

Fruiting body development in *Coprinus cinereus*: regulated expression of two galectins secreted by a non-classical pathway

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Fruiting body formation in the basidiomycete *Coprinus cinereus* is a developmental process that occurs as a response of the mycelium to external stimuli. First, localized, highly branched hyphal structures (knots) are formed as a reaction to nutritional depletion. Hyphal-knot formation is repressed by light; however, light signals are essential for the development of the hyphal knot into an embryonic fruiting body (primordium) as well as karyogamy, meiosis and fruiting body maturation. The role of the different environmental signals in the initial phases of fruiting body development was analysed. It was observed that two fungal galectins, Cgl1 and Cgl2, are differentially regulated during fruiting body formation. *cgl2* expression initiated in early stages of fruiting body development (hyphal knot formation) and was maintained until maturation of the fruiting body, whereas *cgl1* was specifically expressed in primordia and mature fruiting bodies. Immunofluorescence and immunoelectron microscopy studies detected galectins within specific fruiting body tissues. They localized in the extracellular matrix and the cell wall but also in membrane-bound bodies in the cytoplasm. Heterologous expression of Cgl2 in *Saccharomyces cerevisiae* indicated that secretion of this protein occurred independently of the classical secretory pathway.

Keywords: basidiomycete, *Coprinus*, fruiting body, galectin, secretion

INTRODUCTION

Fruiting body development in the heterothallic basidiomycetous fungus *Coprinus cinereus* is a process involving various hyphal–hyphal (cell–cell) interactions and occurs as a response of the mycelium to external stimuli such as light, temperature and depletion of nutrients (Matthews & Niederpruem, 1972; reviewed by Moore, 1998; Kües, 2000). Fruiting bodies form on the dikaryon, a mycelium that is generated through mating of compatible primary mycelia (monokaryons) obtained from germination of the haploid basidiospores produced in the hymenium of fruiting bodies. Mating of monokaryons is governed by the two mating-type loci A

and B (reviewed by Casselton & Olesnický, 1998; Hiscock & Kües, 1999). A stable dikaryon can only be generated by the mating of two monokaryons that have a different specificity in these two mating-type loci. Rare mutations in these loci lead to the formation of self-compatible *Amut Bmut* homokaryons that mimic a dikaryon. In particular, such homokaryons can have the ability to form fruiting bodies (Swamy *et al.*, 1984), demonstrating the regulatory function of the mating-type loci in fruiting body development.

In the initial stage of fruiting body (basidiocarp) development, hyphae form localized, highly branched structures termed hyphal knots (Matthews & Niederpruem, 1972; reviewed by Kües, 2000) (Fig. 1). Without a light stimulus, hyphal knots develop into globose structures, called sclerotia. These long-term survival structures are hyphal aggregates consisting of an inner medulla and a melanized rind (Moore & Jirjis, 1976; Moore, 1981; Cléménçon, 1997; Kües *et al.*, 1998) (Fig. 1). When exposed to light, hyphal knots develop into a compact hyphal mass, termed an initial (reviewed by

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Abbreviation: ECM, extracellular matrix.

The GenBank accession number for the sequence reported in this paper is AF130360

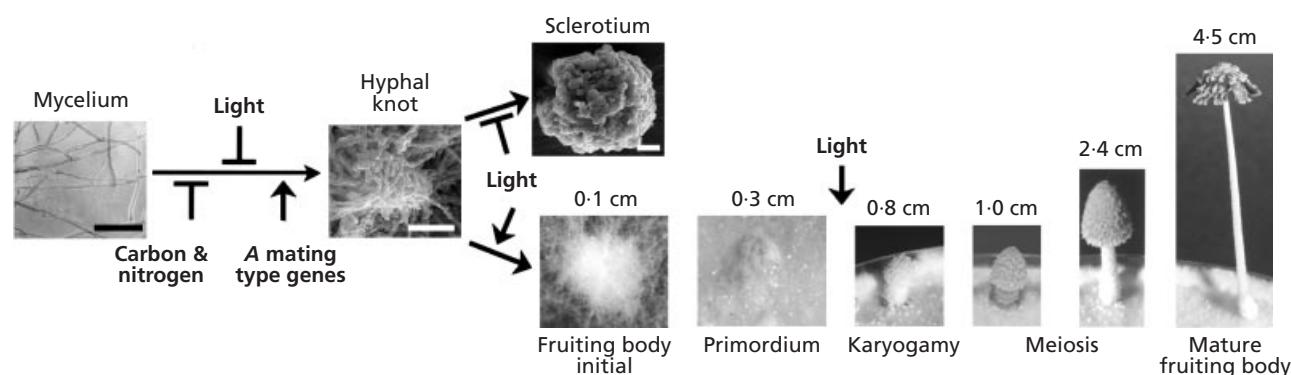


Fig. 1. The pathway of sclerotia and fruiting body development of *C. cinereus* homokaryon AmutBmut. The different developmental structures and the environmental and genetic factors that regulate the differentiation processes are shown. On the mycelium (left) the developmental processes initiate, leading to sclerotium or fruiting body formation (right). Signals that initiate (arrows) or repress (bars) the different steps in development are indicated. Bars within the pictures represent 20 µm; the size of the different developmental structures is indicated.

Moore *et al.*, 1979; Kües, 2000) (Fig. 1). The hyphae of the initial eventually differentiate to form a primordium that is essentially an embryonic fruiting body. At this stage, structures composing the stipe and cap (pileus) of the immature fruiting body are clearly discernible (for reviews see Cléménçon, 1997; Moore, 1995, 1998) (Fig. 1).

During fruiting body development, there is a dramatic change in the growth characteristics of the fungus. The mycelium, consisting of a network of single hyphae, starts to produce multi-hyphal structures, plectenchymatic tissue that requires extensive hyphal-hyphal interactions. Cellular differentiation processes lead to the formation of highly specialized cells like basidia or cystidia in the hymenium of the mature fruiting body (Cléménçon, 1997; Moore, 1998). Hyphal-hyphal interactions are most likely mediated by structural factors present on the cell wall and the extracellular matrix (ECM) of hyphae within the fruiting body. However, very few structural factors that might mediate such interactions are known in the basidiomycetes.

Studies of the biosynthesis and architecture of the fungal cell wall, in particular of the cell wall of the ascomycete *Saccharomyces cerevisiae*, revealed that the outermost layer of the wall is composed of oligosaccharides covalently linked to asparagine, serine or threonine residues of cell-wall proteins (mannans) (reviewed by Kapteyn *et al.*, 1999). Higher fungi can have more complex type oligosaccharides (xylomannan and galactomannan) at their surface (reviewed by Gooday, 1995). This suggests that hyphal interactions might be mediated by lectins, a family of oligosaccharide-binding proteins. Indeed, many basidiomycetes produce low-molecular-mass lectins (see reviews by Guillot & Konska, 1997; Wang *et al.*, 1998) and two lectins, Cgl1 and Cgl2, isolated from fruiting bodies of the basidiomycete *C. cinereus*, were partially characterized (Charlton *et al.*, 1992; Cooper *et al.*, 1997). These lectins show binding specificity towards β -galactosides and do not share sequence homology with any other known fungal lectin.

Surprisingly, the Cgl lectins were found to be homologous to the family of galectins, and were the first galectins identified outside the animal kingdom (Cooper *et al.*, 1997). Galectins, or S-type lectins, specifically bind β -galactoside sugars in a calcium-independent manner and share sequence homology within the carbohydrate-recognition domain. These criteria differentiate the galectins from all other lectins (see review by Barondes *et al.*, 1994). In animals, galectins are involved in many different cellular processes such as muscle differentiation, olfactory development, embryo implantation, metastasis, apoptosis and mRNA splicing (Barondes *et al.*, 1994). Mammalian galectin-1 and galectin-3 were shown to be secreted and interact with components of the ECM (Cooper & Barondes, 1990; Sato *et al.*, 1993). They lack typical secretory signal sequences and are secreted via non-classical secretory pathways (Sato *et al.*, 1993; Cleves *et al.*, 1996; reviewed by Hughes, 1999).

The presence of galectin proteins in fruiting bodies of *C. cinereus* prompted us to speculate that galectins are involved in cellular aggregation, not only in the animal kingdom but also in fungi. To address a potential role of galectins in fruiting body development, we defined more closely the initial developmental phases of fruiting body formation, then analysed the expression and localization of these proteins during this process. We show that expression of the galectins is highly regulated during fruiting body development and can be correlated to external signals that control this process. Importantly, the fungal galectins are ECM proteins that are secreted by a pathway independent of the normal secretory pathway.

METHODS

Culture conditions. *C. cinereus* strains are listed in Table 1. Generation of the *trp1*⁺ and *trp1*⁺ *A_{on}* transformants of the monokaryons PG78 (A6 B42 *trp*-1.1,1.6), FA2222 (A5 B6 *trp*-1.1,1.6) and 218 (A3 B1 *trp*-1.1,1.6) was as described previously (Kües *et al.*, 1998). Strains were grown on solid YMG or YMG/T medium (Rao & Niederpruem, 1969; Granado *et al.*,

Table 1. *Coprinus cinereus* strains used in this study

Strain	Genotype	Reference
AmutBmut	<i>A43mut B43mut pab-1</i>	Swamy <i>et al.</i> (1984), May <i>et al.</i> (1991)
PG78/trp1	<i>A6 B42 pab-1 trp-1.1,1.6 ::trp-1⁺</i>	Kües <i>et al.</i> (1998)
PG78/a1	<i>A6 B42 pab-1 trp-1.1,1.6 ::trp-1⁺ ::a1-2</i>	Kües <i>et al.</i> (1998)
FA2222/trp1	<i>A5 B6 trp-1.1,1.6 acu-1 ::trp-1⁺</i>	Kües <i>et al.</i> (1998)
FA2222/a1	<i>A5 B6 trp-1.1,1.6 acu-1 ::trp-1⁺ ::a1-2</i>	Kües <i>et al.</i> (1998)
218/trp1	<i>A3 B1 trp-1.1,1.6 ::trp1⁺</i>	Kües <i>et al.</i> (1998)
218/a2	<i>A3 B1 trp-1.1,1.6 ::trp1⁺ ::a2-1</i>	Kües <i>et al.</i> (1998)

1997) in dark and light conditions as described by Kertesz-Chaloupková *et al.* (1998). Fruiting body development was followed by growing strains at 37 °C on YMG/T in the dark for 5 d and then incubating the cultures at 25 °C in an alternating 12 h light/dark cycle (Granado *et al.*, 1997). For the nutritional studies, homokaryon AmutBmut was grown at 37 °C in continuous dark on *Coprinus* minimal medium supplemented with 5 mg para-aminobenzoic acid ml⁻¹ (Granado *et al.*, 1997), containing various concentrations of glucose or asparagine. For all of the experiments the cultures were grown from single explants inoculated in the centre of 90 mm Petri dishes.

The *Saccharomyces cerevisiae* wild-type strain BJ1991 (*MAT α trp1 leu2 ura3 prb1-1122 pep4-3 gal2*) (Jones, 1977) and strain PA33-6a (*MAT α leu2 ura3 trp1 his3 sec18-1*) (Bickle *et al.*, 1998) were grown and maintained on minimal media [0.67% yeast nitrogen base (Difco Laboratories), 2% glucose and the appropriate supplements] at 25 °C unless otherwise noted.

Cosmid isolation and plasmid construction. The galectin region encompassing *cgl1* and *cgl2* was isolated from a cosmid library of homokaryon AmutBmut (Bottoli *et al.*, 1999). A 7068 bp *Bam*HI fragment of galectin cosmid 27A9 harbouring both *cgl1* and *cgl2* genomic DNA was subcloned into pBluescript KS- (Stratagene) and sequenced completely on both strands (Microsynth). Fragments of a *Hind*III digest containing all of *cgl1* and most of the *cgl2* sequences (Fig. 2) were also subcloned into pBluescript KS- to yield plasmids pBCG1 and pBCG2, respectively.

All plasmids were propagated in *Escherichia coli* strain XL1-Blue (Stratagene). Plasmid pYCG2 was created by placing the translatable region of *cgl2* and parts of the adjacent 3' intron [DNA from *Coprinus* strain JR52 \times PR2301 (Charlton *et al.*, 1992)] under the control of the yeast *ADH2* promoter of the multicopy yeast vector pYADE4 (Brunelli & Pall, 1993). Plasmids were transformed into yeast by the lithium acetate procedure (Ito *et al.*, 1983).

Isolation of galectins and generation of antibodies. Galectins were isolated from fruiting bodies of homokaryon AmutBmut. Lyophilized fungal tissue was ground with a mortar and pestle and the resulting powder was resuspended in 50 mM Tris/HCl pH 7.0, 150 mM NaCl. This suspension was centrifuged at 10000 g to remove particulate material. The protein concentration of the supernatant was determined with a Bradford assay (Bio-Rad) and adjusted to ≤ 1 mg ml⁻¹. The sample was applied to an affinity column composed of lactosyl-Sepharose (Pharmacia) with a bed volume of 5 ml at a flow rate of 1 ml min⁻¹. Following complete elution of the flow-through peak, a 0.1 M lactose solution was applied to the column and the lectins eluted from the column were collected, dialysed and

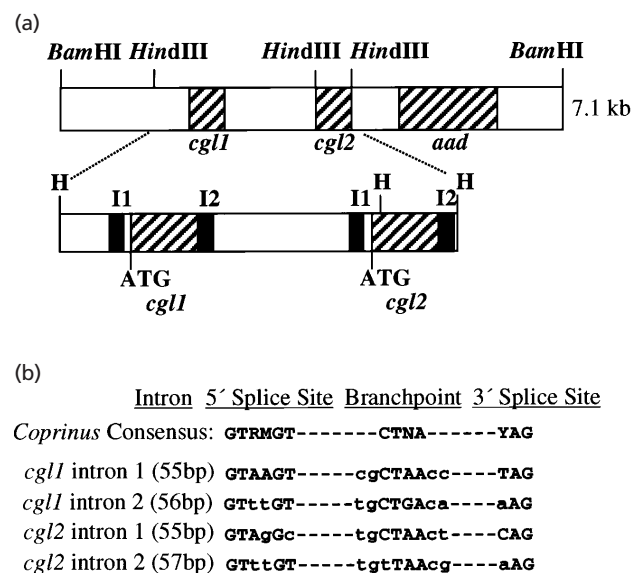


Fig. 2. *cgl1* and *cgl2* are closely linked loci. (a) The genomic 7.1 kb *Bam*HI fragment from homokaryon AmutBmut is shown with the location of *cgl1* and *cgl2* as well as a putative aryl-alcohol dehydrogenase gene (*aad*). The location of the protein-coding region is indicated by a hatched bar, introns (I1 and I2) are indicated. (b) The sequences of the 5' splice site, the putative branch-acceptor sequence and the 3' splice site of the introns in the galectin loci are compared to the consensus sequences derived from *Coprinus* introns (Seitz *et al.*, 1996).

concentrated by lyophilization. The pure galectins were sent to ReadySysteme AG where α -galectin (α -Cgl) antibodies were raised in rabbits.

Detection of galectins by Western blotting. Protein was extracted from lyophilized mycelium or tissue as outlined above. Five microgrammes of total saline-soluble protein for each sample were separated on a 15% SDS-PAGE gel (Laemmli, 1970) using the Protean II xi cell (Bio-Rad). Proteins were transferred to Immobilon P membranes (Millipore) in 48 mM Tris/HCl (pH 9.2), 39 mM glycine, 0.0375% SDS, 20% methanol) using a Trans-Blot Semi-Dry Transfer Cell (Bio-Rad). The membrane was incubated for 20 min in TBS-T [20 mM Tris/HCl, pH 7.5, 0.5 M NaCl, 0.1% (w/w) Tween-80] containing 10% (w/v) dry milk powder at room temperature. Binding of the α -galectin antibodies was performed for 16 h in TBS-T plus 1% (w/v) dry milk powder using a 1:3000 dilution of the serum. The membrane was

washed three times in TBS-T and then incubated in TBS-T plus 1% (w/v) dry milk powder and Protein A coupled to horseradish peroxidase (125 ng ml⁻¹; Sigma). Horseradish peroxidase was detected by ECL chemiluminescence (Amersham Life Science).

Determination of mRNA levels by RT-PCR. Total RNA was isolated from powdered, lyophilized *Coprinus* tissues by a guanidinium isothiocyanate procedure (Chomczynski & Sacchi, 1987) and genomic DNA of homokaryon AmutBmut was obtained by the method of Zolan & Pukkila (1986). The cDNA templates were generated using a DNase I and cDNA synthesis kit (Gibco-BRL) starting with 2 µg total RNA and an oligo-poly(dT)₁₅ primer. An equal portion of each cDNA as well as 10 ng samples of pBCG1, pBCG2 and total genomic DNA of *C. cinereus* homokaryon AmutBmut were added to 50 µl PCR reaction mixtures [20 mM Tris/HCl pH 8.3, 1.5 mM MgCl₂, 15 mM each dNTP and 1 U *Taq* polymerase (MBI Fermentas)]. The *cgl1*- (CGL1F, 5'-ACAGCAGGAC-CAAGGGT-3'; CGL1R, 5'-GTACCGACAGCTAGCAA-GCA-3'), *cgl2*- (CGL2F, 5'-AACAGCAGGCTCAAGAAC-3'; CGL2R, 5'-AATATGTTGGTGTGGCT-3') and β -tubulin- [*tub*] (Matsuo *et al.*, 1999) (β -tubF, 5'-CTCGTCTCCAC-TCTTCATG-3'; β -tubR, 5'-CGTCCTGGTATTGCTGG-TACTCAGC-3') specific primers all flanked introns and were used at a final concentration of 0.5 µM. The reaction mixtures were subjected to 1 cycle of 5 min at 95 °C, 2 min at 50 °C and 2 min at 72 °C, followed by 29 cycles of 30 s at 95 °C, 30 s at 50 °C and 2 min at 72 °C. Aliquots of the samples were then separated on 2% agarose gels.

Immunolocalization of galectin protein. For light microscopy, small 1 to 2 µm thick hand-cut sections of fungal tissue were fixed for 2 h at 4 °C in 20 mM Tris/HCl (pH 7.2), 0.5 M NaCl, 4% (v/v) paraformaldehyde. Following fixation, the samples were dehydrated in a series of ethanol washes and then washed with xylene. The samples were embedded in Paraplast Plus (Sigma) overnight in a 1:1 solution of wax/xylene. The following day the wax/xylene mixture was melted at 60 °C and replaced with fresh molten wax. The samples were kept at 60 °C for 1 h and then the wax was replaced with fresh molten wax. After repeating this last step two more times, the samples were placed into blocks containing fresh molten wax and allowed to harden. The embedded samples were trimmed and 5–8 µm sections were cut from the samples (Leica sectioner), and the ribbon of sections was placed onto a water droplet on slides coated with polylysine (Sigma) and incubated at 42 °C until the sections had stretched. The water was removed from the slides and the samples were incubated at 42 °C overnight to firmly affix the samples to the polylysine-coated slides. The paraffin was removed from the sections through a series of xylene washes and the samples were rehydrated through a decreasing series of ethanol washes. Once rehydrated, the samples were blocked for 20 min in 10% milk powder in 20 mM Tris/HCl (pH 7.5), 0.5 M NaCl, 0.5% (w/w) Tween-80 (blocking buffer). The α -Cgl antibodies and pre-immune serum were incubated on the sections in blocking buffer for 2 h at room temperature. The sections were rinsed in blocking buffer and then incubated with the secondary antibody (rhodamine-conjugated goat anti-rabbit antibody) in blocking buffer for 1 h in the dark. The sections were rinsed in blocking buffer and then in 20 mM Tris/HCl (pH 7.5), 0.5 M NaCl, 0.5% Tween-80 and a drop of antifade reagent (Bio-Rad) was placed on each of the slides. The immunofluorescence was observed using a Zeiss Axiophot Photomicroscope with a Zeiss filter set 15.

For electron microscopy, tissue samples were fixed in 2.5% formaldehyde/0.5% glutaraldehyde in 0.1 M cacodylate

buffer. The samples were dehydrated in an increasing series of ethanol washes and then embedded in LR white resin (Sigma). The embedded samples were trimmed and sectioning was performed on a Reichert-Jung Ultracut E microtome with a diamond knife (Diatome). Ultrathin sections were transferred to 200 mesh carbon coated copper grids (Marivac). To immunolabel the sections, they were equilibrated in 20 mM Tris/HCl (pH 7.5), 0.5 M NaCl, 0.5% Tween-80, then blocked in 20 mM Tris/HCl (pH 7.5), 0.5 M NaCl, 0.5% Tween-80, 0.2% (v/v) Tween-20, 0.2% (w/v) glycine, 2% BSA (TBS/TG/BSA) and then washed in 20 mM Tris/HCl (pH 7.5), 0.5 M NaCl, 0.5%, 0.2% (v/v) Tween-20, 0.2% (w/v) glycine (TBS/TG). The sections were incubated in α -Cgl antibodies (1/100 dilution) or pre-immune serum (1/100 dilution) in TBS/TG/BSA for 16 h. The sections were washed in TBS/TG and then incubated in gold-conjugated protein A (in TBS/TG/BSA). After washing the grids in TBS/TG, then TBS, bound antibodies were fixed to the sections for 5 min in 3% (v/v) glutaraldehyde, 2% formaldehyde (v/v) in 25 mM Tris/HCl (pH 8.0). The sections were washed in water, air-dried, stained in saturated uranyl acetate in 70% ethanol for 15 min and washed in 70% ethanol and water. The grids were allowed to air dry and were viewed with a Hitachi electron microscope.

Immunoprecipitations and low-pH extraction of yeast cell-wall associated proteins. Yeast cells were grown to stationary phase in plasmid-selective minimal medium. Cells (2.5 OD₅₄₆ units) were harvested by centrifugation, resuspended in 0.5 ml minimal medium and incubated at either 25 °C or 37 °C for 30 min (to set the *sec18^{ts}* block). Trans³⁵S label [250 µCi (9250 kBq); ICN Radiochemicals] and protease inhibitors (1 µg chymotrypsin ml⁻¹, 1 µg pepstatin ml⁻¹ and 1 mM PMSF final concentration) were added to the cells. Following 30 min incubation at the appropriate temperature with shaking, the labelling reactions were stopped by the addition of an excess of non-radioactive methionine and cysteine. After a 1.5 h chase, the cells were pelleted, washed in 10 mM NaN₃, resuspended in low-pH extraction buffer (50 mM sodium citrate, pH 3.7, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF) and incubated at 37 °C for 45 min. The cells were vortexed for 5 s and the extracted proteins were separated from the intact cells by centrifugation at 10000 g for 10 s. The supernatant was centrifuged at 10000 g for 1 min to ensure that it was free of cells and proteins were precipitated by adding a one-tenth volume of 100% TCA. After centrifugation, both the cells and TCA precipitated samples were resuspended in 200 µl 50 mM Tris/HCl pH 7.5, 1% SDS. The cells were lysed by vortexing for 1 min with glass beads. Both the total cell extracts and the low-pH extracted cell-wall proteins were boiled for 3 min. Half of the total cell extract was used for immunoprecipitation with a mixture of α -Cgl and α -hexokinase antibodies, the other half with α -CPY antibodies. The cell-wall extracts were incubated with a mixture of α -Cgl and α -hexokinase antibodies. Immunoprecipitations, SDS-PAGE and fluorography were carried out as previously described (Franzoso *et al.*, 1991; te Heesen *et al.*, 1992). α -Cgl and α -hexokinase antibodies were used at a 1:500 dilution while the α -CPY antibodies were used at a 1:100 dilution.

RESULTS

The two galectins are expressed from adjacent loci

Previous studies showed that there are two distinct *Coprinus* galectin genes, *cgl1* and *cgl2* (Cooper *et al.*, 1997). Southern blot experiments (data not shown) and specific PCR analysis (Bottoli *et al.*, 1999), revealed that

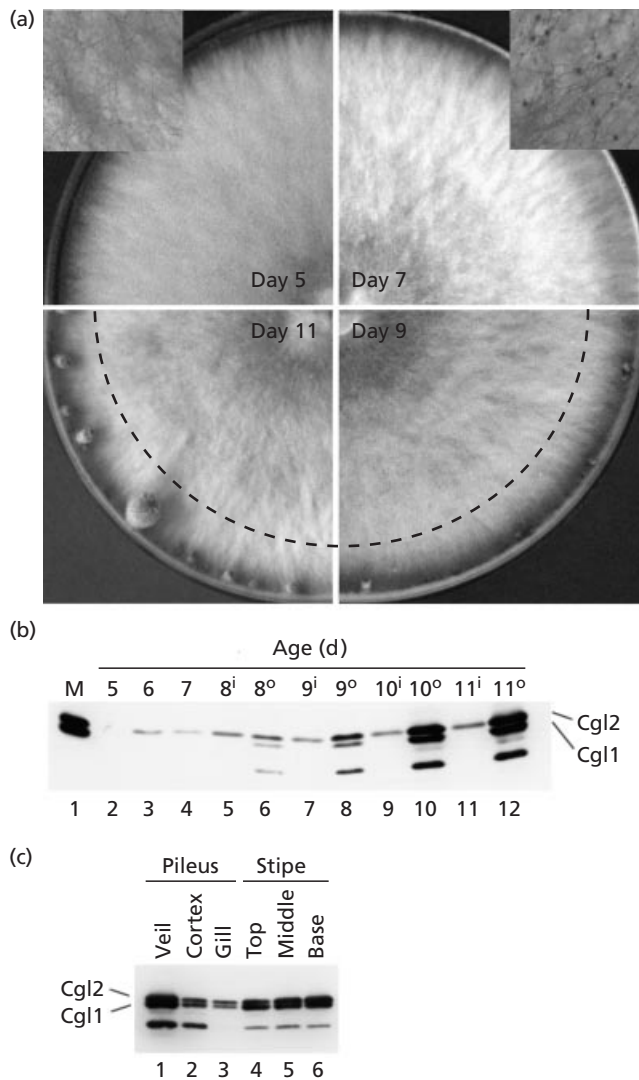


Fig. 3. Expression of *cgl1* and *cgl2* during fruiting body development. (a) Homokaryon AmutBmut was grown on solid YMG/T medium at 37 °C in constant dark for 5 d and then placed at 25 °C in a 12 h alternating light/dark regime. At day 5 (top left), the mycelium did not contain any differentiated structures, whereas at days 6 and 7, hyphal knots were present (top right). At days 8 and 9 (bottom right), 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 lane), whereas hyphal-knot formation was observed over the whole mycelium. The insets illustrate the mycelial structure of the culture, photographed through a stereo-microscope. (b) Analysis of galectin expression. The aerial mycelium and tissues were harvested, proteins extracted and separated by SDS-PAGE, transferred to nitrocellulose and probed with α -Cgl antibodies. The age of the culture is given above each lane and the positions of Cgl1 and Cgl2 are indicated. The total mycelium was harvested from cultures at days 5–7 (lanes 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) Tissue-specific expression in developing fruiting bodies. Meiotic-stage fruiting bodies (2.5 cm) were dissected and examined for galectin expression by Western blot analysis.

both *cgl1* and *cgl2* were present on the same genomic 7.1 kb *Bam*HI fragment of strain AmutBmut. An AmutBmut-derived cosmid library was constructed in a SuperCos derivative (Bottoli *et al.*, 1999) and the chromosomal region encompassing the *Coprinus* galectin gene was isolated. The 7.1 kb *Bam*HI fragment encoding the galectin genes was subcloned and sequenced (GenBank accession no. AF130360). The arrangement of the galectin genes deduced from this sequence is shown in Fig. 2(a). The galectin genes are transcribed from two adjacent, closely linked loci in the same direction (Fig. 2a).

The protein-coding sequences of the two galectin genes were not interrupted by introns, but two introns containing the typical *Coprinus* 5'- and 3'- splice site and branch-acceptor sequences (Seitz *et al.*, 1996) were identified in the 5' and the 3' untranslated regions (Fig. 2b). The removal of each intron from the primary RNA transcripts was verified by RT-PCR using specific primers flanking each of the introns (data not shown for the 5' introns, see below for evidence of splicing of the 3' introns).

Differential regulation of *cgl1* and *cgl2* expression during fruiting body development

To assess the level of galectin proteins during fungal development, we generated α -Cgl antibodies against galectin protein purified from fruiting bodies of strain AmutBmut. This serum recognized the two galectin proteins in Western blot experiments, and it was possible to separate the faster moving Cgl1 from Cgl2 by SDS-PAGE (Cooper *et al.*, 1997) (Fig. 3c, lane 1). These antibodies made it possible to examine Cgl1 and Cgl2 expression during fruiting body development of the homokaryotic strain AmutBmut.

To induce fruiting body development, we exposed dark-grown cultures to a 12 h light/dark cycle. Under these conditions, fruiting body development initiated with the formation of hyphal knots in the dark period. Upon light induction, these hyphal knots can differentiate into initials and primordia, where all the tissues that comprise the mushroom are present (see reviews by Moore, 1998; Kües, 2000) (Fig. 1). After completion of karyogamy in the probasidia, maturation to the fully developed fruiting body occurs (Lu, 1974) (Fig. 1). When grown for 5 d at 37 °C in the dark, cultures had not formed any developmental structures (Fig. 3a) and did not produce any galectins (Fig. 3b, lane 2). However, when such cultures were placed at 25 °C in a light/dark cycle, hyphal knots appeared the next day and Cgl2 protein was detected (day 6, Fig. 3b, lane 3). Hyphal knots differentiated into fruiting body initials and primordia 3 d after first exposure to light (day 8). Whereas hyphal knots developed in the 12 h alternating light/dark cycle over the whole mycelium, with increasing numbers towards the edge of the plate, initials and primordia grew almost exclusively at the edge of the

plate (Fig. 3a). This made it possible to separate the mycelium into inner non-fruiting and outer fruiting zones (Fig. 3a). When galectins were extracted from the non-fruiting area, only Cgl2 was detected (Fig. 3b, lanes 5, 7, 9 and 11), whereas the mycelial samples with the initials and primordia produced high levels of both Cgl1 and Cgl2 (Fig. 3b, lanes 6, 8, 10 and 12). In extracts derived from fruiting mycelium, we also detected a smaller protein of about 12 kDa (Fig. 3b) that, unlike the Cgls, reacts with the horseradish-peroxidase-coupled protein A in the absence of α -Cgl antibodies (P. J. Walser, R. C. Bertossa, U. K  es & M. Aebi, unpublished). This protein did not bind to lactosyl-Sepharose (data not shown) and is thus not another galectin.

To determine the expression of galectins in the mature fruiting body, different tissues of this structure were examined (Fig. 3c). The basidiocarp is composed of two major parts, the stipe (stalk) and the pileus (cap). Present on the surface of the pileus is a fluffy protective tissue called the veil. Under the veil tissue of the pileus is a rigid layer of cells called the pileus-cortex that supports the gill tissue containing the trama, subhymenium and hymenium (Cl  men  on, 1997). Both Cgl1 and Cgl2 were observed in the different tissues and both were present in relatively equal quantities, with the highest levels in the veil cells and the lowest levels in the gill tissue (Fig. 3c).

These data suggest that the two galectin genes are differentially expressed during fruiting body development: Cgl2 expression was correlated with the formation of hyphal knots and was also found in developing fruiting bodies, whereas Cgl1 was found exclusively in fruiting mycelium and was present at high levels in the tissue of fruiting bodies.

Differential regulation of *cgl1* and *cgl2* expression by environmental signals

Our data suggested that expression of the galectin proteins correlated with the induction of differentiation processes in the mycelium. Since differentiation of the mycelium is a response to external stimuli, we modulated the developmental processes by specific growth conditions. Thereby, it was possible to define more precisely the signals required for fruiting body development and to monitor the expression of the two galectin genes.

Recent results showed that hyphal-knot formation is repressed by blue light (K  es *et al.*, 1998) (Fig. 1). When kept in constant dark, fully grown cultures develop hyphal knots as a response to nutritional depletion. If exposed to light at 25   C, hyphal knots can develop into fruiting body initials (Fig. 1). If maintained in darkness at 37   C, these hyphal knots will differentiate into sclerotia (Fig. 1). When cultures were grown in continuous light at 37   C, hyphal-knot development was repressed. Hence, neither sclerotia nor fruiting bodies are formed on such cultures (K  es *et al.*, 1998). We

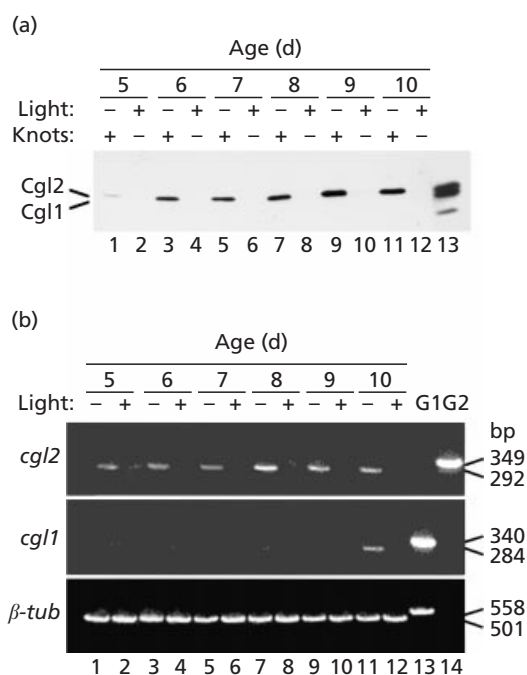


Fig. 4. *cgl2* is the primary galectin expressed during hyphal-knot development and both *cgl1* and *cgl2* are repressed by light. Cultures of homokaryon AmutBmut were grown on YMG/T at 37   C in either continuous dark (  ) or continuous light (+) over a period of 10 d. (a) Protein samples were isolated from the cultures and analysed for galectin expression. The presence (+) or absence (  ) of hyphal knots in the cultures is indicated above each lane. The positions of Cgl1 and Cgl2 are indicated. Purified galectins were applied as a marker (lane 13). (b) From the same mycelial material as (a) total RNA was extracted, cDNA was synthesized and analysed by PCR using gene-specific primers for *cgl2* (top), *cgl1* (middle) and β -tub (bottom) on agarose gels. Each of the primer pairs flanked a single short intron (the 3' intron for both *cgl1* and *cgl2*). Genomic DNA (G1, pBCG1 with *cgl1*, lane 13, and G2, pBCG2 with *cgl2*, lane 14) was analysed as a control. For the β -tub control, total genomic DNA of homokaryon AmutBmut was used (lane 13). The expected size of the amplified genomic fragment and the amplified cDNA fragment is indicated for each locus.

followed the expression of galectins in cultures grown at 37   C either in continuous dark or continuous light (Fig. 4). Galectin expression was observed only in cultures grown in continuous darkness, whereas continuous light repressed induction of both hyphal-knot formation and galectin expression. Galectin expression in dark-grown cultures coincided with the appearance of hyphal knots on the cultures (Fig. 4). With increasing age, the number of hyphal knots per culture increased, levelling off after 8 d incubation. By day 8, sclerotia were starting to develop from the hyphal knots, and by day 10 most hyphal knots had developed into the light-brown immature and dark-brown mature sclerotia. In the dark-grown cultures, Cgl2 was the predominant galectin detected, while only very low levels of Cgl1 were observed in the oldest cultures containing mature sclerotia. We analysed transcript levels of *cgl1* and *cgl2* by RT-PCR using *cgl1*- and *cgl2*-specific primers

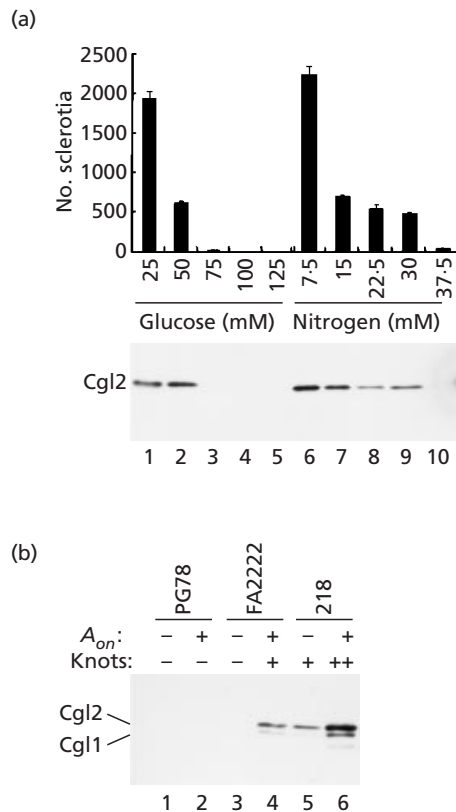


Fig. 5. Galectin expression is repressed by high levels of glucose and asparagine in the medium and regulated by the *A* mating-type locus. (a) Strain AmutBmut was cultured at 37 °C in continuous darkness for 12–15 d on minimal medium. Increasing levels of glucose (carbon source) or asparagine (nitrogen source) were present in the medium. At the time of harvest, the number of sclerotia per plate was counted (5 replicates for each concentration). The mean values are given with standard errors. The aerial mycelium of the same samples was harvested and the presence of galectins was assayed by Western blot analysis. The position of Cgl2 is indicated. (b) Three different monokaryons of *Coprinus* transformed with *trp1*⁺ vector DNA (*A_{on}*–) and with additional plasmid DNA carrying compatible *A* mating-type genes (*A_{on}*+) (Kües *et al.*, 1998) were cultured for 10 d on YMG/T at 37 °C in continuous darkness. Proteins were extracted and analysed for the presence of galectins and the occurrence (+) or absence (–) of hyphal knots in the cultures.

flanking the 3' introns (Fig. 4b). This analysis indicated that *cgl2* was expressed in all cultures that formed knots and sclerotia, while *cgl1* was only weakly expressed in the older cultures with mature sclerotia, but isolated sclerotia did not contain detectable levels of Cgl1 and Cgl2 protein (data not shown). In cultures grown in continuous light neither *cgl1* nor *cgl2* transcripts were detected (Fig. 4b).

High levels of carbon and nitrogen repress both galectin expression and knot development

Fruiting body development is not only controlled by light, but also by other environmental factors, since starvation conditions initiate this process (Madelin,

1960; Moore & Jirjis, 1976). When cultures were grown in complete darkness to induce hyphal-knot and sclerotia formation, we observed that increasing either the carbon source (glucose) or the nitrogen source (asparagine) in the medium repressed hyphal-knot and sclerotia formation (Fig. 5a), without affecting the morphology of the mycelium (data not shown). Increasing the glucose concentration in the medium was more effective than raising the nitrogen level. Interestingly, the level of Cgl2 expressed in the cultures correlated with the number of sclerotia present: high glucose or asparagine levels in the medium repressed Cgl2 expression (Fig. 5a). RT-PCR assays confirmed that the regulation of *cgl* expression by the carbon and the nitrogen source occurred at the transcriptional level (data not shown). Again, we were unable to detect Cgl1 protein in these dark-grown cultures, although with a sensitive RT-PCR assay we observed very low levels of *cgl1* expression (data not shown).

The *A* mating-type gene products modulate galectin expression

Besides the environmental factors that control differentiation in *Coprinus*, recent studies showed that the genetic disposition of the mycelium is also essential for the induction of differentiation processes. In particular, the *A* mating-type locus controls developmental processes. Monokaryotic strains transformed with a heterologous *A* mating-type gene exhibit altered developmental characteristics (Kertesz-Chaloupková *et al.*, 1998; Kües *et al.*, 1998). Monokaryons such as PG78 (*A*6 *B*42) and FA2222 (*A*5 *B*6) do not form hyphal knots in the dark and are therefore unable to develop higher-order structures. When transformed with compatible *A* mating-type genes [*A_{on}* phenotype displaying unfused clamp cells (Casselton & Olesnick, 1998; Kües, 2000)], strain PG78 is still incapable of hyphal-knot development, whereas *A_{on}* transformants of strain FA2222 form hyphal knots and sclerotia. Another monokaryon, strain 218 (*A*3 *B*1), produces hyphal knots in the dark in the absence of a compatible *A* mating-type gene. However, when a compatible *A* mating-type gene is introduced, the strain generates abundant hyphal knots (Kües *et al.*, 1998). The differential behaviour of the monokaryons made it possible to determine the role of the *A* mating-type locus on the regulation of galectin expression (Fig. 5b). Strain PG78 transformed with vector DNA or a compatible *A*43-derived *A* mating-type gene (*a1-2*) was unable to induce hyphal-knot formation and no galectin expression was detected (Fig. 5b, lanes 1 and 2). In strain FA2222, introduction of the heterologous *a1-2* gene induced both hyphal-knot formation and expression of *cgl2* that are absent in the vector control (Fig. 5b, lanes 3 and 4). Low levels of Cgl2 were also observed in monokaryon 218, but transformation with the *A*42-derived *a2-1* gene resulted in a strong increase of both hyphal knots and *cgl2* expression. Interestingly, the *A_{on}* transformants of strains FA2222 and 218 also produced low levels of Cgl1. The data show that the *A* mating-type locus is involved in the regulation

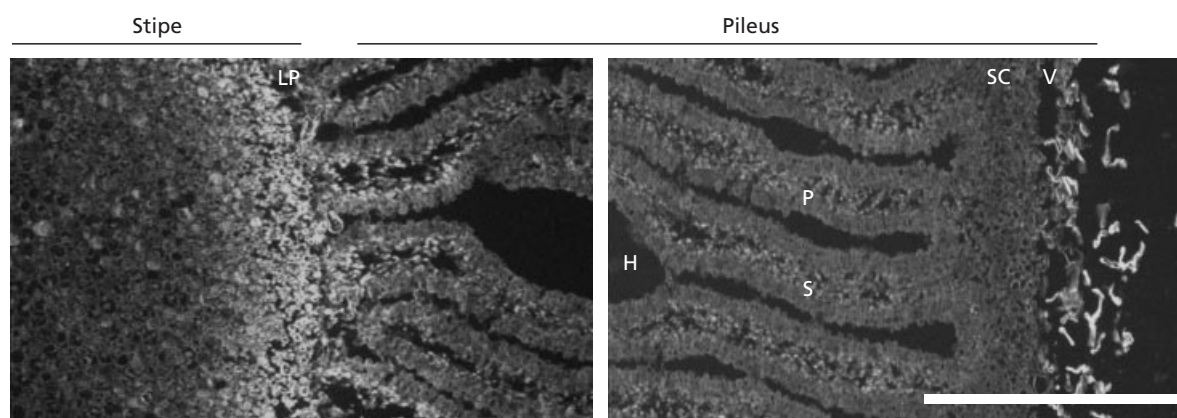


Fig. 6. Galectins are differentially localized in the fruiting bodies of *C. cinereus*. Semi-thin cross-sections (5–8 μm) from fruiting body tissue fixed in paraformaldehyde and embedded in paraffin wax, stained with $\alpha\text{-Cgl}$ and goat anti-rabbit rhodamine-conjugated antibodies. In this micrograph the stipe is to the left showing the lipsanoblem (LP), while the pileus is to the right. The hymenium (H) of primary (P) and secondary (S) gills are specified. The subcutis (SC) is covered by the sparse veil cells (V). Bar, 100 μm .

of *cgl1* and *cgl2* expression. Again, we noted a highly coordinated regulation of hyphal-knot formation and *cgl2* expression (Fig. 5b).

Localization of galectins within fruiting body tissues

Galectin expression was initiated by the induction of hyphal-knot formation, but galectins are most abundant in fruiting body tissues. To determine the localization of the galectins more precisely, an immunohistochemical approach was taken. A horizontal section through a developing cap (in a stage shortly after karyogamy) is shown in Fig. 6. Galectin labelling was observed throughout the fruiting body, within most tissues of the stipe and pileus. However, there were large differences in the level of labelling between some of the different tissues or cell types. The highest level of expression observed was within a group of cells that form the outer portion of the stipe, a region called the lipsanoblem (Cl  men  on, 1997), and strong labelling extended into the primary gills. Another region of intense labelling was along the veil cells of the pileus. These highly vacuolated cells showed high levels of labelling along the surface of the cells. Moderate labelling was observed in the central hyphae of the stipe, and also on the hyphae of the pileus-cortex. The lowest level of labelling was observed on the basidia present along the hymenium. All of the staining observed appeared to be present at the surface and in between the hyphae.

Visualization of galectin localization with immuno-electron microscopy

Our immunofluorescence analyses suggested that galectins reside on the surface of the cells within the fruiting body, but it was necessary to achieve higher resolution to pinpoint their localization within the tissues. We performed immuno-electron microscopy analyses using $\alpha\text{-Cgl}$ antibodies with tissue samples obtained from primordia and fruiting bodies. Within the stipe, dense

staining of the extracellular matrix was observed and staining was also apparent along the surface of the cell wall. The highest concentration of staining was observed along the surface and between the hyphae of the veil tissue, and between the hyphae that comprise the lipsanoblem (Fig. 7a–d). Consistently high staining was observed within the secondary cell wall at the dolipore septa of all tissues examined (Fig. 7d). In addition to the extracellular labelling, internal structures were stained with the $\alpha\text{-Cgl}$ antibodies, in particular in cytoplasmic vesicles of hyphae from the stipe (Fig. 7e). These vesicles were found to be between 0.35 μm and 0.75 μm in size and were enclosed by a single membrane structure (Fig. 7f). Finally, galectins were also observed within darkly staining regions within the cytoplasm, usually adjacent to the cell membrane (Fig. 7g). These structures were generally smaller than the vesicle structures and were approximately 0.14–0.2 μm in size.

Cgl2 is secreted via a non-classical secretory pathway

Even though we showed that the *Coprinus* galectins were secreted proteins, both proteins lack a classical secretory signal sequence and are not N-glycosylated despite the presence of a potential N-linked glycosylation site (Cooper *et al.*, 1997). The mammalian galectin-1 and galectin-3 are secreted proteins, but leave the cell independently of the classical secretory pathway (Sato *et al.*, 1993; Cleves *et al.*, 1996; Hughes, 1999): expression of the human galectin-1 in yeast showed that this protein was still secreted when the normal secretory pathway was blocked. Therefore, we took advantage of the highly developed experimental system in yeast and analysed the secretion of Cgl2 in *S. cerevisiae*.

Cell-wall proteins from stationary-phase yeast cells harbouring a *cgl2* expression vector were extracted by incubating the cells in low-pH (pH 3.7) buffer at 37 $^{\circ}\text{C}$. With this very mild extraction procedure, it was possible to solubilize Cgl2 without releasing the highly expressed

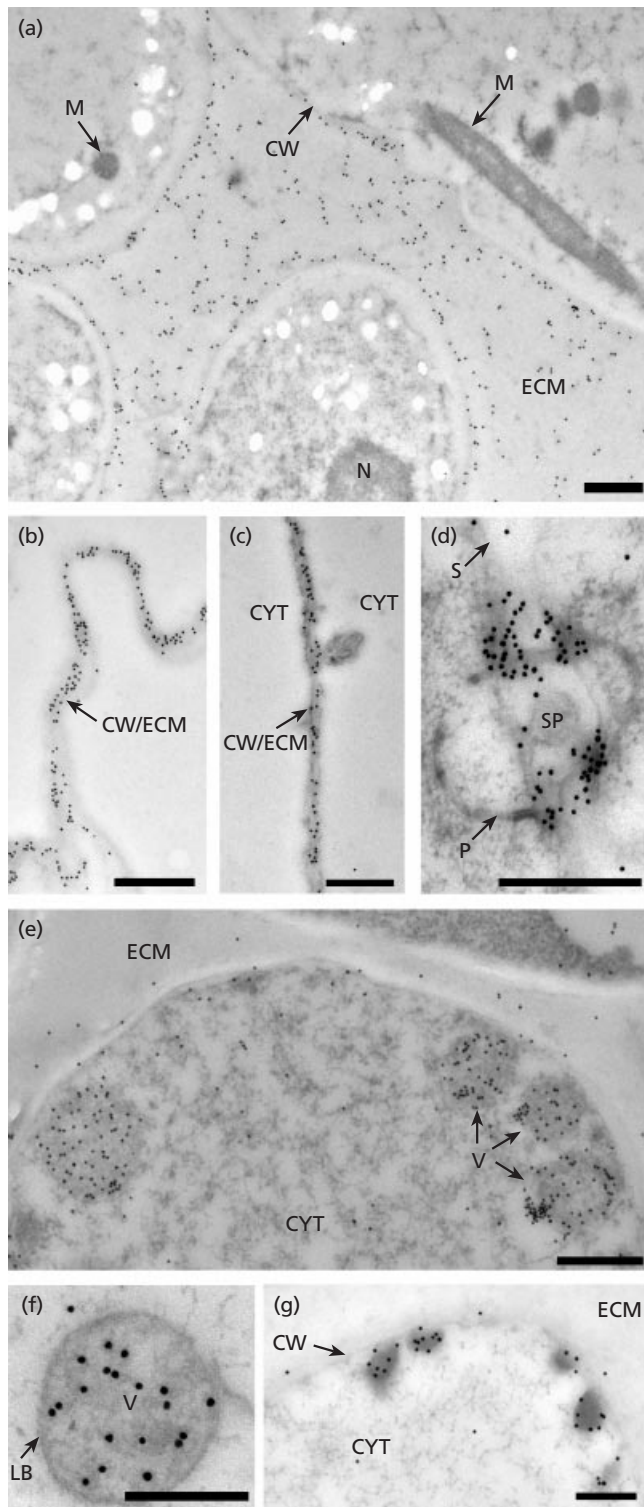


Fig. 7. Galectins are secreted in *Coprinus*. Electron micrographs of thin sections of *Coprinus* tissue embedded in LR white resin and preserved by glutaraldehyde/formaldehyde are shown. The sections were immunostained with anti-Cgl antibodies and 15 nm colloidal-gold conjugated protein A. (a) Galectin staining pattern on primordium tissue indicating the presence of mitochondria (M), nuclei (N), cell wall (CW) and the extracellular matrix (ECM). (b, c) Pileus tissue from meiotic fruiting bodies showing heavy galectin labelling within the

cytoplasmic protein hexokinase (Fig. 8a). We concluded that Cgl2 was secreted when expressed in *S. cerevisiae* and remained attached to, or trapped within, the cell wall. Based on the time course of extraction shown in Fig. 8(a), we chose 45 min as an optimal extraction time for further experiments.

To address the role of the classical secretory pathway in Cgl2 secretion, we followed the export in a yeast strain carrying the *sec18-1* mutation that results in a block of protein transport from the endoplasmic reticulum to the Golgi compartment at a non-permissive temperature (37 °C) (Eakle *et al.*, 1988). Wild-type and *sec18* cells were grown to stationary phase and incubated in fresh medium at 25 °C or 37 °C for 30 min. Newly synthesized proteins were then labelled by the addition of ^{35}S -labelled amino acids for 30 min, followed by a chase with an excess of non-radioactive amino acids. Cell-wall proteins were extracted; hexokinase and Cgl2 were immunoprecipitated from both total and cell-wall protein extracts using specific antibodies and analysed by SDS-PAGE (Fig. 8b). Cgl2 was secreted independently of the incubation temperature, suggesting that export of Cgl2 in yeast did not require the SEC18 protein. We also followed the maturation of the vacuolar protease CPY. In the endoplasmic reticulum of wild-type yeast, CPY is glycosylated (p1CPY, 67 kDa), modified in the Golgi and when translocated to the vacuole, a propeptide is cleaved away to yield mature CPY (mCPY, 63 kDa). In the *sec18* strain grown at 37 °C, p1CPY but no mCPY was detected, indicating the classical secretory pathway was blocked (Fig. 8c). These results demonstrate that the *C. cinereus* galectin is secreted by a pathway independent of the SEC18 protein, as is the case for human galectin-1 (Cleves *et al.*, 1996).

DISCUSSION

Regulation of fruiting body development and expression of galectins

Fruiting body formation is the most fascinating but also the most complex developmental process in the life cycle of *C. cinereus*. Environmental signals such as depletion of nutrients and light (Madelin, 1956a, b) stimulate the organism to produce a localized, highly ordered, multi-hyphal structure that requires extensive hyphal-hyphal interactions. Fruiting body formation initiates with the

highly vacuolated cells of the cortex and outer-veil cells respectively. CYT, cytoplasm. (d) Galectin localization within the septal pore (SP) region (dolipore) of the hyphae. Both sides of the septal pore are covered with a membrane structure called the parentheses (P). (e–g) Galectins are associated with membrane-bound vesicles (V) in the cytoplasm of hyphae from the stipe. (e) Galectin staining pattern of stipe tissue from meiotic fruiting bodies showing the presence of darkly staining bodies with galectin localized within them. (f) An enlarged view of another such vesicle showing the presence of a membrane (LB). (g) Small darkly staining regions associated with the cell membrane that show staining. Bars, 0.5 µm (a–d), 0.25 µm (e, f), 0.5 µm (g).

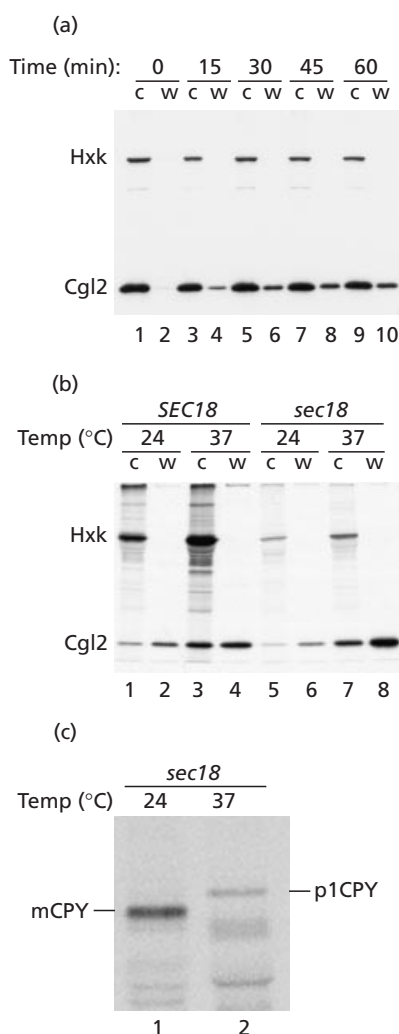


Fig. 8. Galectin secretion in yeast. (a) Strain BJ1991 transformed with pYAD-cgl2 was grown at 30 °C in liquid minimal medium to stationary phase. Cells were harvested and treated with low-pH extraction buffer at 37 °C for the times indicated. Protein extracts from each time point were prepared. Both total cellular (c) and cell-wall (w) protein samples for each time point were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with α -Cgl and Hxk antibodies. (b) Galectin secretion is independent of the classical secretory pathway. BJ1991 (SEC18) and PA33-6A (sec18) strains were incubated in stationary phase at 25 °C or 37 °C for 30 min and radiolabelled with 35 S-labelled amino acids at 25 °C and 37 °C for 30 min. Cell (c) and cell-wall (w) associated protein samples were prepared as before, an aliquot was immunoprecipitated with α -Cgl and Hxk antibodies, separated by SDS-PAGE (12.5%) and the presence of Cgl2 and Hxk was visualized by autoradiography. (c) Aliquots of the cell-associated protein samples from strain PA33-6A (sec18) incubated either at 25 °C (lane 1) or 37 °C (lane 2) were immunoprecipitated with anti-CPY antibodies, separated by SDS-PAGE (7%) and the positions of the endoplasmic reticulum-localized pro-CPY (p1CPY) and the vacuolar CPY (mCPY) was visualized by autoradiography.

formation of hyphal knots, areas of intense localized hyphal branching in the aerial mycelium (Matthews & Niederpruem, 1972; Kües, 2000). Formation of hyphal knots is repressed by light (Kües *et al.*, 1998), but light is

required as a positive signal to initiate further development of a hyphal knot into a fruiting body initial and, later in the process, to trigger karyogamy and meiosis (Lu, 1974; reviewed by Elliot, 1994; Kües, 2000). Using defined growth conditions, we were able to dissect the pathway of development into distinct steps requiring defined signals for initiation (Fig. 1) and, most importantly, we showed that distinct developmental processes correlate with the specific expression of two fungal galectins. While *cgl2* expression is coordinated with hyphal-knot development, *cgl1* is specifically expressed during fruiting body formation (Fig. 3). From this analysis it is apparent that *cgl2* expression is initiated as a result of the primary signals (depletion of nutrients) required for hyphal-knot formation, whereas *cgl1* expression is fruiting body-pathway specific. The Cgl proteins can now be used as markers to study the process of development in *Coprinus* at the molecular level. With the two Cgl proteins as a read-out system, it will be possible to define the different signalling pathways that control fruiting body development at a molecular level. Light, nutritional resources and the A mating-type products have in this study been shown to control expression of galectin genes. It is possible that light-activated factors, nutritional cues and the A mating-type proteins may control *cgl2* and also *cgl1* expression directly. Unfortunately, factors involved in the light-receptor pathway and in nutritional signalling have not yet been identified in *Coprinus*, nor have the binding site(s) of the *Coprinus* A mating-type products. In the future, we hope that the galectin genes and their promoters may provide insight into how these factors influence gene expression in *Coprinus*.

Although the expression of *cgl2* was regulated by the same factors that regulate hyphal-knot development, and the protein was localized to the hyphal knots (data not shown), we do not know whether Cgl2 protein is required for hyphal-knot formation. We have isolated mutant strains that form hyphal knots but are unable to enter the fruiting body-specific differentiation pathway (Y. Liu, M. Aebi & U. Kües, unpublished) and some of the mutant strains do not express galectins. These mutant strains indicate that Cgl2 expression might not be necessary for hyphal-knot formation. However, all mutant strains unable to form fruiting body primordia were also deficient in the expression of Cgl1 protein, suggesting that this protein is involved in fruiting body formation. Specific knock-out experiments are in progress to determine whether the two proteins are essential for the developmental processes.

Cellular and subcellular localization of the galectins

Within the fruiting bodies of *Coprinus* the galectins were localized throughout most tissues, but primarily within the veil and the lipsanoblem (Fig. 6). These two tissues are distinct, yet both are subjected to enormous levels of stress during fruiting body development. During early stages of primordial development the veil tissue covers the entire embryonic fruiting body. As the fruiting body grows, the veil tissue is stretched and

eventually torn. The only remnants of the veil in the mature fruiting body is a sparse web-like tissue present on the surface of the pileus (Cl  men  on, 1997). Another tension is generated on the lipsanoblem of the stipe due to cap expansion. As shown in Fig. 6, this tissue is directly connected to the primary gills and thereby provides an anchor required during expansion of the cap mediated by tangential growth of the meristemoid tissue of the cap (Reijnders, 1979; for review see Moore, 1998). The same tissue of the stipe is also involved in the process of rapid stipe elongation (see review by Kamada, 1994) where a strong hyphal adherence is essential for stability of the stipe. It is tempting to speculate about the function of the two galectin proteins. The localization of the proteins in the ECM suggests that they are involved in hyphal–hyphal interactions, mediated by their ability to bind specific carbohydrates of the cell wall. Homodimer formation, a prerequisite of this hypothesis, has been demonstrated for the Cgl2 protein (Cooper *et al.*, 1997). The tension applied on the outer stipe and cap tissues requires an increased connectivity of the hyphae forming them. The high level of galectins in the extracellular matrix of the lipsanoblem suggests that they contribute to a strong hyphal–hyphal interaction.

Secretion of the galectins

Immunolocalization as well as the expression of Cgl2 protein in yeast demonstrate that the *Coprinus* galectins are secreted, yet both Cgl1 and Cgl2 lack the hydrophobic N termini typical of proteins secreted by the classical secretory pathway. In addition, the fungal galectins are not post-translationally modified (Cooper *et al.*, 1997). In animals, galectin-1 and galectin-3 are secreted independently of the classical secretory pathway (Cooper & Barondes, 1990; Sato *et al.*, 1993; Mehul & Hughes, 1997; reviewed by Hughes, 1999). Our analysis of Cgl2 secretion in yeast suggests that as found for human galectin-1 (Cleves *et al.*, 1996), the export does not occur via the classical Sec18p-dependent pathway. It has been suggested that alternate routes of secretion maintain the separation of ligands from their receptors until a proper contextual environment is reached outside the cell (Bejcek *et al.*, 1989; Dunbar *et al.*, 1989). Since all glycosylated derivatives emerge from the endoplasmic reticulum/Golgi, the alternate mechanism of secretion of mammalian galectin-1 has been postulated to prevent intracellular binding of galectin-1 to its galactose receptor (Cooper *et al.*, 1991) and to provide a means for developmental modulation of secretion by external factors present in the extracellular matrix (Hughes, 1999). In mammalian systems, other proteins known to be secreted by non-classical mechanisms include basic fibroblast growth factor (bFGF) (Yu *et al.*, 1993), interleukin-1   [IL-1   (Rubartelli *et al.*, 1990)] and thioredoxin (Rubartelli *et al.*, 1992). Both bFGF and IL-1   are present within cytoplasmic vesicles, and in the case of bFGF, these vesicles have been shown to fuse with the plasma membrane, discharging bFGF from the cell (Qu *et al.*, 1998). Our subcellular immunolocalization experiments indicated that the *Coprinus*

galectins were also localized within cytoplasmic vesicles and in darkly staining aggregates associated with the cell membrane (Fig. 7). As is the case with bFGF and IL-1  , it is possible that the fungal galectins are secreted via cytoplasmic vesicles.

In filamentous fungi, classical protein secretion occurs predominantly at the growing hyphal tip (W  sten *et al.*, 1991; Moukha *et al.*, 1993). The finding that the *Coprinus* galectins are capable of being secreted via a non-classical route indicates that galectin secretion may not be limited to the hyphal tip. Such a system would enable the fungus to modulate its entire hyphal surface at specific periods during development. If galectins play a role in hyphal–hyphal interactions in the fruiting body, this may be an important mechanism to consider, because fungal morphogenesis is often achieved by rapid growth of cells already present within the tissue and not by cellular division (see the reviews by Gooday, 1975; Kamada, 1994; Moore, 1998). Thus, hyphal growth occurs along the lateral walls of the hyphae and not at the tip. During such growth, cell-wall biogenesis occurs equally over the cell-wall surface (reviewed by Gooday, 1975, 1982). Non-classical secretion might provide means of delivering large amounts of cell-wall components (including galectins) to the ECM, independent of hyphal-tip growth.

Celerin *et al.* (1997) identified fungal collagens (fungal fimbriae), indicating that components of the ECM evolved prior to the divergence of animals and fungi. Our localization of the fungal galectins to the ECM strengthens this point and shows that two characteristic proteins of the animal ECM also exist in fungi. Such conservation of the components of the ECM suggests that the interactions present between the ECM are also conserved. In animal systems, galectins bind certain collagens (Warfield *et al.*, 1997; Ochieng *et al.*, 1998; Sasaki *et al.*, 1998). The fungal collagens have glycoproteinaceous subunits (Castle *et al.*, 1992) and homologues have been identified in *C. cinereus* (Castle & Boulianne, 1991). It will be interesting to see if their animal counterparts, the fungal galectins, interact with fungal collagen in the ECM of the fruiting body.

ACKNOWLEDGEMENTS

We thank Stephan Schr  der and Stephan te Heesen for providing yeast antisera, Tobias Schmelze for gifts of yeast strains and expression vectors, Rinaldo Bertossa and Piers Walser for unpublished data and Piers Walser for critical reading of the manuscript. This work was supported by Swiss National Science Foundation grants 3100-040350 (to M.A.) and 31-46'940.96 (to U.K.), the Natural Sciences and Engineering Research Council of Canada grant A4693 (to B.C.L.) and the ETH Z  rich.

REFERENCES

- Barondes, S. H., Cooper, D. N., Gitt, M. A. & Leffler, H. (1994). Galectins: structure and function of a large family of animal lectins. *J Biol Chem* **269**, 20807–20810.
- Bejcek, B. E., Li, D. Y. & Deuel, T. F. (1989). Transformation by v-

- sis occurs by an internal autoactivation mechanism. *Science* **245**, 1425–1499.
- Bickle, M., Delley, P. A., Schmidt, A. & Hall, M. N. (1998).** Cell wall integrity modulates RHO1 activity via the exchange factor ROM2. *EMBO J* **17**, 2235–2245.
- Bottoli, A., Kertesz-Chaloupková, K., Boulianne, R. P., Granado, J. D., Aebi, M. & Kües, U. (1999).** Rapid isolation of genes from an indexed library of *C. cinereus* in a novel *pab1*⁺-cosmid. *J Microbiol Methods* **35**, 129–141.
- Brunelli, J. P. & Pall, M. L. (1993).** A series of yeast shuttle vectors for expression of cDNAs and other DNA sequences. *Yeast* **9**, 1299–1308.
- Casselton, L. & Olesnick, N. S. (1998).** Molecular genetics of mating recognition in basidiomycete fungi. *Microbiol Mol Biol Rev* **62**, 55–70.
- Castle, A. J. & Boulianne, R. P. (1991).** Fimbrial proteins of *Ustilago violacea*, *Coprinus cinereus*, and *Schizophyllum commune*. *MSA Newsletter* **42**, 8.
- Castle, A. J., Boulianne, R., Xu, J. & Day, A. W. (1992).** Post translational modification of fimbrial protein from *Ustilago violacea*. *Can J Microbiol* **38**, 1144–1149.
- Celerin, M., Ray, J. M., Schisler, N. J., Day, A. W., Stetler-Stevenson, W. G. & Laudenbach, D. E. (1997).** Fungal fimbriae are composed of collagen. *EMBO J* **15**, 4445–4453.
- Charlton, S., Boulianne, R., Chow, Y. & Lu, B. C. (1992).** Cloning and differential expression during the sexual cycle of a meiotic endonuclease-encoding gene from the basidiomycete *Coprinus cinereus*. *Gene* **122**, 163–169.
- Chomczynski, P. & Sacchi, N. (1987).** Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**, 156–159.
- Cléménçon, H. (1997).** *Anatomie der Hymenomyceten: eine Einführung in die Cytologie und Plectologie der Krustenpilze, Porlinge, Keulenpilze, Leistlinge, Blätterpilze und Röhrlinge. Anatomy of the Hymenomycetes: an Introduction to the Cytology and Plectology of Crust Fungi, Bracket Fungi, Club Fungi, Chanterelles, Agarics and Boletes.* Teufen, Switzerland: Flück-Wirth.
- Cleves, A. E., Cooper, D. N. W., Barondes, S. H. & Kelly, R. B. (1996).** A new pathway for protein export in *Saccharomyces cerevisiae*. *J Cell Biol* **133**, 1017–1026.
- Cooper, D. N. W. & Barondes, S. H. (1990).** Evidence for export of a muscle lectin from cytosol to extracellular matrix and for novel secretory mechanism. *J Cell Biol* **110**, 1681–1691.
- Cooper, D. N. W., Massa, S. M. & Barondes, S. H. (1991).** Endogenous muscle lectin inhibits myoblast adherence to laminin. *J Cell Biol* **115**, 1437–1448.
- Cooper, D. N. W., Boulianne, R. P., Charlton, S., Farrell, E. M., Sucher, A. & Lu, B. C. (1997).** Fungal galectins: sequence and specificity of two isolectins from *Coprinus cinereus*. *J Biol Chem* **272**, 1514–1521.
- Dunbar, C. E., Browder, T. M., Abrams, J. S. & Nienhuis, A. W. (1989).** COOH-terminal-modified interleukin-3 is retained intracellularly and stimulates autocrine growth. *Science* **245**, 1493–1496.
- Eakle, K. A., Bernstein, M. & Emr, S. D. (1988).** Characterization of a component of the yeast secretion machinery: identification of the *SEC18* gene product. *Mol Cell Biol* **8**, 4098–4109.
- Elliot, C. G. (1994).** *Reproduction in Fungi.* London: Chapman & Hall.
- Franzusoff, A., Rothblatt, J. & Schekman, R. (1991).** Analysis of polypeptide transit through the yeast secretory pathway. *Methods Enzymol* **194**, 662–674.
- Gooday, G. W. (1975).** The control of differentiation in fruit bodies of *Coprinus cinereus*. *Rep Tottori Mycol Inst* **12**, 151–160.
- Gooday, G. W. (1982).** Metabolic control of fruitbody morphogenesis in *Coprinus cinereus*. In *Basidium and Basidiocarp*, pp. 157–174. Edited by K. Wells & E. K. Wells. New York: Springer.
- Gooday, G. W. (1995).** Cell walls. In *The Growing Fungus*, pp. 43–74. Edited by N. A. R. Gow & G. M. Gadd. London: Chapman & Hall.
- Granado, J., Kertesz-Chaloupková, K., Aebi, M. & Kües, U. (1997).** Restriction enzyme-mediated DNA integration in *Coprinus cinereus*. *Mol Gen Genet* **256**, 28–36.
- Guillot, J. & Kanska, G. (1997).** Lectins in higher fungi. *Biochem Syst Ecol* **25**, 203–230.
- te Heesen, S., Janetzky, B., Lehle, L. & Aebi, M. (1992).** The yeast WBP1 is essential for oligosaccharyltransferase activity *in vivo* and *in vitro*. *EMBO J* **11**, 2071–2075.
- Hiscock, S. J. & Kües, U. (1999).** Cellular and molecular mechanisms of sexual incompatibility in plants and fungi. *Int Rev Cytol* **193**, 165–295.
- Hughes, R. C. (1999).** Secretion of the galectin family of mammalian carbohydrate-binding proteins. *Biochim Biophys Acta* **1473**, 172–185.
- Ito, H., Fukuda, Y., Murata, K. & Kimura, A. (1983).** Transformation of intact yeast cells treated with alkali cations. *J Bacteriol* **153**, 3153–3162.
- Jones, E. W. (1977).** Bipartite structure of the *ade3* locus of *Saccharomyces cerevisiae*. *Genetics* **85**, 209–223.
- Kamada, T. (1994).** Stipe elongation in fruit bodies. In *The Mycota*, vol. 1, *Growth, Differentiation and Sexuality*, pp. 367–379. Edited by J. G. H. Wessels & F. Meinhardt. Berlin: Springer.
- Kapteyn, J. C., Van Den Ende, H. & Klis, F. M. (1999).** The contribution of cell wall proteins to the organization of the yeast cell wall. *Biochim Biophys Acta* **1426**, 373–383.
- Kertesz-Chaloupková, K., Walser, P. J., Granado, J. D., Aebi, M. & Kües, U. (1998).** Blue light overrides repression of asexual sporulation by mating-type genes in the basidiomycete *Coprinus cinereus*. *Fungal Genet Biol* **23**, 95–109.
- Kües, U. (2000).** Life history and developmental processes in the basidiomycete *Coprinus cinereus*. *Microbiol Mol Biol Rev* **64**, 316–353.
- Kües, U., Granado, J. D., Hermann, R., Boulianne, R. P., Kertesz-Chaloupková, K. & Aebi, M. (1998).** The A mating type and blue light regulate all known differentiation processes in the basidiomycete *Coprinus cinereus*. *Mol Gen Genet* **260**, 81–91.
- Laemmli, U. K. (1970).** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lu, B. C. (1974).** Meiosis in *Coprinus*. V. The role of light on basidiocarp initiation, mitosis and hymenium differentiation in *Coprinus lagopus*. *Can J Bot* **52**, 299–305.
- Madelin, M. F. (1956a).** The influence of light and temperature on fruiting of *Coprinus lagopus* Fr. in pure culture. *Ann Bot* **20**, 467–480.
- Madelin, M. F. (1956b).** Studies on the nutrition of *Coprinus lagopus* Fr., especially as affecting fruiting. *Ann Bot* **20**, 307–330.
- Madelin, M. F. (1960).** Visible changes in the vegetative mycelium of *Coprinus lagopus* Fr. at the time of fruiting. *Trans Br Mycol Soc* **43**, 105–110.

- Matsuo, T., Yamamoto, Y., Muraguchi, H. & Kamada, T. (1999).** Effects of amino-acid substitutions in β -tubulin on benomyl sensitivity and microtubule functions in *Coprinus cinereus*. *Mycoscience* **40**, 241–250.
- Matthews, R. R. & Niederpruem, D. J. (1972).** Differentiation in *Coprinus lagopus*. I. Control of fruiting and cytology of initial events. *Arch Mikrobiol* **87**, 257–268.
- May, G., Le Chevanton, L. & Pukilla, P. J. (1991).** Molecular analysis of the *Coprinus cinereus* mating type A factor demonstrates an unexpectedly complex structure. *Genetics* **128**, 529–538.
- Mehul, B. & Hughes, R. C. (1997).** Plasma membrane targeting, vesicular budding and release of galectin 3 from the cytoplasm of mammalian cells during secretion. *J Cell Sci* **110**, 1169–1178.
- Moore, D. (1981).** Developmental genetics of *Coprinus cinereus*: genetic evidence that carpophores and sclerotia share a common pathway of initiation. *Curr Genet* **3**, 145–150.
- Moore, D. (1995).** Tissue formation. In *The Growing Fungus*, pp. 423–466. Edited by N. A. R. Gow & G. M. Gadd. London: Chapman & Hall.
- Moore, D. (1998).** *Fungal Morphogenesis*. Cambridge: Cambridge University Press.
- Moore, D. & Jirjis, R. I. (1976).** Regulation of sclerotium production by primary metabolites in *Coprinus cinereus*. *Trans Br Mycol Soc* **66**, 377–382.
- Moore, D., Elhiti, M. M. Y. & Butler, R. D. (1979).** Morphogenesis of the carpophore of *Coprinus cinereus*. *New Phytol* **66**, 377–382.
- Moukha, S. M., Wösten, H. A. B., Asther, M. & Wessels, J. G. H. (1993).** *In situ* localization of the secretion of lignin peroxidases in colonies of *Phanerochaete chrysosporium* using a sandwiched mode of culture. *J Gen Microbiol* **139**, 969–978.
- Ochieng, J., Leite-Browning, M. L. & Warfield, P. (1998).** Regulation of cellular adhesion to extracellular matrix proteins by galectin-3. *Biochem Biophys Res Commun* **246**, 788–791.
- Qu, Z., Kayton, R. J., Ahmadi, P., Liebler, J. M., Powers, M. R., Planck, S. R. & Rosenbaum, J. T. (1998).** Ultrastructural immunolocalization of basic fibroblast growth factor in mast cell secretory granules: morphological evidence for bFGF release through degranulation. *J Histochem Cytochem* **46**, 1119–1128.
- Rao, P. S. & Niederpruem, D. J. (1969).** Carbohydrate metabolism during morphogenesis of *Coprinus lagopus*. *J Bacteriol* **100**, 1222–1228.
- Reijnders, A. F. M. (1979).** Developmental anatomy of *Coprinus Persoonia* **10**, 383–424.
- Rubartelli, A., Cozzolino, F., Talio, M. & Sitia, R. (1990).** A novel secretory pathway for interleukin-1 β , a protein lacking a signal sequence. *EMBO J* **9**, 1503–1510.
- Rubartelli, A., Bajetto, A., Allavena, G., Wollman, E. & Sitia, R. (1992).** Secretion of thioredoxin by normal and neoplastic cells through a leaderless secretory pathway. *J Biol Chem* **267**, 24161–24164.
- Sasaki, T., Brakebusch, C., Engel, J. & Timpl, R. (1998).** Mac-2 binding protein is a cell-adhesive protein of the extracellular matrix which self-assembles into ring-like structures and binds beta1 integrins, collagens and fibronectin. *EMBO J* **17**, 1606–1613.
- Sato, S., Burdett, I. & Hughes, C. (1993).** Secretion of the baby hamster kidney 30-kDa galactose-binding lectin from polarized and nonpolarized cells: a pathway independent of the endoplasmic reticulum-golgi complex. *Exp Cell Res* **207**, 8–18.
- Seitz, L. C., Teliang, K., Cummings, W. J. & Zolan, M. E. (1996).** The *rad9* gene of *Coprinus cinereus* encodes a proline-rich protein required for meiotic chromosome condensation and synapsis. *Genetics* **142**, 1105–1117.
- Swamy, S., Uno, I. & Ishikawa, T. (1984).** Morphogenic effects of mutations at the A and B incompatibility factors in *Coprinus cinereus*. *J Gen Microbiol* **130**, 3219–3224.
- Wang, H., Ng, T. B. & Ooi, V. E. C. (1998).** Lectins from mushrooms. *Mycol Res* **102**, 897–906.
- Warfield, P. R., Makker, P. N., Raz, A. & Ochieng, J. (1997).** Adhesion of human breast carcinoma to extracellular matrix proteins is modulated by galectin-3. *Invasion Metastasis* **17**, 101–112.
- Wösten, H. A. B., Moukha, S. M., Sietsma, J. H. & Wessels, J. G. H. (1991).** Localization of growth and secretion of proteins in *Aspergillus niger*. *J Gen Microbiol* **137**, 2017–2023.
- Yu, Z. X., Biro, S., Fu, Y. M., Sanchez, J., Smale, G., Sasse, J., Ferrans, V. J. & Casscells, W. (1993).** Localization of basic fibroblast growth factor in bovine endothelial cells: immunohistochemical and biochemical studies. *Exp Cell Res* **204**, 247–259.
- Zolan, M. & Pukkila, P. (1986).** Inheritance of DNA methylation in *Coprinus cinereus*. *Mol Cell Biol* **6**, 195–200.

Received 23 February 2000; accepted 9 May 2000.