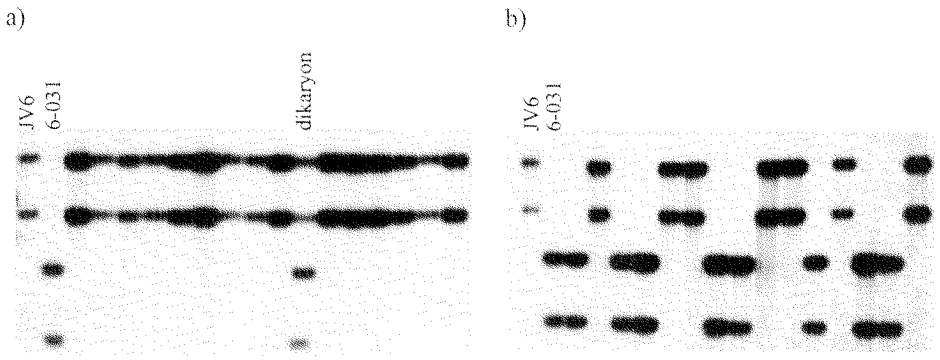
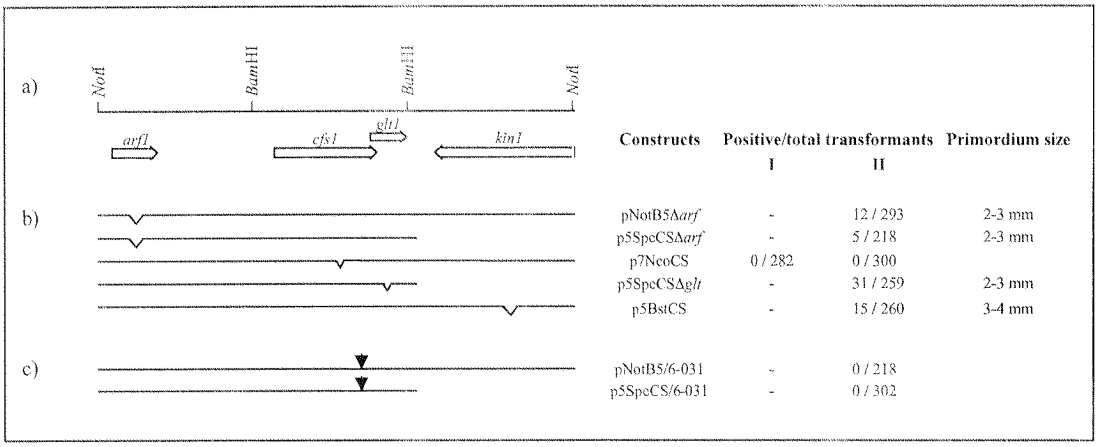


Figure 4. Inheritance of the JV6 and 6-031 *Bgl*II-RFLP patterns obtained for the 3.5 kb *Bam*HI fragment of plasmid pBam3.5 in the F1 progeny of cross JV6 x 6-031. The first two lanes shown in figures a) and b) represent the hybridization of the 3.5 kb *Bam*HI fragment to genomic DNA of monokaryon JV6 and mutant 6-031. All other lanes show the patterns of individual strains of the JV6 x 6-031 progeny. a) All clones of the progeny that initiated fruiting had the JV6 RFLP pattern, except for a dikaryon that had both parental patterns. b) The JV6 and 6-031 patterns were randomly found in clones of the progeny that were unable to initiate fruiting body formation.

4.4.5 Characterization of the 10.6 kb *Not*I-B region

The quantitative and qualitative differences in complementation activities with different plasmids led us to sequence the whole genomic *Not*I-B region, which is 10526 bp in size. A GeneBank BLAST search with this sequence revealed four potential coding regions, whose deduced protein sequences showed highest similarities to the human ADP-ribosylation factor-like protein 2 ARL2 (67% identity and 82% similarity over a length of 185 aa; accession number P36404), the cyclopropane fatty acid synthase CFA of *Escherichia coli* (32% identity and 48% similarity over a length of 367 aa; accession number P30010), the galacturonosyl transferase Cap1E in *Streptococcus pneumoniae* (32% identity and 49% similarity over a length of 91 aa; accession number L36873) and the C-terminal part of the kinesin-like protein UNC-104 in *Caenorhabditis elegans* (32% identity and 51% similarity over a length of 271 aa; accession number P23678) (Fig. 5a).



Constructs	Positive/total transformants I	Positive/total transformants II	Primordium size
pNotB5Δ*arf*	-	12 / 293	2-3 mm
p5SpeCSΔ*arf*	-	5 / 218	2-3 mm
p7NcoCS	0 / 282	0 / 300	
p5SpeCSΔ*glt*	-	31 / 259	2-3 mm
p5BstCS	-	15 / 260	3-4 mm
pNotB5/6-031	-	0 / 218	
p5SpeCS/6-031	-	0 / 302	

Figure 5. Gene *cfs1* is essential for fruiting body initiation. a) Sequence analysis of pNotB5 revealed four potential *C. cinereus* open reading frames (*arf1*, *cfs1*, *glt1* and *kin1*), of which one (*kin1*) is only partially present on the *Not*I-B fragment. b) Plasmids containing a deletion (Δ) in *arf1*, *glt1* and *kin1* still permitted initiation of fruiting body formation when transformed into mutant 6-031. Positive transformants developed 2-4 mm primordia with a normal morphology. In contrast, a deletion in *cfs1* abolished the ability to induce fruiting body formation. c) A T to G transversion (arrow) was found in the *cfs1* allele of mutant 6-031. Plasmids carrying this point mutation did not activate fruiting body formation in mutant 6-031. Conditions for experimental setups I and II were as described in Fig. 2. -, not performed.

A transcription analysis of the entire 10.6 kb *Not*I-B fragment detected three transcripts (Fig. 6), corresponding in location to the deduced coding regions for the ARL2-like protein (gene *arf1*), for the potential CFA (gene *cfs1*) and for the UNC-104 like kinesin (gene *kin1*). Weak transcripts for *arf1* were detected in Northern blots of total RNA. When using poly(A)$^+$ RNA to increase the sensitivity, transcripts for *arf1*,

cfs1 and *kin1* were well detected but we never observed a transcript specific to the region translating into a Cap1E-like sequence (putative gene *glt1*).

The *arf1* transcript is about 0.58 kb in size (Fig. 6b). *arf1* is expressed in all developmental stages tested (mycelia grown in constant dark and constant light, dark-grown mycelium containing primary hyphal knots, primordia with a size of 1 mm and 1 cm) at similar level, but decreases slightly at the stage of primary hyphal knot formation (Fig. 6c). The expression of the 1.75 kb sized *cfs1* transcript (Fig. 6b) is poor in the dark

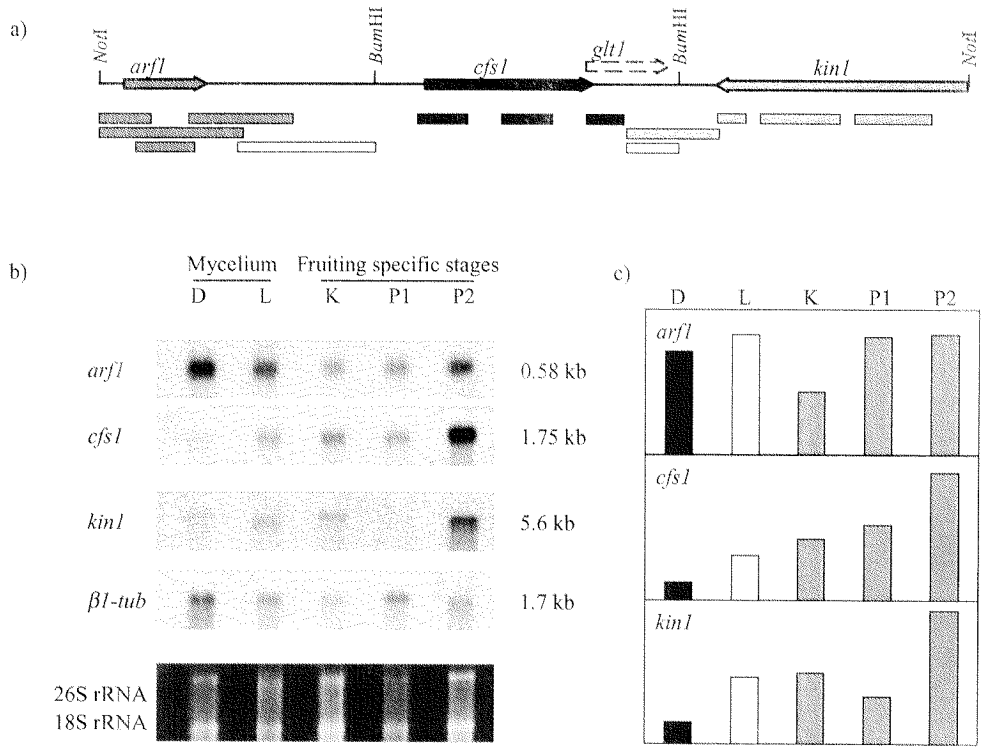


Figure 6. Transcription profile of the *Not*I-B region. a) Three transcripts, *arf1*, *cfs1* and *kin1* (represented by hatched, black and dotted arrows, respectively) were detected in Northern blot analysis by various DNA probes (shown as hatched, black and dotted boxes, respectively). The arrow head indicates the direction of transcription. No signal was detected with two probes (open boxes) in the intergenic *arf1-cfs1* and *cfs1-kin1* regions. Since no specific transcript was detected, the putative gene *glt1* is indicated by a dashed arrow. b) Gene expression was analyzed with poly(A)$^+$RNA isolated from vegetative mycelium grown on YMG/T agar at 37°C for 4 days in the dark (sample D) or 7 days in the light (sample L). Letter K denotes RNA isolated from cultures grown for 6 days at 37°C in the dark that developed numerous primary hyphal knots within the aerial mycelium. Letter P1 and P2 indicate RNA extracted from isolated 1mm sized primordia at a prekaryogamy stage and from isolated 1 cm sized primordia undergoing meiotic divisions, respectively. Primordia were harvested at day 3 (P1) and day 5 (P2) after fully grown YMG/T cultures were incubated at 25°C under standard fruiting conditions. 26S and 18S rRNA were shown to indicate the quality of poly(A)$^+$RNA samples. c) Densitometric quantification of the transcripts. For calibration, the β1-tubulin (*β1-tub*) transcript per poly(A)$^+$RNA was used as standard. Transcript levels are given in arbitrary units. Black and open columns indicate dark (D) and light (L) mycelia, respectively; gray columns, the fruiting specific stages (K, P1 P2).

(Fig. 6c). When culture forms primary hyphal knots, light enhances *cfs1* transcription, which continues to increase from fruiting body initiation to primordial maturation (Fig. 6c). Gene *kin1* has a 1.7 kb transcript (Fig. 6b), which is poorly expressed in all stages except in primordia at the meiotic stage, just about to undergo rapid stipe elongation and cap expansion (Fig. 6c).

4.4.6 Gene *cfs1* is essential for fruiting body initiation and primordia maturation

The transformation data showed in Fig. 2 did not yet allow to definitely assign the fruiting restoring ability of the 10.6 kb *Not*I-B fragment and the 3.5 kb *Bam*HI fragment to a specific gene. Moreover, the higher frequency of transformants initiating fruiting and the qualitative progress in primordia development with larger DNA fragments indicate that more than one of the cloned genes may contribute together or sequentially to fruiting body initiation. Southern blot analysis, using genomic DNA from mutant 6-031 and homokaryon AmutBmut digested with a number of restriction enzymes (*Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, *Kpn*I, *Nru*I, *Pvu*II, *Sca*I, *Sph*I, *Ssp*I, *Xho*I) and three DNA probes that together covered the whole *Not*I-B fragment (a 4.4 kb *Not*I-*Kpn*I fragment, a 3.5 *Bam*HI fragment and a 3.5 kb *Spe*I-*Not*I fragment), excluded the possibility of a large deletion in this region in mutant 6-031, since no difference in band pattern was observed between the two strains (not shown). Moreover, each probe detected only single bands (not shown), indicating the single copy nature of all cloned genes.

Next, we constructed plasmids with deletions within the coding region of *arf1*, *cfs* or *kin1* and within the putative gene *glt1* (see Material and methods) and tested them by transforming mutant 6-031. Only plasmid p7NcoCS, carrying a deletion in *cfs1*, lost the ability to restore fruiting body initiation in the mutant (Figure 5b), defining *cfs1* as the gene active in fruiting body initiation. Since interruptions within other genes did not reduce transformation efficiency down to a value of 1% and since primordia formed were of 3-5 mm in size (not shown), it is possible that the general chromosomal environment of gene *cfs1* rather than the presence of other functional genes influences *cfs1* gene expression and accounts for the developmental differences observed in our transformation experiments.

The final proof that *cfs1* is an essential gene in fruiting body initiation came from isolation of the *cfs1* allele from mutant 6-031. Two DNA fragments covering the entire 3.5 kb *Bam*HI fragment with *cfs1* in mutant 6-031 were PCR-amplified and sequenced.

A single T to G transversion was found in the mutant's *cfs1* allele, which leads to a Y441D amino-acid substitution in the C-terminus of the deduced protein sequence (Fig. 7). When introducing this T to G transversion into the cloned *Not*I-B fragment and a smaller 7 kb *Not*I-*Spe*I fragment, neither of the two resulting plasmids pNotB5/6-031 and p5SpeCS/6-031 were able to restore fruiting body initiation (Fig. 6c), indicating that the point mutation is indeed the cause of the inability to initiate fruiting body formation in mutant 6-031.

4.4.7 The *cfs1* gene encodes a protein highly similar to bacterial cyclopropane fatty acid synthases

The cDNA of the *cfs1* gene is 1776 bp in size with a 66 bp long nucleotide sequence upstream of the first start codon ATG and a 300 bp long nucleotide sequence downstream of the stop codon TGA. Comparison of the genomic DNA and cDNA sequences revealed an open reading frame (ORF) of 1407 bp interrupted by 10 introns (not shown). These introns are 53 to 70 bp in size and have the typical *C. cinereus* 5'- and 3'-splice sites and branch-receptor sequences (Seitz *et al.*, 1996). The promoter region of *cfs1* contains a CAAT element 60-57 bp upstream of the transcription initiation site. No classical TATA box is found, but an AATAAAAA sequence is 37-30 bp upstream of the transcription initiation site. 456 bp are upstream to the transcription initiation site in the 3.5 kb *Bam*HI fragment having the smallest sequence regularly but inefficiently active in transformation (Fig. 2). This sequence should mediate at least some promoter activity. Within the *cfs1* coding region, 332 bp downstream of the start codon ATG in the second exon is the *Eco*RI restriction site used to construct plasmid pEco4.4 and pEco4.4-pab with 5' truncated *cfs1* copies. Homologous recombination at the natural *cfs1* locus within the *C. cinereus* genome might therefore explain the occasional transformants with well developed primordia obtained with these two plasmids (not shown).

The *cfs1* ORF encodes a polypeptide of 469 amino acid residues with a predicted molecular mass of 52 kDa. The codon usage of the *cfs1* gene agrees well with the general *C. cinereus* codon usage, except that codons ACG, CCA, CCG and GCG are under-represented (0 ‰, 2.1 ‰, 2.1 ‰ and 4.3 ‰ compared to the general usage of 11.4 ‰, 16.9 ‰, 13.8 ‰ and 15.9 ‰, respectively) and that codons GCU and UAC are over-represented (42.6 ‰ and 31.9 ‰ compared to 28.5 ‰ and 13.8 ‰, respectively).

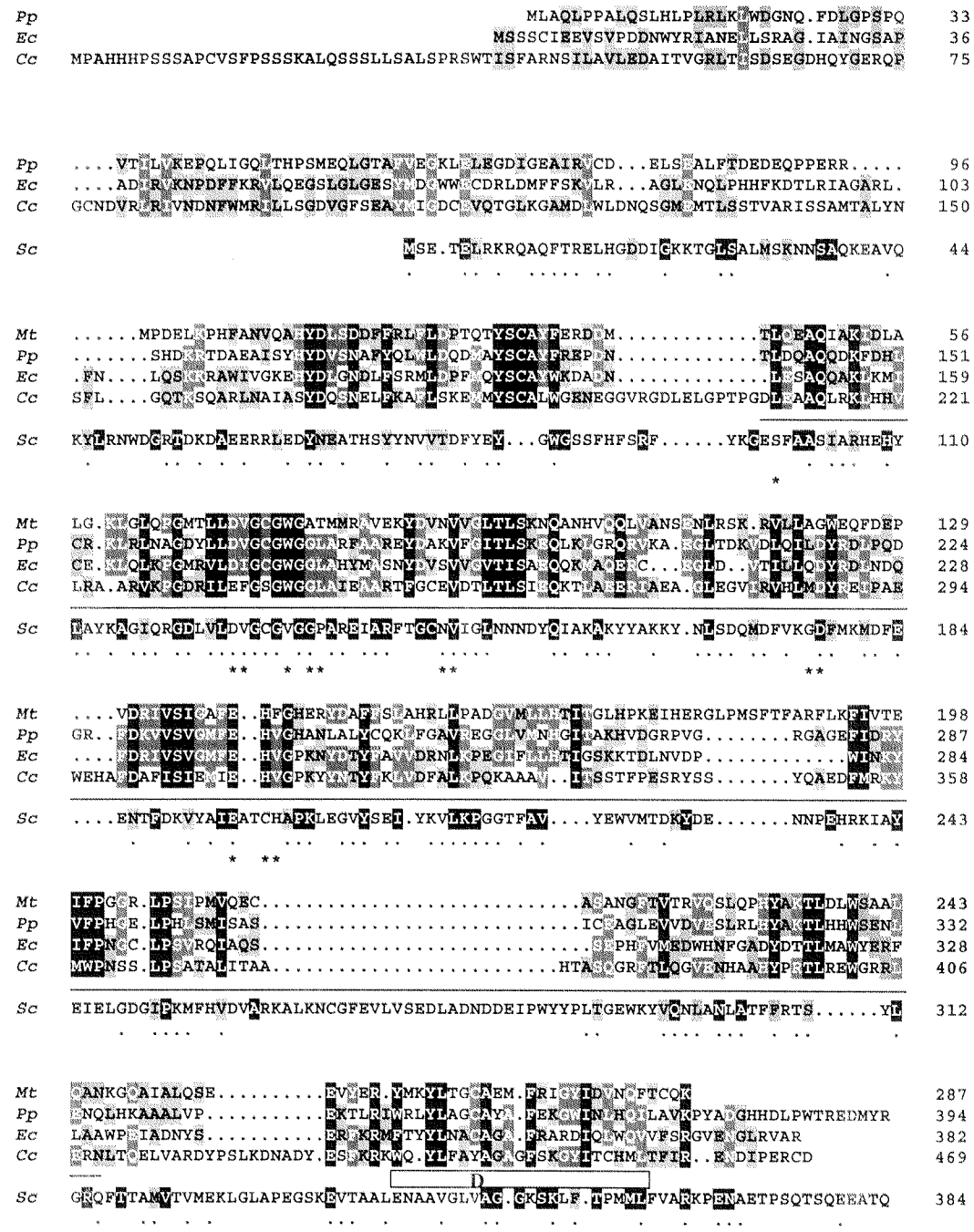


Figure 7. Sequence alignment of *C. cinereus* Cfs1 (*Cc*) to bacterial cyclopropane fatty acid synthases (*Mt*, *Pp*, *Ec*) and to yeast Δ24-sterol methyltransferase ERG6. *Pp*: protein ylp3 of the Gram$^-$ bacterium *Pseudomonas putida* (P31049); *Ec*: CFA of the Gram$^-$ bacterium *E. coli* (P30010); *Mt*: CFA1 of the Gram$^+$ bacterium *Mycobacterium tuberculosis* (Q11195); *Sc*: ERG6 of yeast *S. cerevisiae* (S42003). In the ERG6 protein sequence, amino acids identical to the *C. cinereus* Cfs1 sequence are shaded in black, similar ones in gray. Amino acids in the ERG6 sequence that are identical or similar to one or more bacterial proteins are marked by dots. A line marks the structurally conserved SAM-dependent MTase fold. * indicates those amino acids shown in other proteins to contact SAM (Fauman, *et al*, 1999). The open box at the C-terminal ends of the proteins indicates potential transmembrane regions in Cfs1. The letter D indicates the amino acid exchange found in Cfs1 of mutant 6-031 at position Y441.

The Cfs1 protein has an overall high identity (25-32% identity and 43-49% similarity over the whole protein length) to a number of bacterial cyclopropane fatty acid synthases (Fig. 7), a special subfamily of the S-adenosyl-L-methionine (SAM) dependent C-methyltransferases (MTases) (Fauman *et al.*, 1999). A database search for related proteins also identified two potential but not yet characterized eukaryotic protein products from the plant *Arabidopsis thaliana* (31% identity and 48% similarity over a length of 409 aa, 15% identity and 25% similarity over the whole protein length; accession number BAB02771.1) and from the worm *C. elegans* (32% identity and 48% similarity over a length of 323 aa, 22% identity and 38% similarity over the whole protein length; accession number T18571) (Fig. 7). In the yeast *Saccharomyces cerevisiae*, the most similar protein is ERG6, a Δ24-sterol-C-methyltransferase (31% identity and 48% similarity over a length of 236 aa; 19% identity and 33% similarity over the whole protein length, accession number S42003; Fig. 7). Consistent with a function as SAM-dependent methyltransferase, a SAM-binding motif is present in Cfs1 between aa 209 and 338 (Fig. 7). Protein profile programs (PSA and PSIpred) predict this region to adopt the typical 6 helices-7 strands configuration of SAM-binding domains (referred to as a SAM-dependent MTase fold) (Fauman *et al.*, 1999). All but one amino acid shown to contact SAM in structurally characterized SAM-dependent MTases (Fauman *et al.*, 1999) are conserved in Cfs1 (Fig. 7). Programs TMpred and TMHMM both predict potential transmembrane domains in the C-terminus of the *C. cinereus* Cfs1 protein (aa 435-453 and aa 437-456), closely behind the SAM-binding motif (Fig. 7). The Y441D amino acid substitution found by sequencing in the last second exon of the *cfs1* allele of mutant 6-031 is localized in this region (Fig. 7). This substitution overturns the prediction of a transmembrane domain and gives rise to higher hydrophilicity (Goldman/Engelman/Steitz prediction) at the C-terminal region in the mutant protein (not shown). Overall, the wild-type Cfs1 protein appears to be hydrophilic without any long hydrophobic region.

4.5 Discussion

In this study, we characterized the first mutant of the basidiomycete *C. cinereus* with a specific defect in fruiting body initiation, at the transition stage from primary to secondary hyphal knots. Through mutant complementation, we cloned gene *cfs1* that, by homology to heterologous genes, encodes a potential cyclopropane fatty acid

synthase. Consistent with a function in fruiting body initiation, transcription of the *cfs1* is low in the dark, but induced by light. The gene is specifically active at the light dependent stage of secondary hyphal knot formation and in subsequent primordium development. When transforming the subcloned *cfs1* gene into the fruiting defective mutant 6-031, we observed quantitative and qualitative differences in complementation activities with *cfs1*-carrying DNA fragments of different length. Interruptions within the open reading frames of the neighboring genes (*arf1*, *kin1* and the putative gene *glt1* present in the large DNA fragments) had no effect on the complementation activity of *cfs1*, indicating that the likely cause for the differences in transformation activity is rather that the larger chromosomal environment may play a role in proper *cfs1* gene expression. The two *cfs1* neighboring genes, *arf1* and *kin1*, have also been shown to be differentially transcribed. The transcription of *arf1* is somewhat decreased at the onset of *cfs1* expression during primary hyphal knots formation, and *kin1* is specifically transcribed at subsequent stages of primordia formation when *cfs1* transcription is the highest. It is possible that the transcription profiles of *arf1* and *kin1* influence the expression of *cfs1* during early and later stages of fruiting body development.

Occurrence of cyclopropane fatty acid synthases and their products in bacteria and eukaryotes. Cyclopropane fatty acid (CFA) synthases have been found in several bacteria with the CFA synthase of *E. coli* being the most extensively studied (Wang *et al.*, 1992). In *E. coli*, the enzyme catalyzes the formation of a cyclopropane ring by transferring a methyl group from SAM to a *cis*-double bond in the unsaturated fatty acid (UFA) chains of membrane phospholipids. *cis*-9,10-methylenehexadecanoic acid (17CFA), *cis*-9,10-methyleneoctadecanoic acid (MOA, DHSA = dihydrosterculic acid, C19) and *cis*-11,12-methyleneoctadecanoic acid (lactobacillic acid, C19) are characteristic bacterial CFAs [for review see (Grogan and Cronan, 1997)].

Unlike *E. coli*, in the cell envelope of *Mycobacterium tuberculosis* cyclopropane mycolic acids [ranging in sizes from C60 to C90, (Minnikin, 1982)] are formed by five CFA synthases (Glickman *et al.*, 2000). Cyclopropane mycolic acids are specific to slow-growing pathogenic mycobacteria and are shown to be necessary for cording, persistence and virulence of *M. tuberculosis* (Glickman *et al.*, 2000). In *E. coli*, the CFA synthase is not essential for growth under an assortment of experimental conditions (Grogan and Cronan, 1986). However, it was recently shown that a function of CFA synthase is to improve the survival of *E. coli* cells in low pH environment (Chang and

Cronan, 1999). In other bacteria such as *M. tuberculosis*, several *Lactobacillus, Pseudomonas* and *Xenorhabdus* species, *Clostridium botulinus*, *Halomonas salina*, *Lactococcus lactis*, *Proteus vulgaris* and *Yersinia enterocolitica*, the production of CFAs are also related to stress conditions. High temperature, high osmolality, high salt, high ethanol, low nitrogen and low phosphorus content and the presence of phenolic compounds in culture media, low pH, limited oxygen conditions, as well as oxygen saturation of the medium are all factors causing an increase in bacterial CFA content (Bodnaruk and Golden, 1996; Broadbent and Lin, 1999; Couto *et al.*, 1996; Evans *et al.*, 1998; Guillot *et al.*, 2000; Jacques, 1981; Jacques, 1982; Maheshwari and Nishimura, 1994; Monteoliva *et al.*, 1993; Rozes and Peres, 1998; Valderrama *et al.*, 1998; Yuan *et al.*, 1995). In consequence of CFA production, membrane properties, especially membrane fluidity, alter with enhanced bacterial stress tolerance (Broadbent and Lin, 1999; Brown *et al.*, 1997; Chang and Cronan, 1999; Couto *et al.*, 1996; Sajbidor, 1997). Phospholipids containing cyclopropane fatty acids have a broader transition temperature range than those containing unsaturated fatty acids, which confers more resistance of the membrane lipid matrix to environmental perturbations (Perly *et al.*, 1985). Cyclopropane-containing membranes enhance stability by suppressing segmental mobility of hydrocarbon chains, thus providing increased rigidity with respect to external shock (Dufourc *et al.*, 1983; Dufourc *et al.*, 1984).

CFA-containing phospholipids have been found in a wide variety of $Gram^+$ and $Gram^-$ bacterial species, but only in very few phylogenetically unrelated eukaryotic organisms. In eukaryotes, the chemical structures of CFAs are far more diverse than in bacteria. DHSA has been identified in five genera of trypanosomatid protozoa (*Crithidia*, *Herpetomonas*, *Leishmania*, *Leptomonas* and *Phytomonas*) independent of whether having endosymbiotic or other microbial associates (Fish *et al.*, 1981). CFA synthase activity has been demonstrated in *Crithidia fasciculata* (Li *et al.*, 1993) and *Herpetomonas* (Holz *et al.*, 1983). The plasmodial slime mold *Physarum polycephalum* has a unique CFA termed PHYLA (a lysophosphatidic acid composed of cyclic phosphate and cyclopropane-containing hexadecanoic acid) (Murakami-Murofushi *et al.*, 1992). Unsaturated CFAs in the cellular slime mold *Polysphondylium pallidum* (17CFA and lactobacillic acid) were shown to originate from ingested *E. coli* that contain saturated CFAs (Saito and Ochiai, 1998). Most interestingly, the saturated CFAs content in vegetative cells is relatively high, substantially decreasing at the development transition from amoebae to aggregation-competent cells, while the

unsaturated CFAs concomitantly increase (Saito and Ochiai, 1998). Cyclopropanated and brominated C18 straight-chain unsaturated fatty acids have been isolated from lichens (*Cladonia furcata*, *Lecanora fructulosa*, *Leptogium saturninum*, *Parmelia linctina*, *P. comtseliadalis*, *Peltigera canina* and *Xanthoria* spp.) containing an ascomycete and either a green alga (*Trebouxia* spp.) or a cyanobacterium (*Nostoc* spp.) (Rezanka and Dembitsky, 1999). Cyclopropyl hydroxy-eicosanoids were found in the red alga *Constantinea simplex* (Nagle and Gerwick, 1990), cyclopropanated C19 straight-chain fatty acid (cladocroic acid) in the deep water sponge *Cladocroce incurvata* (D'Auria *et al.*, 1993) and cyclopropane containing eicosanoid (C20) in the caribbean soft coral *Plexaura homomalla* (White and Jensen, 1993). CFAs with 17, 18 and 19 carbon atoms occur in females and eggs, but interestingly not in males, of the spirostreptid millipedes *Graphidostreptus tumuliporus* (Karsch), *Archispirostreptus syriacus*, *A. gigas* and *Orthoporus ornatus* (Oudejans and van der Horst, 1978; van der Horst *et al.*, 1972). A long chain CFA containing 25 carbon atoms was found in leaves of the early spring plants snow drop (*Galanthus nivalis* L.) and cow parsley (*Anthrisus silvestris* Hoffm. [L.]) (Kuiper and Stuiver, 1972). *cis*-9,10-methyleneheptadecanoic acid (dihydromalvalic acid, C18) and DHSA are reported in roots of the plants *Ceiba pentandra* L. (Malvaceae) and *Sterculia foetida* L. (Sterculiaceae) (Kaimal and Lakshminarayana, 1970) and in seeds oil of many plant species, especially in immature seeds from Malvales (Fisher and Cherry, 1983; Grondin *et al.*, 1997; Yano *et al.*, 1971). 17CFA and DHSA are present in roe and in both immature and mature female and male fish *Fundulus heteroclitus* (L.) (Cosper and Ackman, 1983) [at least some of the CFAs might originate from the resident bacterial gut population (Cosper *et al.*, 1984)]. Feeding experiments in rats show that CFA from the diet can be specifically absorbed by intestinal tissues (Greter *et al.*, 1979). However, we do not yet know the origin of the 2,3-methylenehexadexadecanoic acid and 2,3-methyleneoctadecanoic acid from sheep rumen tissues (Body, 1972), the 17CFA from submitochondrial particles of bovine heart, mammalian heart and liver tissues (Sakurada *et al.*, 1999), the *cis*-3,4-methyleneoctadecanoic acid (a urinary metabolite of DHSA (Greter *et al.*, 1979) and the *trans*- and *cis*-3-cyclopropane octanoylcarnitines (Libert *et al.*, 1997) from human urine.

The role of cyclopropane fatty acids in eukaryotes. In no case, a specific role for CFAs in eukaryotes has been identified and their function remains obscure. The stress-resistance relationship observed in bacteria evoked to attribute the cold-hardiness and

drought resistance in certain plants (Kuiper and Stuiver, 1972) and desiccation tolerance in millipedes (particularly in eggs and early larval stages) to the presence of CFAs (Oudejans *et al.*, 1976; van der Horst and Oudejans, 1978). Accumulation of C17:0 CFA in fluorescent pseudomonads has been correlated with antifungal activity, but there is no direct evidence for fatty acids acting on fungal growth (Ellis *et al.*, 2000). Most interestingly, MOA from the human pathogen *Helicobacter pylori* has been shown to directly activate protein kinase C (PKC, an enzyme implicated in the control of epithelial proliferative activity and in the process of malignant transformation) in gastric epithelial cells, as well as to enhance histamine- and dibutyryl cyclic AMP-stimulated acid secretion in parietal cells and in increasing DNA synthesis in gastric epithelial cells (Beil *et al.*, 1998a; Beil *et al.*, 1998b). In contrast, PHYLPA from *Physarum polycephalum* inhibits proliferation of human fibroblast cells through arresting cells at the G(1) or G(2) phase and elicits an increase in cAMP in fibroblast cells (Murakami-Murofushi *et al.*, 1993). PHYLPA has also been shown in *vitro* to selectively inhibit members of the DNA polymerase-α family (Murakami-Murofushi *et al.*, 1995).

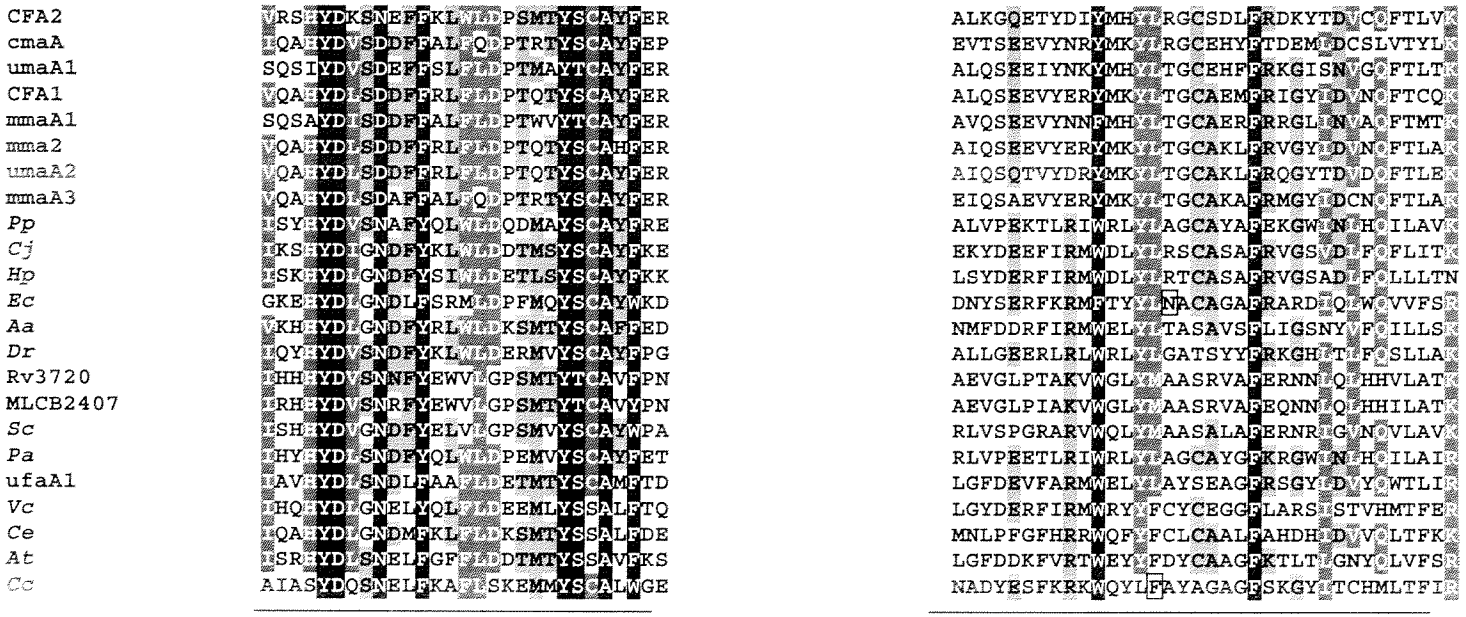
No cyclopropanated moiety has so far been reported in higher fungal lipids (Solberg, 1989). However, in the basidiomycete *Agaricus bisporus* and other higher fungi, the unsaturated linoleic acid is the major constituent of fatty acids (Bonzom *et al.*, 1999; Byrne and Brennan, 1975; Solberg, 1989). The related oleic acid is shown in *E. coli* to be a substrate for the action of CFA synthase (Marinari *et al.*, 1974). In this study we have shown that the *C. cinereus cfs1* gene is superfluous for vegetative mycelial growth, but essential for fruiting body initiation. Thus, our study for the first time demonstrates a function of CFAs in fungal development and offers a possibility to study the CFAs in relation to developmental processes in eukaryotes. *C. cinereus* and the developmental mutants we have generated provide an easy system to elucidate molecular mechanisms of the potential CFA synthase Cfs1 and its products. This research becomes very promising since the phenotype of mutant 6-031 is linked to a specific developmental process.

It is possible that by the action of Cfs1 the physical properties of cellular membranes alter and that this is the trigger to initiate sexual morphogenesis in the fungus. In fact, two types of membrane interactive compounds induced, in feeding experiments, fruiting body development in Basidiomycetes such as *Pleurotus ostreatus*, *Schizophyllum commune* and *C. cinereus* (see chapter 1; for review see (Kües and Liu, 2000). One type of such compounds are surfactants, *e.g.* sucrose esters of fatty acids (Magae, 1998) and

plant saponins that stimulate the primordium formation in *P. ostreatus* in a dosage dependent manner (Magae, 1999). Others belong to a group of cerebrosides, which induce precocious fruiting in *S. commune* and *C. cinereus* (Kawai, 1989; Mizushina *et al.*, 1998). Membrane alteration has therefore been postulated to be a stress signal that promotes the fungus to shift from vegetative to reproductive growth (Magae, 1999).

Protein motifs: the SAM-binding motif and other conserved regions. Within the *C. cinereus* Cfs1 protein, we identified the most prominent motif, a potential SAM-binding domain, also referred to as a "SAM-dependent methyltransferase (MTase) fold" (Fauman *et al.*, 1999). This motif has been found in a large family of SAM-dependent MTases, which transfer a C-unit from SAM to either sulfur (S), carbon (C), nitrogen (N) or oxygen (O) atoms in DNA, RNA, proteins and lipids (Fauman *et al.*, 1999). CFA synthases are C-MTases that, in bacteria, transfer a methyl group from SAM to a C atom in unsaturated membrane localized phospholipids (Grogan and Cronan, 1997). Generally, C-MTases are relatively rare when compared to O- and N-MTases (Fauman *et al.*, 1999). C-MTases modify small molecules such as tryptophan and sterols, act in tetrapyrrole synthesis or methylate macromolecules including DNA and RNA (Fauman *et al.*, 1999). Among these various C-MTases, plant and fungal Δ^{24}-sterol C-MTase are the closest related to CFA synthases, possibly because their enzyme activities are both linked to lipid bilayers (Leber *et al.*, 1994; Taylor and Cronan, 1979). Low homology is found over the whole protein length for example between ERG6 of *S. cerevisiae* and Cfs1 of *C. cinereus* and the bacterial CFA synthases, with the highest conservation in the SAM-binding motif (Fig. 7). In ERG6, directly upstream of the SAM-dependent MTase fold is a sequence (DFYEYGWGSSFHFS) referred to as region I that is highly specific to all Δ^{24}-sterol C-MTase (defined consensus motif YEXGWG) and that has sterol binding activity (Nes *et al.*, 1999; Nes *et al.*, 1998). This sequence is not present in *C. cinereus* Cfs1 and other bacterial CFA synthases (Fig. 7). Instead, a highly conserved sequence (region I), only found in (potential) CFA synthases, occupies the corresponding position in these members of C-MTases (Fig. 7 and 8). Considering the function of region I in Δ^{24}-sterol C-MTase in sterol-binding, it is tempting to speculate that the conserved sequence found in all CFA synthases is a binding domain for the unsaturated CFAs localized within the lipid bilayer.

In *E.coli*, CFA synthase is a soluble protein found in the cell cytoplasm that uses



Region I:

$V/I_{83}XXH_{87}\mathbf{Y_{100}D_{100}}V/L/I_{91}S_{70}\mathbf{N/D_{100}}D/N_{65}F_{74}\mathbf{F/Y_{100}}XL/I_{74}W/F_{83}L_{91}D_{83}P_{57}S/T_{74}M/L_{74}T/S_{61}\mathbf{Y_{100}S/T_{100}}C_{87}\mathbf{A_{100}}Y/F_{65}\mathbf{F/W_{100}}XX$

Region II: $XXXXQ/E_{70}XXXR/K_{65}\mathbf{XY/W/F_{100}}XXY_{96}L/M_{83}XXC_{70}A_{61}XX\mathbf{F_{100}}K/R_{61}XG_{61}XL/I/V_{78}D/N_{81}V/L_{78}XQ_{78}XXXK/R_{96}$

Figure 8. Two conserved regions in potential CFA synthases. Region I in all bacterial and eukaryotic CFA synthases is directly N-terminal to the SAM-binding domain (Fig. 7). Region II is at the C-terminus of all proteins behind the SAM-binding domain. The consensus sequences of both regions are shown with the percentage of amino acid usage (numbers in subscript; in bold: positions with 100 % aa conservation). In region II, the boxed cysteine residue (C354) of the *E. coli* CFA synthase (*Ec*, accession number P30010) is thought to be enzyme active site (Wang, *et al*, 1992). The boxed tyrosine (Y441) of the *C. cinereus* Cfs1 sequence (*Cc*) is substituted by an aspartic acid (D) in mutant 6-031. Proteins CFA2 (Q11196), umaA1 (CAA17424), CFA1 (Q11195), mmaA1 (B70614), mma2 (A70614), umaA2 (CAA17425), mmaA3 (AAC44618), Rv3720 (CAA18042) and ufaA1 (H70830) are from *M. tuberculosis* (strain H37RV). CmaA (AAC44876) is from *M. bovis*. BCG and MLCB2407 (CAA19156) are from *M. leprae*. Other bacterial proteins are *Pp* from *Pseudomonas putida* (P31049), *Cj* from *Campylobacter jejuni* (CAB73437), *Hp* from *Helicobacter pylori* strain 26695 (H64571), *Aa* from *Aquifes aeolicus* (F70449), *Dr* from *Deinoccus radiodurans* strain R1 (AAF11731), *Sc* from *Streptomyces coelicolor* A3(2) (CAB89463), *Pa* from *P. aeruginosa* (AAG08931) and *Vc* from *Vibrio cholerae* (AAF94281). Eukaryotic proteins are *Ce* from *C. elegans* (T18571) and *At* from *A. thaliana* (BAB02771).

SAM as a soluble and UFA-containing phospholipid as an insoluble substrate (Taylor and Cronan, 1979). Free UFAs are not converted by the enzyme (Cronan *et al.*, 1974; Thomas and Law, 1966). The substrate C-double bond, positioned at 9-11 carbon units from the glycerol backbone of the phospholipid molecule (Marinari *et al.*, 1974; Ohlrogge *et al.*, 1976), is located deeply within the hydrophobic core of the membrane bilayer (Gally *et al.*, 1979; Seelig and Seelig, 1980). CFA synthase has been shown to specifically interact with membranes containing either unsaturated or cyclopropanated acyl chains (Taylor and Cronan, 1979). Biochemical data suggest that the enzyme gains access to both inner and outer leaflets of intact UFA-containing phospholipids (Marinari *et al.*, 1974). Inhibitor studies with sulfhydryl-modifying reagents and C-terminal truncation (50 aa) suggest that the C-terminus and especially C354 (Fig. 7) have a role in interaction with the membrane (Wang *et al.*, 1992). Most interestingly, in mutant 6-031 we found a Y441D substitution in the corresponding C-terminal region of Cfs1 (termed region II, Fig. 8) and we have shown that Y441 is essential for Cfs1 function. Computer programs predict the wild-type Cfs1 of *C. cinereus* being a cytoplasmic enzyme, like the CFA synthase of *E. coli* (Taylor and Cronan, 1979), with two transmembrane domains in the C-terminus. This suggests that the C-terminus functions in anchoring the Cfs1 protein to the membrane and/or represents part of the catalytic domain.

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Curriculum vitae

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