PROTEINS EXPRESSED DURING HYPHAL AGGREGATION FOR FRUITING BODY FORMATION IN BASIDIOMYCETES

KIRAN K.R. LAKKIREDDY[1], MÓNICA NAVARRO-GONZÁLEZ[1], RAJESH VELAGAPUDI[1,2], URSULA KÜES[1]

[1]Georg-August-Universität Göttingen, Büsgen-Institut, Molekulare Holzbiotechnologie und technische Mykologie
Büsgenweg 2, 37077 Göttingen
Germany

[2] Present address: Department of Medicine, University of Massachusetts Medical School,
Worcester, MA 01605
USA

kiran-kumar.reddy@forst.uni-goettingen.de, mnavarr@gwdg.de, velagapudidms@yahoo.co.in, ukuees@gwdg.de

ABSTRACT

The first visible step in fruiting body development in basidiomycetes is the formation of small hyphal knots by localized intense branching of hyphae of restricted length accompanied by hyphal aggregation. In *Coprinopsis cinerea*, the first not yet fruiting-specific step of hyphal branching occurs in the dark, the second step requires a light signal. Hyphal aggregation implies cell-cell contacts and protein interactions on the outer cell walls are anticipated. Few protein candidates were identified and discussed in the past for such function. Amongst were the galectins of *C. cinerea* and the Aa-Pri1 protein (aegerolysin) of *Agrocybe aegerita* that are specifically expressed during the step of hyphal aggregation as well as during subsequent primordia development. In this study, we follow up the distribution of genes for proteins with lectin and/or hemolysin function in the steadily growing number of available genomes of basidiomycetes. Neither galectin genes nor genes for other lectins nor *Aa-pri1*-like genes nor other hemolysin genes are present in all mushroom species, making an essential role for such functions in hyphal aggregation unlikely.

Keywords: Lectin, hemolysin, mushroom formation, hyphal knots, predator defence

INTRODUCTION

Vegetative mycelial growth of filamentous fungi basically consists of tip growth of leading hyphae with sporadic subterminal initiation of a sidebranch that then also undergoes tip elongation for further growth [1]. Such simple mycelial growth can thus locally be considered as just two-dimensional. Fruiting body development in contrast is a complex process which changes from simple two-dimensional vegetative growth of the mycelium to formation of a compact three-dimensional aggregated structure in which differentiation of specific cap and stipe tissues takes place [2]. The first visible structure is the hyphal knot generated by intense localized formation of stunted, growth-restricted sidebranches that interweave and eventually aggregate with each other. In *Coprinopsis cinerea*, we distinguish primary from secondary hyphal knots (Figs. 1, 2) which form within dark and subsequently upon (blue) light illumination, respectively [3, 4]. Initiation of hyphal aggregation is controlled by the mating type genes [5, 6] and environmental factors – which in addition to light are temperature, nutrients, humidity, and aeration [2, 7] –, but little is yet known on the cellular processes leading to aggregation. Different proteins have however been implicated in basidiomycetes in functioning in hyphal aggregation, based on observations of coincidental expression of their genes with initiation of fruiting and subsequent primordia development [7, 8].

Many different types of sugar moieties-binding lectins are known to occur in mushrooms as candidate proteins for mediating cellular aggregation [8, 9]. Galectins are β-

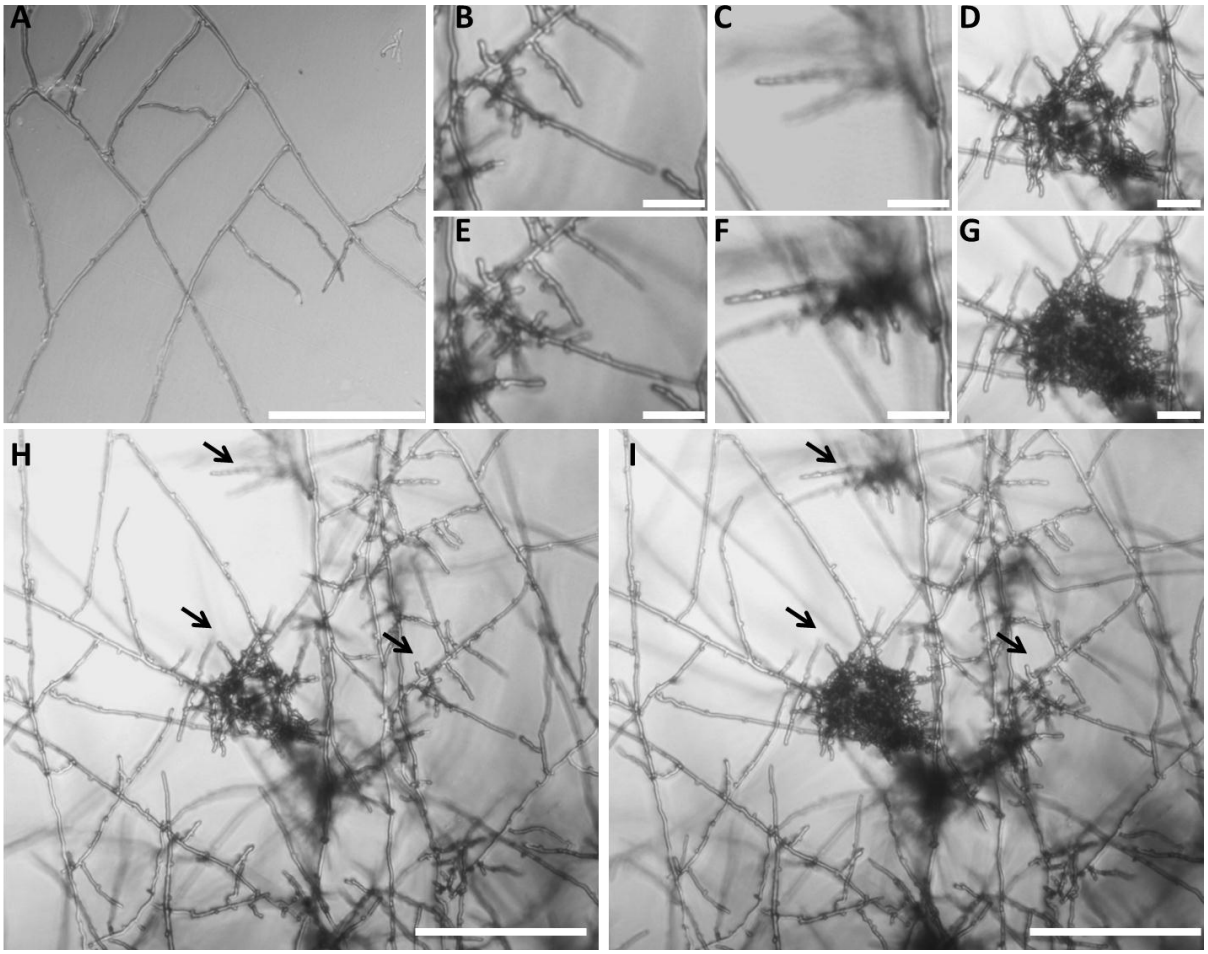

Figure 1: Primary hyphal knot formation of *C. cinerea* homokaryon AmutBmut in an YMG/T agar culture: Mycelial lattice after 24 h of growth (A). Primary hyphal knots within the lattice after 40 h (B-D,H) and 60 h of growth (E-G,I). Arrows in I and H point to structures shown enlarged in B-D and E-G, respectively. Size bar = 20 µm (B-G), = 100 µm (A,H,I).

galactoside binding lectins characterized by a specific sugar-binding domain [10]. In *C. cinerea*, expression of galectins (CGL1, CGL2) and a galectin-related lectin (CGL3) starts at the stages of primary, respectively secondary hyphal knot formation and continues throughout primordia formation. Galectins are secreted and localize to cell walls and the extracellular matrix (ECM) of mushroom tissues. Highest expression is found in the outer cap and outer stipe tissues [4, 11-13]. Although a function in cell-cell aggregation had been postulated [4], more recent studies showed that the proteins are not essential and point to a role in protection against grazing arthropods and nematodes [14-16]. Related mushroom-specific galectins exist in *Agrocybe aegerita* (syn. *cylindracea*) [17, 18], *Heterobasidion annosum* (*irregulare*) [8], *Laccaria bicolor* and *Laccaria amethystina* [12, 19]. Application of isolated *A. aegerita* galectin AAL in fresh cultures reduced mycelial growth rates and induced mycelial cord formation. Most interestingly, application on own and on also foreign (*Auricularia polytricha*) established mycelium resulted in formation of aggregates and primordia differentiation [20, 21]. Also *Agrocybe* lectins have anti-nematode activities [22].

Members of another family of β-galactoside binding lectins [FB (fungal fruit body) lectin super-family] occur in *Athelia* (*Sclerotium*) *rolfsii* (SRL; SLR-like), *Agaricus bisporus* (ABL), *Xerocomus chrysenteron* (XCL), *Pleurotus cornucopiae* (PCL-M, PCL-F), *Boletus edulis* (BCL) and *Paxillus involutus* [23-30]. Functions in aggregation in sclerotia formation and in inhibition of sclerotia germination have been reported for SRL in *A. rolfsii* [31].

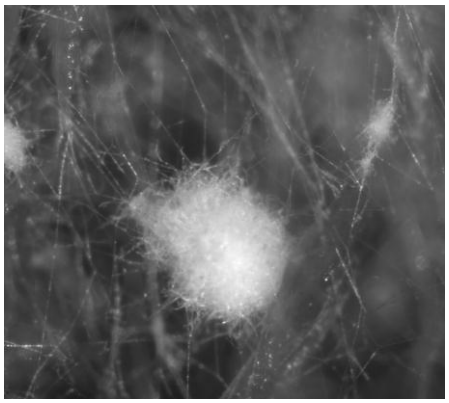

Figure 2: View on an about 100 µm sized secondary hyphal knot (center) grown on horse dung and neighbouring primary hyphal knots to the left and right.

PCL-F is envisaged to contribute to aggregation during fruiting although lectin-deficient *P. cornucopiae* mushrooms of normal shape appear to exist [32, 33], SRL, SLR-like and XCL showed anti-nematodal and insecticidal activities [24,26,34]. Structurally, ABL and XCL resemble actinoporins [27, 35], a family of membrane-integrating pore-forming toxins that act hemolytic [36]. LSLa is one of three closely related lectins (LSLa to LSLc) from *Laetiporus sulphureus* and represents another small characterised mushroom protein with combined lectin and pore-forming activities. This hemolysin divides into an N-terminal lectin-domain and a C-terminal porin domain of the haemolytic aerolysin protein family [37]. Further mushroom lectins are represented by ricin B-type proteins from *Clitocybe nebularis* [38], *Pleurotus squamosus* [39], *Pleurocybella porrigens* [40], and *Marasmius oreades* [41], and the immunomodulatory lectin FIP-fve from *Flammulina velutipes* [42]. Lectin PVL from *Psathyrella* (*Lacrymaria*) *velutina* is an integrin-like protein with seven internal repeats expressed both in mycelium and in mushrooms. There is a homolog in *C. cinerea* [43].

Aegerolysins (for which *A. aegerita* Aa-Pri1 = aegerolysin was name-giving) belong to another family of pore-forming hemolysins. Lectin-like interactions are not described but these proteins interact with lipid rafts in cellular membranes [44]. *Pleurotus ostreatus* ostreolysin and pleurotolysin A (with pleurotolysin B from a two-component system), *Pleurotus eryngii* erylysin A (interacting with erylysin B) and possibly *Pleurotus nebrodensis* nebrodeolysin are other closely related members of this family [45-48]. Postulated to be aggregation factors [49, 50], application of ostreolysin to *P. ostreatus* mycelium was found to be fruiting inducing [45]. In coincidence, aegerolysins in *Agrocybe* and *Pleurotus* species are expressed at initiation of fruiting and during fruiting body development [49, 50]. Also in *Moniliophthora perniciosa*, aegerolysin genes expressed along with fruiting body formation have been described [51]. Flammutoxin from *F. velutipes* is another type of pore-forming hemolysin specifically expressed during fruiting [52].

MATERIALS AND METHODS

Strain, culture conditions and microscopy. The self-compatible *C. cinerea* homokaryon AmutBmut able to form fruiting bodies due to mutations in both mating type loci [4] was cultivated on YMG/T complete medium or on horse dung as described [53]. For microscopy of hyphal development, observation windows were made within fully grown fungal YMG/T cultures by cutting out agar pieces of about 1 cm^2. Cultures were further incubated at 28^oC in the dark for about 50 h and hyphal growth within the windows was monitored at intervals of 3 to 4

hours, using an inverse Axiovert (Zeiss, Göttingen, Germany) microscope in a dark room with a yellow filter placed into the light beam of the microscope.

Sequence analysis. *L. velutina* PVL (GenBank ABB17278), *C. cinerea* CGL1 (AAB04141; CCG1_05003), *F. velutipes* FIP-fve (ADB24832), ricin B-like lectins of *C. nebularis* (ACK56062), *P. squamosus* (BAC87876), and *L. sulphureus* (LSLa; 1W3A_A), FB lectins of *A. bisporus* (ABL; Q00022), *P. cornucopiae* (PCL-F1; AB056470), and *X. chrysenteron* (XCL; AAL73235), *A. aegerita* aegerolysin (AAC02265), *P. eryngii* erylysin A (BAI45247) and B (BAI45248), and *F. velutipes* flammutoxin (BAA76510) were used in tblastn searches (expect 1.0E-0; word size 3; no filter) of basidiomycete genomes available in July 2011. From the MycoCosm page (http://genome.jgi-psf.org/programs/fungi/index.jsf) of the Joint Genome Institute (JGI) were used Pucciniomycotina *Melampsora laricis-populina* v1.0, *Puccinia graminis*, *Rhodotorula graminis* strain WP1 v1.1, and *Sporobolomyces roseus* v1.0, Ustilagomycotina *Malassezia globosa* and *Ustilago maydis*, Agaricomycotina *A. bisporus* var. *bisporus* (H97) v2.0, *A. bisporus* var. *burnettii* JB137-S8, *Auricularia delicata* SS-5 v1.0, *Ceriporiopsis subvermispora* B, *Coniophora puteana* v1.0, *C. cinerea* Okayama 7, *Cryptococcus neoformans* var. *grubii* H99, *Dacryopinax* sp. DJM 731 SSP-1 v1.0, *Dichomitus squalens* v1.0, *Fomitiporia mediterranea* v1.0, *Fomitopsis pinicola* SS1 v1.0, *Ganoderma* sp. 10597 SS1 v1.0, *Gloeophyllum trabeum* v1.0, *H. annosum* (*irregulare*) v2.0, *L. bicolor* v2.0, *Phanerochaete carnosa* v1.0, *Phanerochaete chrysosporium* v2.0, *Phlebia brevispora* HHB-7030 SS6 v1.0, *P. ostreatus* PC9 v1.0 and PC15 v2.0, *Postia placenta* MAD-698, *Punctularia strigosozonata* v1.0, *Schizophyllum commune* v1.0, *Serpula lacrymans* S7.3 v2.0 and S7.9 v1.0, *Stereum hirsutum* FP-91666 SS1 v1.0, *Trametes versicolor* v1.0, *Tremella mesenterica* Fries v1.0, and *Wolfiporia cocos* MD-104 SS10 v1.0, from the fungal site (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=fungi) of the National Centre for Biotechnology Information (NCBI) *Microbotryum violaceum* p1A1 Lamole, *Puccinia triticina* 1-1 BBBD Race 1, *Mixia osmundae* IAM 14324, *Melassezia restricta* CBS 7877, *Cryptococcus gattii* R265 and WM276, and *M. perniciosa* FA553, and from the Munich Information Center for Protein Sequences (MIPS) *Sporisorium reilianum* (http://mips.helmholtz-muenchen.de/genre/proj/sporisorium/). Where required, computer-defined gene coordinates were manually corrected and genes annotated on respective pages at JGI (except *S. lacrymans* 1627212: delete N-terminal 54 aa). *M. perniciosa* ABRE01005301 (NCBI) was also amended: join 834-1117,1172-1310. For other accession numbers see figures. Using ClustalX (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html) and GeneDoc version 2.6.002 (http://www.psc.edu/biomed/genedoc/), sequences were aligned Phylogenetic trees were calculated by neighbour joining by MEGA version 4.0 [54].

RESULTS AND DISCUSSION

Hyphal knot formation and early hyphal aggregation. Fig. 1A shows at first two-dimensional vegetative mycelial growth (known as lattice formation [3]) of *C. cinerea* homokaryon AmutBmut when entering a new surface. About 20-30 hours later, hyphal knots of different developmental stages can be found at distinct places within the mycelium (Fig. 1B-D, H). Clearly, intense initiation of formation of sidebranches occurs highly localized over a restricted length (about 50 µm) at one leading hypha or, in closest distance, at two or three neighbouring hyphae. Although the sidebranches stop growing after a few µm (up to about 30 µm as estimated from Fig. 1B-G), the close vicinity of areas of intense branching at neighbouring hyphae allow sidebranches from different leading hyphae to intermingle (Fig. 1D,E).

Table 1: Potential lectins and/or hemolysins in mushroom forming Agaricomycotina

Species	Lectins					Pore-forming lectins			Pore-forming hemolysins		
	***F. velutipes* FIP-fve**	***L. velutina* PVL**	***C. cinerea* CGL1**	***C. nebularis* ricin B-like lectin**	***P. squamosus* ricin B-like lectin**	***L. sulphureus* LSLa**	***D. squalens* 158296 *G. trabeum* 129557**	**FB lectins ABL, PCL-F1, XCL**	***A. aegerita* aegerolysin, *P. eryngii* erylysin A**	***P. eryngii* erylysin B**	***F. velutipes* flammutoxin**
A. bisporus var. *bisporus*	-	-	-	-	-	-	-	75698 194888 194894	-	-	-
A. bisporus var. *burnettii*	-	-	-	-	-	-	-	114704	-	-	-
A. delicata	57009	-	-	-	-	-	-	67476 85006 115017 115035 117944	-	-	199645
C. subvermispora	172151[§] 172155[§] 172156[§]	-	-	-	117217 117225 125532	172144[+]	-	-	172089	-	-
C. puteana	-	-	-	-	-	-	-	-	-	-	-
C. cinerea	-	CC1G _03091*	CC1G _00723 _05003 _05505	CC1G _10077 _10083	CC1G _10075 _10077 _10083	CC1G _08369[+] _10318 _11805	CC1G _08369[+] _10318 _11805	-	-	-	-
Dacryopinax sp.	-	-	-	-	-	-	-	-	-	-	-
D. squalens	53695 101883 125142	-	-	-	148933 172700 172722 172724 172726 172744 201382 201385 201389[Δ]	158296	158296	-	69680	-	90817
F. mediterranea	-	-	-	-	-	-	-	-	-	-	160286 160275 187205 187206
F. pinicola	-	-	-	-	82284 82401 82402 82405 82406 82407 82435 82645 89059	124282 281656[+]	124282	-	-	-	82673 95762
Ganoderma sp.	-	-	-	-	-	-	-	-	-	-	-
G. trabeum	-	-	-	-	-	129557	129557	-	-	-	-
H. irregulare	-	-	58543	-	-	-	-	-	148469	38497	65755
L. bicolor	-	692684	236913 312069 723752	-	-	576524[+] 461940	461940	185716	-	-	-
M. perniciosa	-	-	-	-	EEB99847[Δ] EEB94816[Δ]	ABRE01 005301*	-	-	EEB90416 EEB92328[Δ] EEB93043[Δ] EEB93315[Δ] EEB95579[Δ] EEB96271	EBB89936 ABRE01 017070[§]	-
P. carnosa	192435	-	-	-	211794 261044	-	-	257886 263561	-	-	-
P. chrysosporium	-		-	-	-	-	-	6917 140897	-	-	-
P. brevispora	79844 117676	-	-	-	-	-	-	71190	-	-	-
P. ostreatus PC9	-	-	-	-	122379	-	-	107763	72745	133806	67050 117864
P. ostreatus PC15	-	-	-	-	1119533[+]	-	-	1044138	1090164	1090161	168572 1091975
P. placenta[#]	135177 135180	-	-	92379 135173	92379 135173 135175 135176[Δ] 135181 135182 135188 135191	135167 135168	135167 135168	46158 46169 57081 135165	-	-	135146 135148

* model corrected; [+] model from gene with frameshift/deletion/early stop codon; [§] gene remnant?; [#] contains hits from alleles; [Δ] incomplete gene; [¶] putative family 5 glycoside hydrolase with two N-terminal ricin B-like motifs

Table 1: Potential lectins and/or hemolysins in mushroom forming Agaricomycotina (continued)

Species	Lectins					Pore-forming lectins			Pore-forming hemolysins		
	***F. velutipes* FIP-fve**	***L. velutina* PVL**	***C. cinerea* CGL1**	***C. nebularis* ricin B-like lectin**	***P. squamosus* ricin B-like lectin**	***L. sulphureus* LSL a**	***D. squalens* 158296 *G. trabeum* 129557**	**FB lectins ABL, PCL-F1, XCL**	***A. aegerita* aegerolysin *P. eryngii* erylysin A**	***P. eryngii* erylysin B**	***F. velutipes* flammutoxin**
P. strigosozonata	-	-	-	-	-	-	-	134444 143781 154836 154837$^{\Delta}$	101965	-	-
S. commune	-	-	-	-	103548	-	-	-	-	-	74780
S. lacrymans S7.3	-	-	-	-	162712* 173380 187490	-	-	-	-	-	-
S. lacrymans S7.9	-	-	-	-	442012 457021 477093	-	-	-	-	-	-
S. hirsutum	-	-	-	-	-	-	-	153353 182934	-	-	-
T. versicolor	121721 184741	-	-	-	-	-	-	-	52920	52921	-
T. mesenterica	-	-	-	-	73633¶	-	-	-	-	-	-
W. cocos	-	-	-	-	-	-	-	81600	-	-	-
Total	**12**	**2**	**7**	**4**	**46**	**13**	**9**	**28**	**13**	6	**16**

Formation of further primary and also higher order side branches occurs so that within the developing primary hyphal knot first hyphal aggregation becomes possible (Fig. 1D, E, G-I). When this happens, it becomes difficult to follow up further processes of development by simple light microscopy since the three-dimensional structures are more and more impervious to light. How the step from primary hyphal knot to the secondary hyphal knot and compact aggregation happen is thus still to be clarified. Fig. 2 shows an impression of a secondary hyphal knot with primary hyphal knots growing in the neighbourhood.

Candidate proteins for hyphal aggregation. Proteins proposed to act in hyphal aggregation for mushroom formation (see Introduction) were used to search the genomes of in total 40 different species (7 Pucciniomycotina; 4 Ustilagomycotina; 29 Agaricomycotina). Allelic genomes of two different monokaryons were available for analysis of *P. ostreatus* (PC9, PC15) and *S. lacrymans* (S7.3, S7.9) and, due to dikaryon sequencing, also for *P. placenta* [55]. Strikingly, none of the tested proteins detected candidate genes in the tblastn searches with any of the Pucciniomycotina, the Ustilagomycotina, and the yeast-like Agaricomycotina, suggesting that these proteins are specific to the mushroom-forming Agaricomycotina (see results in Table 1). Also remarkable, none of the species had genes for all types of proteins but most had one or more genes for lectins and/or hemolysins. Species from different orders share types of proteins and closely related species in contrast do not. The gene distribution limited to always only a few and often even unrelated species does not argue for an essential function of any of the tested proteins in hyphal aggregation and fruiting body formation.

FIP-fve-like lectins and integrin-like proteins. Genes for FIP-fve-like lectins were found in seven wood-rotting species from the Auriculariales, Polyporales and Corticiales (Table 1). In contrast, genes for lectins with integrin-like repeats such as PVL of the saprotroph *L. velutina* were only detected in the dung fungus *C. cinerea* and the ectomycorrhizal *L. bicolor* from the Agaricales (Table 1, Fig. 3). PVL binds N-acetylglucosamine and N-acetylneuromic acid in dependence of calcium and this may help in defence of bacteria [43]. However, it might not be by accident that this type of lectin is not present in any of the many wood-rotting species analysed, raising the question whether occurrence of this type of lectin is restricted to saprotrophic and mycorrhizal species for example to help, as suggested [43], in colonisation of humic soil containing pectins and polygalacturonic acid from decomposing plant material.

Galectins and FB lectins. Genes for galectins were only found in the Agaricales *C. cinerea* and *L. bicolor* and in *H. irregulare* from the Russulales (Table 1), all of which were reported before [8,13]. In contrast, FB lectins are wider distributed and genes were found in Agaricales (4 of 7 species), Auriculariales (1 of 1 species), Corticiales (4 of 4 species), Polyporales (2 of 7 species) and Russulales (1 of 2 species), irrespectively of whether *A. bisporus* ABL, *P. cornucopiae* PCL-F1 or *X. chrysenteron* XCL were used in tblastn searches (Table 1). The β-galactoside binding galectins and FB lectins are distantly related [8]. Galectins have a carbohydrate recognition domain (CRD) consensus of H-3x-R-(7-11)x-N-(6-7)x-W-2x-E-x-R. The *C. cinerea* galectin-like CGL3 (CC1G_00723) contains an R instead of the sugar-recognizing W. Due to this R, CGL3 binds chitooligosaccharides but, unlike galectins, not lactose [13]. Of the members of the fungal galectin family, only LBG3 has also this residue while others possess the classical galectin CRD motif or have 1-2 changes at other positions (Fig. 4). The W residue of the conserved amino acids in the CRD of the galectins is also found in the FB lectins [8] but other amino acids make contact with the sugars (Fig. 4, [57]). The sugar-binding residues (Fig. 4) are highly conserved in all proteins of the FB lectin super-family analysed in this study, although only the residue G was found in all 28 of them and only 8 of them had a perfect central HNY-4x-D-I/V/L-x-T motif (Fig. 4; not further shown).

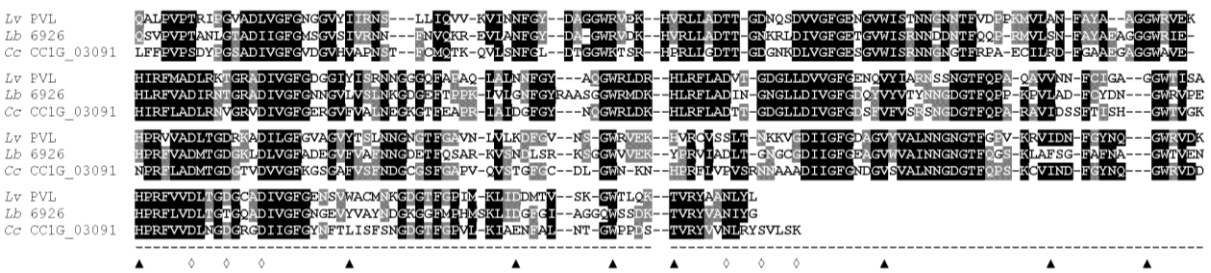

Figure 3: Integrin-like repeats (underlined, 2x per line) from *L. velutina* (*Lv*), *C. cinerea* (*Cc*) and *L. bicolor* (*Lb*) lectins. N-termini of proteins are not shown due to ambiguities in the protein models. Amino acids of sugar and calcium binding are indicated by▲ and ◊, respectively [43].

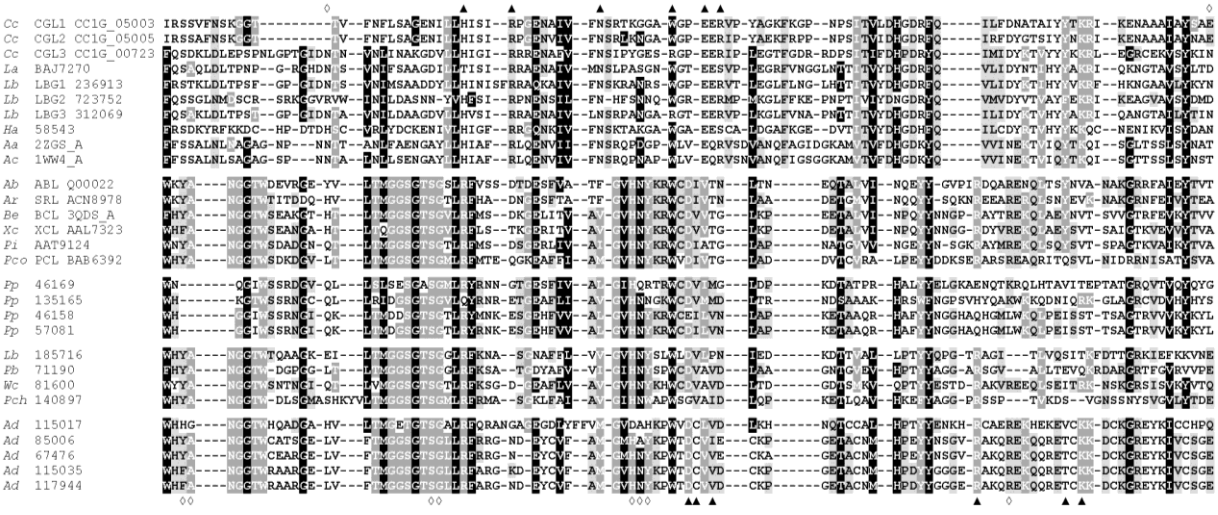

Figure 4: CRD motifs of fungal galectins and galectin-like proteins and of selected FB lectins from four different phylogenetic clades (see Fig. 5). ▲marks primary sugar binding sites for galectins and FB lectins, ◊ extra sites for CGL3 and secondary sites for FB lectins [13, 56].

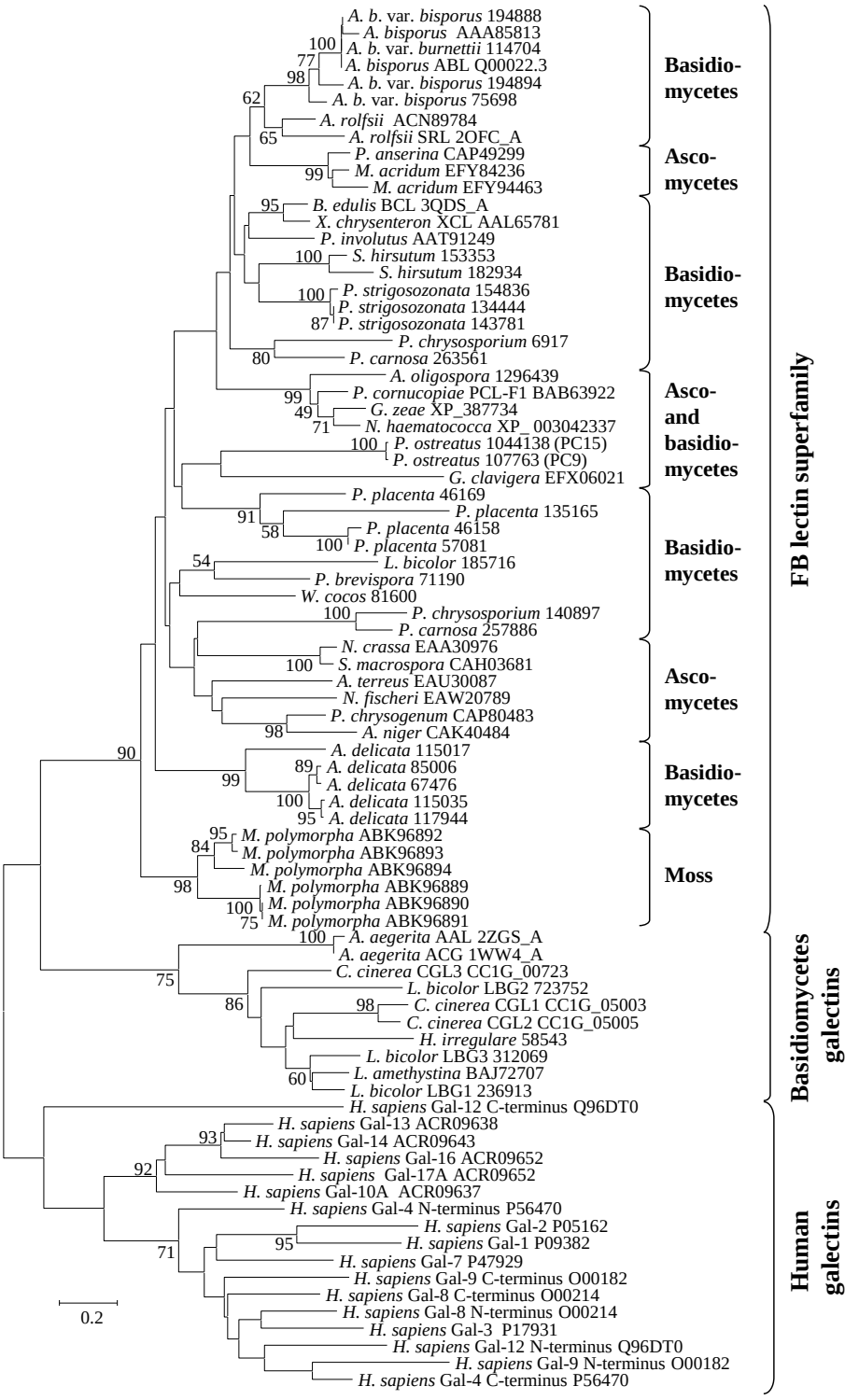

Figure 5: Phylogenetic tree of fungal FB lectins, galectins and galectin-related lectins, FB lectins from the liverwort *Marchantia polymorpha* and human galectins (where present duplicated CRDs were included as N- and C-terminal domain). Fungal species other than those with genomes analysed in study were from the basidiomycetes *A. aegerita*, *A. rolfsii*, *Boletus edulis*, *L. amethystina*, *P. involutus*, *P. cornucopiae*, and *X. chrysenteron*, and from the ascomycetes *Arthrobotrys oligospora*, *Aspergillus niger*, *Aspergillus terreus*, *Gibberella zeae*, *Grosmannia clavigera*, *Metarhizium acridum*, *Metarhizium anisopliae*, *Nectria haematococca*, *Neosatorya fischeri*, *Neurospora crassa*, *Podospora anserina*, and *Sordaria macrospora*. JGI protein IDs and GenBank accession numbers are given in the figure. Bootstrapping values (500 replications) above 50 are shown at tree branchings. Scale bar = number of nucleotide substitutions per site.

A phylogenetic tree was produced from all fungal galectins and FB lectins, using the human galectins and FB lectins found recently in a liverwort as foreign proteins (Fig. 5). The tree suggests that FB lectins might have been evolved from ancestors common to the fungal galectins. The position of the group of moss FB lectins is interesting since it might point to a split of galectins and FB lectins prior to the split of plants and fungi. In support of this, there are also genes for galectin-like proteins in plants [10]. Interesting is further to note that ascomycete and basidiomycete FB lectins intermingle with each other. Duplications of FB lectins happened frequently late in evolution close to speciation. There are four major clades of fungal proteins within the FB super-family (Fig. 5) which correspond largely to differences in the sugar-interacting residues in the CRD motifs (Fig. 4 and not shown). Whether these go along with alterations in sugar binding (efficiencies or sugar types) remains to be elucidated.

Ricin B-like lectins. Of the known fungal ricin B-like lectins, two were used in this study in genome searches. Hits to genes were rare for the *C. nebularis* protein unlike the *P. squamosus* lectin that obtained wider distributed hits, including multiple genes in some of the species and the hits by the *C. nebularis* protein. Genes were found in 4 of 7 Agaricales, 1 of 2 Boletales, 1 of 4 Corticiales, 4 of 9 Polyporales, and in the Tremellales species (Table 1). The products divide in four subgroups of simple ricin B-like lectins plus three other ricin B proteins. Subgroups of the ricin B super-family may have little amino acid identity but they share a β-trefoil structure and contain a conserved Q/NxW motif for sugar binding [38, 40]. All 46 proteins listed in Table 1 had this motif in one or more copies. Of the putative simple lectins, 2 had 1, 14 had 2, 3 had 3, 13 had 4, and 1 had 6 copies [proteins of subgroups I (132 to 182 aa), II and III (256 to 336 aa)] and 6 had 6 to 8 copies [proteins of subgroups III [774 to 838 aa)], respectively. Searching the JGI pages with the keyword ricin revealed many more potential genes for ricin B-like lectins in the fungi than found in our current tblastn searches (also in species with so far no detected gene of interest; Table 1). It apparently will be a major but also a fascinating task to resolve the complete ricin B super-family in the basidiomycetes.

Hemolytic LSL-like lectins. *L. sulphureum* LSLa is special by its N-terminal lectin and C-terminal aerolysin domains [37]. Evidence for such dual proteins was found in 8 different species of the Agaricales and Polyporales (Table 1). These proteins divide into four different groups with highly conserved aerolysin domains and less conserved N-terminal domains (Fig. 6). The N-terminal domain in LSLa adopts a β-trefoil structure exposing specific sugar- contacting residues [37]. These residues are not much conserved between the proteins except *F. pinicola* 124282 (Fig. 6), suggesting that the N-termini of the new proteins have either no lectin function or that they developed novel sugar-binding sites. The N-terminal domains of *L. bicolor* 461940, *C. cinerea* CC1G_11805, and *P. placenta* 135167 were individually used in tblastn searches. Neither gave any further meaningful hits, implying unique evolutionary developments for these domains.

Aegerolysins and flammutoxins. Like for the analysed types of lectins, genes for different types of hemolysins were only found in some species (Table 1). In *D. squalens*, *H. irregulare*, and *P. ostreatus*, genes for different types of hemolysins were detected; other species had only one type in one or more gene copies or no gene for any of the tested kinds of hemolysins. The distribution was independent of the fungal order. Aegerolysin genes occurred in Agaricales, Corticiales, Polyporales, and Russulales and orthologues for flammutoxin in Auriculariales, Agaricales, Hymeochaetales, Polyporales, and Russulales (Table 1).

The closely related *A. aegerita* aegerolysin and the *P. eryngii* erylysin A detected the same set of putative aegerolysin genes (Table 1). Some species contained aegerolysin genes and, in addition, genes for erylysin B-like proteins (Table 1). The latter type of gene never occurred

without an aegerolysin gene. Moreover, where both present, the genes come together in divergently transcribed pairs (not shown), emphasizing a common functional role such as has been suggested in *P. eryngii* and *P. ostreatus* by the experimental finding of dimerization of their products [46, 47]. In *D. squalens*, an erylysin B-like gene was missing but there was a footprint of a former gene upstream to the aegerolysin gene 69680 (not shown).

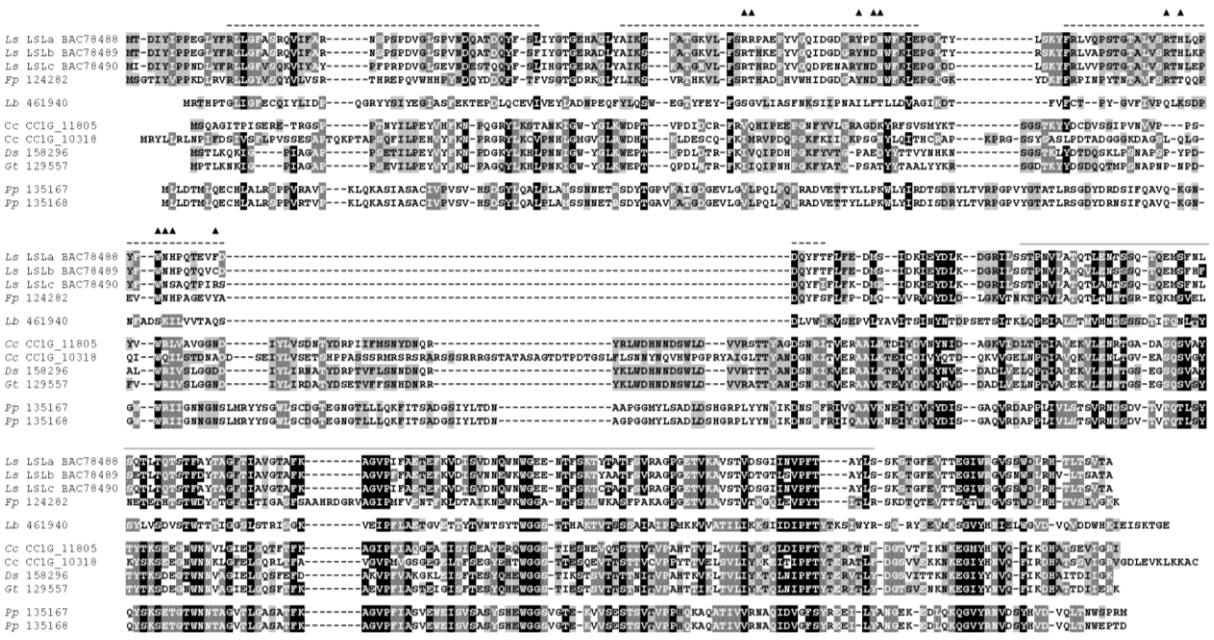

Figure 6: Alignment of putative lectins with aerolysin domains. The β-trefoil module and the sugar-binding residues at the N-terminus of *L. sulphureum* LSLa are marked above the sequence by dashed lines and ▲ and the aerolysin region is indicated by a solid line [37]. Note that from Table 1 only proteins from complete and from intact genes are shown.

CONCLUSIONS

Different lectins and hemolysins have been forwarded as candidate proteins to act in hyphal aggregation, often evidenced by their expression correlating to fruiting (see Introduction). Genome analysis in this study revealed that genes for the tested types of proteins were specific to the mushroom forming species. However, genes for none of the various specific types of proteins occur in all mushroom forming species, although presence of one or more types of lectins and of hemolysins was not rare. The results imply that either these proteins may not be essential for fruiting body initiation and hyphal aggregation or that in evolution different routes have in parallel been developed for the course of events to initiate and continue the fruiting process. Since fruiting body development stands as an essential step at the beginning of sexual spore formation, it appears unlikely that a process central to initiation has been independently invented several times. Experimental evidences we have so far from studies in the literature suggest neither for the different lectins nor for the pore-forming hemolysins a direct function in aggregation.

Pore-forming hemolysins may influence membrane signalling in specific interaction with lipid rafts [44] and, by this, they may indirectly influence the hyphal aggregation process by for example modifying the frequency of fruiting or the environmental conditions under which fruiting occurs. Such effect could explain the observation of induction of fruiting upon application of ostreolysin (in excess?) to vegetative *P. ostreatus* mycelium [45]. Is this a true biological function or an experimental artefact by just exceeding the normal threshold of the

protein? What might be another function of fungal hemolysins? Do they possibly (also) act in defence? Lectins linked to fruiting body development have been seen to act toxic against small putative predators [15, 16, 22, 24, 26, 34]. This may reflect an adoption of a secondary function of these proteins, if there is any in hyphal aggregation. *F. velutipes* LSL-type lectins have an extra pore-forming domain for membrane interaction [37]. FB lectins resemble in structure the bacterial pore-forming haemolytic actinoporins [27, 35]. Lectins may thus interact with membranes [57], possibly via binding to glycolipids [12, 31], thereby effecting signalling [57]. Application of fungal galectins to vegetative mycelium resulting in initiation of fruiting [20,21] might reflect such effect possibly mediated by unnatural high protein concentrations. The importance of membranes in fruiting of Agaricomycotina is supported by the fruiting-inducing effects of various membrane-interacting substances and surfactants when added to vegetative mycelium [2,7] and by the finding of a gene for a cyclopropane fatty-acid synthase being essential for fruiting body initiation in *C. cinerea* [58].

ACKNOWLEDGEMENTS

We are very grateful to the JGI for providing fungal genomes for research. Support by the European Community via the PhD program BIONUTZ (grant 80025676) is acknowledged.

REFERENCES

[1] Buller AHR. (1931). *Researches on Fungi. Vol. IV*. Hafner Publishing Co., New York, USA.
[2] Kües U. (2000). Life history and developmental processes in the basidiomycete *Coprinus cinereus*. *Microbiol. Mol. Biol. Rev.* 64: 316-353.
[3] Matthews TR. & Niederpruem DJ. (1972). Differentiation in *Coprinus lagopus.* I. Control of fruiting and cytology of initial events. *Arch. Mikrobiol.* 87: 257-268.
[4] Boulianne RP. et al. (2000). Fruiting body development in *Coprinus cinereus*: regulated expression of two galectins secreted by a non-classical pathway. *Microbiol*. 146: 1841-1853.
[5] Kües U. *et al.* (1998). The *A* mating type and blue light regulate all known differentiation processes in the basidiomycete *Coprinus cinereus*. *Mol. Gen. Genet*. 260: 81-91.
[6] Kües U. *et al.* (2002). Influence of activated *A* and *B* mating-type pathways on developmental processes in the basidiomycete *Coprinus cinereus*. *Mol. Genet. Genom.* 268: 262-271.
[7] Kües U. & Liu Y. (2000). Fruiting body production in basidiomycetes. *Appl. Microbiol. Biotechnol.* 54: 141-152.
[8] Walser PJ. *et al.* (2003). Extracellular matrix proteins in mushroom development. *Rec. Res. Dev. Microbiol.* 7: 381-415.
[9] Wang HX. *et al*. (1998). Lectins from mushrooms. *Mycol. Res*. 102: 897-906.
[10] Cooper DNW. (2002). Galectinomics: finding themes in complexity. *Biochim. Biophys. Acta* 1572: 209-231.
[11] Bertossa RC. *et al.* (2004). Promoter analysis of *cgl2*, a galectin encoding gene transcribed during fruiting body formation in *Coprinopsis cinerea* (*Coprinus cinereus*). *Fungal Genet. Biol.* 41: 1120-1131.
[12] Walser PJ. *et al*. (2005). Ligand interactions of the *Coprinopsis cinerea* galectins. *Fungal Genet. Biol*. 42: 293-305.
[13] Wälti MA. *et al.* (2008). Structural basis for chitotetraose coordination by CGL3, a novel galectin-related protein from *Coprinopsis cinerea*. *J. Mol. Biol.* 379: 146-159.
[14] Wälti MA. *et al.* (2006). Targeted gene silencing in the model mushroom *Coprinopsis cinerea* (*Coprinus cinereus*) by expression of homologous hairpin RNAs. *Eukaryot. Cell* 5: 732-744.
[15] Butschi A. *et al*. (2010). *Caenorhabditis elegans* N-glycan core β-galactoside confers sensitivity towards nematotoxic fungal galectin CGL2. *PLoS Pathog.* 6: e1000717.

[16] Bleuler-Martínez S. *et al.* (2011). A lectin-mediated resistance of higher fungi against predators and parasites. *Mol. Ecol*. 20: 3056-3070.
[17] Yagi F. *et al.* (2001). *Agrocybe cylindracea* lectin is a member of the galectin family. *Glycoconjugate J.* 18: 745-749.
[18] Yang N. *et al*. (2005). Molecular character of the recombinant antitumor lectin from the edible mushroom *Agrocybe aegerita*. *J. Biochem.* 138: 145-150.
[19] Lyimo B. *et al*. (2011). Primary structure and specificity of a new member of galectin family from the amethyst deceiver mushroom *Laccaria amethystina*. *Biosci. Biotechnol. Biochem.* 45: 62-69.
[20] Sun H. *et al*. (2003). A lectin with mycelia differentiation and antiphytovirus activities from the edible mushroom *Agrocybe aegerita*. *J. Biochem. Mol. Biol.* 36: 214-222.
[21] Luan R. *et al*. (2010). Opposing developmental functions of *Agrocybe aegerita* galectin (AAL) during mycelia differentiation. *Fungal Biol*. 114: 599-608.
[22] Zhao S. *et al*. (2009). Lectins but not antifungal proteins exhibit anti-nematode activity. *Env. Toxicol. Pharmacol.* 28 : 265-268.
[23] Wu AM. *et al*. (2001). Carbohydrate specificity of a lectin isolated from the fungus *Sclerotium rolfsii*. *Life Sciences*. 69: 2039-2050.
[24] Bhat RS. *et al*. (2010). Cloning of *Sclerotium rolfsii* lection gene and its nematicidal activity. *Curr. Sci.* 9: 1185-1186.
[25] Crenshaw RW. *et al.* (1995). Isolation and characterization of a cDNA clone encoding a lectin gene from *Agaricus bisporus*. *Plant Physiol*. 107: 1465-1466.
[26] Trigueros V. *et al*. (2003). *Xerocomus chrysenteron* lectin: identification of a new pesticidal protein. *Biochim. Biophys. Acta*. 1621: 292-298.
[27] Birck C. *et al.* (2004). A new lectin family with structure similarity to actinoporins revealed by the crystal structure of *Xerocomus chrysenteron* lectin. *J. Mol. Biol.* 344: 1409-1420.
[28] Iijima N. *et al.* (2002). Two genes encoding fruit body lectins of *Pleurotus cornucopiae*: Sequence similarity with the lectin of a nematode-trapping fungus. *Biosci. Biotechnol. Biochem*. 66: 2083-2089.
[29] Bovi M. *et al*. (2011). Structure of a lectin with antitumoral properties in king bolete (*Boletus edulis*) mushrooms. *Glycobiol*. 21: 1000-1009.
[30] Le Quere A. *et al*. (2006). Screening for rapidly evolving genes in the ectomycorrhizal fungus *Paxillus involutus* using cDNA microarrays. *Mol. Ecol.* 15: 535-550.
[31] Swamy BM. *et al*. (2004). Immunolocalization and functional role of *Sclerotium rolfsii* lectin in development of fungus interaction with its endogenous receptor. Glycobiol. 14:951-957.
[32] Oguri S. *et al.* (1994). Isolation, crystallization and characterization of a 16.5 kDa protein from fruit bodies of lectin-deficient strain of *Pleurotus cornucopiae*. *Biosci. Biotechnol. Biochem.* 58: 502-506.
[33] Oguri S. *et al.* (1996). A novel developmental stage-specific lectin of the basidiomycete *Pleurotus cornucopiae*. *J. Bacteriol.* 178: 5692-5698.
[34] Jaber K. *et al.* (2007). Effect of a fungal lectin from *Xerocomus chrysenteron* (XCL) on the biological parameters of aphids. *Comm. Appl. Bio. Sci*. 72: 629-638.
[35] Carrizo ME. et al. (2005). The antineoplastic lectin of the common edible mushroom (*Agaricus bisporus*) has two binding sites, each specific for a different configuration at a single epimeric hydroxyl. *J. Biol. Chem.* 280: 10614-10623.
[36] Kristan KC. *et al*. (2009). Molecular mechanism of pore formation by actinoporins. *Toxicon* 54: 1125-1134.
[37] Mancheno JM. *et al*. (2005). Structural analysis of the *Laetiporus sulphureus* hemolytic pore-forming lectin in complex with sugars. *J. Biol. Chem*. 280: 17251-17259.

[38] Pohleven J. *et al*. (2009). Purification, characterization and cloning of a ricin B-like lectin from mushroom *Clitocybe nebularis* with antiproliferative activity against human leukemic T cells. *Biochim. Biophys. Acta* 1790: 173-181.
[39] Tateno H. *et al*. (2004). Cloning, expression in *Escherichia coli* and characterization of the recombinant NeuAcα2, 6Galβ1,4GlcNAc-specific high-affinity lectin and its mutants from the mushroom *Polyporus squamosus*. *Biochem. J.* 382: 667-675.
[40] Suzuki T. *et al*. (2009). Purification, characterization, and cDNA cloning of a lectin from the mushroom *Pleurocybella porrigens*. *Biosci. Biotechnol. Biochem*. 73: 702-709.
[41] Grahn EM. *et al.* (2009). Structural characterization of a lectin from the mushroom *Marasmius oreades* in complex with the blood group B trisaccharide and calcium. *J. Mol. Biol.* 390: 457-466.
[42] Ko J-L. *et al.* (1995). A new fungal immunomodulatory protein, FIP-fve isolated from the edible mushroom, *Flammulina velutipes* and its complete amino acid sequence. *Eur. J. Biochem*. 228: 244-249.
[43] Cioci G. *et al.* (2006) β-Propeller crystal structure of *Psathyrella velutina* lectin: An integrin-like fungal protein interacting with monosaccharides and calcium. *J. Mol. Biol.* 357: 1575-1591.
[44] Berne S. *et al.* (2009). Aegerolysins: structure, function, and putative biological role. *Prot. Sci.* 18 : 694-706.
[45] Berne S. *et al.* (2007). Ostreolysin enhances fruiting initiation in the oyster mushroom (*Pleurotus ostreatus*). *Mycol. Res*. 111: 1431-1436.
[46] Sakurai N. *et al*. (2004). Cloning, expression, and pore-forming properties of mature and precursor forms of pleurotolysin, a sphingomyelin-specific two-component cytolysin from the edible mushroom *Pleurotus ostreatus*. *Biochim. Biophys. Acta* 1679: 65-73.
[47] Shibata T. *et al.* (2010). Isolation and characteization of a novel two-component hemolysin, erylysin A and B, from an edible mushroom, *Pleurotus eryngii*. *Toxicon* 56: 1436- 1442
[48] Lv H. *et al.* (2009). Nebrodeolysin, a novel hemolytic protein from mushroom *Pleurotus nebrodensis* with apoptosis-inducing and anti-HIV-1 effects. *Phytomed*. 16: 198-205.
[49] Fernandez Espinar M-T. & Labarère J. (1997). Cloning and sequencing of the *Aa-Pri1* gene specifically expressed during fruiting initiation in the edible mushroom *Agrocybe aegerita*, and analysis of the predicted amino-acid sequence. *Curr. Genet*. 32: 420-424.
[50] Berne S. *et al*. (2002). *Pleurotus* and *Agrocybe* hemolsins, new proteins hypothetically involved in fungal fruiting. *Biochim. Biophys. Acta* 1570: 153-159.
[51] Pires ABL. *et al.* (2009). Early development of *Moniliophthora perniciosa* basidiomata and developmentally regulated genes. *BMC Microbiol*. 9: 158.
[52] Tomita T. *et al.* (2004). Protein sequence analysis, cloning, and expression of flammutoxin, a pore-forming cytolysin from *Flammulina velutipes* – Maturation of dimeric precursor to monomeric active form by carboxyl-terminal truncation. *J. Biol. Chem.* 279: 54161-54172.
[53] Granado JD. *et al*. (1997). Restriction enzyme-mediated DNA integration in *Coprinus cinereus*. *Mol. Gen. Genet.* 256, 28-36.
[54] Tamura K. *et al*. (2007). MEGA4: Molecular evolutionary genetic analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24: 1596-1599.
[55] Martinez D. *et al.* (2009). Genome, transcriptome, and secretome analysis of wood decay fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion. *Proc. Natl. Acad. Sci. USA* 106: 1954-1959.
[56] Leonidas D. *et al.* (2007). Structural basis for the carbohydrate recognition of the *Sclerotium rolfsii* lectin. *J. Mol. Biol.* 368 :1145-1161.
[57] Gutiérrez-Aguirre I. *et al*. (2006). Membrane binding of zebrafisch actinoporin-like protein: AF domains, a novel superfamily of cell membrane binding domains. *Biochem. J.* 398: 381-392.