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MINI-REVIEW

Entomotoxic and nematotoxic lectins and protease inhibitors from fungal fruiting bodies

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Abstract Fruiting bodies or sporocarps of dikaryotic (ascomycetous and basidiomycetous) fungi, commonly referred to as mushrooms, are often rich in entomotoxic and nematotoxic proteins that include lectins and protease inhibitors. These protein toxins are thought to act as effectors of an innate defense system of mushrooms against animal predators including fungivorous insects and nematodes. In this review, we summarize current knowledge about the structures, target molecules, and regulation of the biosynthesis of the best characterized representatives of these fungal defense proteins, including galectins, beta-trefoil-type lectins, actinoporin-type lectins, beta-propeller-type lectins and beta-trefoil-type chimerolectins, as well as mycospin and mycocypin families of protease inhibitors. We also present an overview of the phylogenetic distribution of these proteins among a selection of fungal genomes and draw some conclusions about their evolution and physiological function. Finally, we present an outlook for future research directions in this field and their potential applications in medicine and crop protection.

Keywords Basidiomycete · Ascomycete · Sporocarp · Glycan · Insect · Nematode

Introduction

Fruiting bodies, also referred to as mushrooms or sporocarps, are important microscopic or macroscopic structures formed during the sexual reproduction cycle of the fungal subkingdom Dikarya (Hibbett et al. 2007; Taylor and Ellison 2010). These fungi are characterized by the formation of dikaryotic hyphae and include the phyla *Ascomycota* and *Basidiomycota*. Fruiting body formation is independent of the lifestyle (saprotrophic or biotrophic) of these fungi and sometimes coupled to the preceding formation of sclerotia—compact masses of hardened mycelium containing food reserves (Martin et al. 2008; Stajich et al. 2010; Teichert et al. 2014; Yin et al. 2012). Since the fruiting bodies produce and disperse the sexual spores, defense of these structures against fungivores, including predators, grazers, and parasites, is essential for fungal reproduction. Thus, dikaryotic fungi employ, in addition to a large repertoire of secondary metabolites, a plethora of proteins acting as deterrents or toxins in defense of their fruiting bodies (Spiteller 2015; Wang et al. 2002). These proteins include lectins that bind to the glycans of glycoproteins or glycolipids in the digestive tract of fungivores (Bleuler-Martinez et al. 2011), protease inhibitors that inhibit digestive proteases of fungivores (Renko et al. 2010), biotin-binding proteins that sequester this essential cofactor (Bleuler-Martinez et al. 2012), pore-forming proteins that cause cell lysis (Mancheno et al. 2010; Ota et al. 2014), RNA toxins (ribotoxins) that cleave or depurinate RNA molecules (Lacadena et al. 2007), and other enzymes including proteases, oxidases, and phospholipases (Erjavec et al. 2012). The first two groups of proteins: lectins and protease inhibitors,

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have been studied most thoroughly, and an ever increasing body of evidence confirms that they are part of a fungal innate defense system against predators and parasites (Künzler 2015; Sabotič et al. 2012). Many reviews on mushroom lectins and protease inhibitors have been published recently but mainly viewing their potential application in human medicine (Dunaevsky et al. 2014; Erjavec et al. 2012; Hassan et al. 2015; Kobayashi and Kawagishi 2014; Sabotič and Kos 2012; Wong et al. 2010; Xu et al. 2011). The present review focuses on fruiting body lectins and protease inhibitors that have been characterized at the genetic, molecular, structural, and functional levels and that exhibit toxicity to nematodes and/or insects (Table 1). Their common characteristics include (i) small size, solubility, and resistance toward extreme pH and temperature; (ii) the lack of a signal sequence for classical secretion; (iii) the lack or low number of cysteine residues and disulphide bridges; and (iv) the lack of glycosylation, of which (ii) to (iv) are indicators of their cytoplasmic localization. Their exceptional characteristics make these fungal defense proteins attractive reagents for protecting crops against plant pests as well as in veterinary and human medicine against parasites.

Lectins

Lectins, also known as (hem)agglutinins, are defined as proteins containing at least one domain that binds to a specific carbohydrate (glycan) structure without modifying it (Sharon and Lis 2004). Binding of the carbohydrate is usually achieved by multiple weak interactions that result in high specificity, avidity, and/or affinity of the lectin for the carbohydrate ligand (Andre et al. 2015). Lectins were first classified according to their carbohydrate-binding specificity and, later, according to their sequence homology and evolutionary relatedness. The latter classification is based on the conservation of amino acid sequence motifs and the three-dimensional structures of the carbohydrate recognition domains (CRDs) (Varki 2009).

Based on the overall organization, three types of lectins are distinguished, namely merolectins, hololectins, and chimerolectins (Peumans and Van Damme 1995). Merolectins or monovalent lectins are small proteins with a single CRD. Hololectins are composed of two or more, usually homologous, CRDs on the same or on different polypeptide chains. The multivalency of carbohydrate-binding sites of hololectins is responsible for their ability to agglutinate cells or precipitate glycoconjugates (Brewer et al. 2002). Chimerolectins are fusion proteins composed of a CRD and an unrelated domain with a well-defined catalytic or biological activity that acts independently of the carbohydrate-binding domain.

The physiological functions of lectins are almost infinite, with the common denominator that they act as recognition molecules in cell-molecule and cell-cell interactions (Sharon and Lis 2004; Varki 2009). In fungi, lectins have been implicated in defense against fungivores (Bleuler-Martinez et al. 2011), in fungal developmental processes including fruiting body formation (Luan et al. 2010; Swamy et al. 2004; Wang et al. 1998), in molecular recognition during mycorrhization or parasitism (Guillot et al. 1994; Guillot and Konska 1997; Wang et al. 1998), and in storage of nutrients (Kellens and Peumans 1990). Most experimental evidence is in support of the defensive function of fungal lectins, which is mediated by binding of the lectin to non-self glycans on the cells of the target organism (Künzler 2015). However, despite the recent reports of the successful identification of target glycoconjugates in insects and nematodes, the mechanism of action of many of these lectins is still unclear and the mechanisms may differ for the various lectins. All the characterized fungal lectins are multivalent and thus likely to crosslink glycoconjugates on the cell surface. The lattices between lectins and glycoconjuates may affect the turnover of the involved glycoconjugates by either preventing or triggering their internalization. Alternatively, the formation of these lattices may lead to activation of intracellular signaling pathways, e.g., by lectin-mediated oligomerization of (glycosylated) signaling receptors (see Künzler 2015 and references therein).

In the last 15 years, numerous mushroom lectins have been isolated and characterized, revealing mushrooms as a rich source of lectins with unique carbohydrate-binding specificities (Goldstein and Winter 2007; Hassan et al. 2015). Six different structural families of mushroom lectins: galectin, β-trefoil-type, β-propeller-type, actinoporin-type, cyanovirin-N-type, and immunoglobulin-type, have been identified to date (Varrot et al. 2013). Here, we summarize the current knowledge about the families containing members with nematotoxic and/or entomotoxic activities.

Galectins

Galectins constitute a family of β-galactoside-binding hololectins with a characteristic fold and a signature of carbohydrate-binding residues that occurs in animals and fungi but is apparently absent from bacteria and plants (Di Lella et al. 2011). The genome of the model mushroom *Coprinopsis cinerea* contains a tandem gene array encoding two highly homologous galectins: CGL1 and CGL2 (Cooper et al. 1997) (Table 1). In addition to these two proteins, the *C. cinerea* genome codes for a homologous, galectin-like protein, CGL3, that binds LacdiNAc and chitobiose but not β-galactosides (Walti et al. 2008). Besides these proteins from *C. cinerea*, galectins from two different *Agrocybe* species: ACG from *Agrocybe cylindracea* and AAG from *Agrocybe aegerita*, have been characterized (Yagi et al. 2001; Yang et al. 2009) (Table 1). The latter two share 88 % sequence identity

Table 1 Overview of entomotoxic and/or nematotoxic lectins and protease inhibitors from mushrooms

Name	Origin	Size of monomer	Fold (PDB code)	Oligomer	No. of carbohydrate-binding sites per oligomer	In vitro specificity[a]	In vivo specificity in *C. elegans*	Toxicity	Reference
CGL2	*Coprinopsis cinerea*	150 aa	Galectin (1UL9)	Homotetramer	4	Gal-β1,4-Glc Gal-β1,4-GlcNAc Gal-β1,3-GalNAc	Gal-β1,4-Fuc	Entomotoxic Nematotoxic Amoebicidal	(Bleuler-Martinez et al. 2011; Boulianne et al. 2000; Butschi et al. 2010; Cooper et al. 1997; Titz et al. 2009; Walser et al. 2004; Yan et al. 2012)
AAG	*Agrocybe aegerita*	159 aa	Galectin (2ZGL)	Homodimer	2	Gal-β1,4-Glc	na	nd	(Luan et al. 2010; Yang et al. 2009; Yang et al. 2005a; Yang et al. 2005b; Yang et al. 2005c)
ACG	*Agrocybe cylindracea*	160 aa	Galectin (1WW7)	Homodimer	2	Gal-β1,4-GlcNAc Neu5Ac-α2,3-Gal-β1,4-GlcNAc	na	nd	(Ban et al. 2005; Imamura et al. 2011; Kuwabara et al. 2013; Liu et al. 2008; Yagi et al. 2001; Yagi et al. 1997)
RSA	*Rhizoctonia solani*	142 aa	β-trefoil (4G9M)	Homodimer	4	Gal GalNAc	na	Entomotoxic	(Hamshou et al. 2013; Hamshou et al. 2010b; Hamshou et al. 2012; Skamnaki et al. 2013)
SSA	*Sclerotinia sclerotiorum*	153 aa	β-trefoil (2X2S)	Homodimer	2	Gal GalNAc	na	Entomotoxic Amoebicidal	(Bleuler-Martinez et al. 2011; Sulzenbacher et al. 2010; Van Damme et al. 2007)
CNL	*Clitocybe nebularis*	149 aa	β-trefoil (3NBC)	Homodimer	2	Gal GalNAc Gal-β1,4-Glc GalNAc-β1,4-GlcNAc GalNAc-α1,3(Fuc-α1,2)Gal-β1,4-GlcNAc	Not clear	Entomotoxic Nematotoxic Amoebicidal	(Bleuler-Martinez et al. 2011; Pohleven et al. 2011; Pohleven et al. 2009; Pohleven et al. 2012)
CCL2	*Coprinopsis cinerea*	142 aa	β-trefoil (2LIE)	Homodimer[b]	2	Gal-β1,4(Fuc-α1,3)-GlcNAc GlcNAc-β1,4(Fuc-α1,3)-GlcNAc	α1,3-Fucosylated N-glycan cores	Nematotoxic	(Schubert et al. 2012; Yan et al. 2012)
MpL	*Macrolepiota procera*	141 aa	β-trefoil (4ION)	Homodimer	2	Gal-β1,4-GlcNAc Gal-β1,3-GlcNAc	Not clear	Nematotoxic	(Žurga et al. 2014)
BEL β-trefoil	*Boletus edulis*	146 aa	β-trefoil (4I4R)	Homodimer	6	Gal GalNAc Gal-β1,4-Glc Gal-β1,3-GalNAc	na	nd	(Bovi et al. 2013)
AAL	*Aleuria aurantia*	313 aa	β-propeller (1OFZ)	Homodimer	10	Fuc-α1,2-X Fuc-α1,3-X Fuc-α1,6-X	Not clear	Entomotoxic Nematotoxic Amoebicidal	(Bleuler-Martinez et al. 2011; Fujihashi et al. 2003; Fukumori et al. 1990; Kochibe and Furukawa 1980; Olausson et al. 2010; Olausson et al. 2008; Tateno et al. 2009; Wimmerova et al. 2003)
AOL_1	*Aspergillus oryzae*	310 aa	β-propeller	Homodimer	12	Fuc-α1,6-GlcNAc Fuc-α1,2-Gal-β1,4-GlcNAc	na	nd	(Matsumura et al. 2008; Tateno et al. 2009)
AFL	*Aspergillus fumigatus*	315 aa	β-propeller (4UOU)	Homodimer	12	Terminal Fuc	na	nd	(Houser et al. 2015; Houser et al. 2013)
PVL	*Psathyrella velutina*	401 aa	β-propeller (2BWR)	Monomer	6	GlcNAc-β1,4-X Neu5Ac	na	nd	(Cioci et al. 2006; Ueda et al. 1999a; Ueda et al. 2002; Ueda et al. 1999b; Ueda et al. 2003)
LbTec2	*Laccaria bicolor*	227 aa	β-propeller	Homotetramer[b]	24	3-O-Me-Man 4-O-Me-Man[b] 2-O-Me-Fuc	3-O-Me-Man 2-O-Me-Fuc on N-glycan antenna	Nematotoxic	(Wohlschlager et al. 2014)
XCL	*Xerocomus chrysenteron*	143 aa	Actinoporin (1XI0)	Homotetramer	4+4	Gal-β1,3-GalNAc and GlcNAc	Core1-O-glycans and terminal GlcNAc on α1,3-mannose of N-glycans	Entomotoxic Nematotoxic	(Birck et al. 2004; Bleuler-Martinez et al. 2011; Damian et al. 2005; Francis et al. 2003; Jaber et al. 2008; Jaber et al. 2007; Jaber et al. 2006; Marty-Detraves et al. 2004; Trigueros et al. 2003; Yan et al. 2012)

Table 1 (continued)

Name	Origin	Size of monomer	Fold (PDB code)	Oligomer	No. of carbohydrate-binding sites per oligomer	In vitro specificity[a]	In vivo specificity in *C. elegans*	Toxicity	Reference
ABL (ABA)	*Agaricus bisporus*	143 aa	Actinoporin (1Y2T)	Homotetramer	4+4	Gal-β1,3-GalNAc and GlcNAc	na	nd	(Carrizo et al. 2005; Crenshaw et al. 1995; Nakamura-Tsuruta et al. 2006; Presant and Kornfeld 1972; Yu et al. 1993; Yu et al. 1999)
SRL	*Sclerotium* (*Athelia*) *rolfsii*	143 aa	Actinoporin (2OFC)	Homodimer	2+2	Gal-β1,3-GalNAc and GlcNAc	na	nd	(Chachadi et al. 2011; Inbar and Chet 1994; Leonidas et al. 2007; Vishwanathreddy et al. 2014)
BEL	*Boletus edulis*	143 aa	Actinoporin (3QDS)	Homotetramer	4+4	Gal-β1,3-GalNAc	na	nd	(Bovi et al. 2011)
TAP1	*Sordaria macrospora*	143 aa	Actinoporin	nd	nd	Gal-β1,3-GalNAc	Not clear	Entomotoxic Nematotoxic	(Bleuler-Martinez et al. 2011; Nowrousian and Cebula 2005; Yan et al. 2012)
AOL_2	*Arthrobotrys oligospora*	145 aa	Actinoporin	Homodimer	nd	nd	na	nd	(Balogh et al. 2003; Rosen et al. 1996a; Rosen et al. 1992; Rosen et al. 1996b; Rosen et al. 1997)
MOA	*Marasmius oreades*	293 aa	β-trefoil chimeric (2IHO)	Homodimer	6	Gal-α1,3-Gal/GalNAc-β	Gal-α1,3-GalNAc on GSLs	Entomotoxic[b] Nematotoxic	(Cordara et al. 2011; Grahn et al. 2007; Grahn et al. 2009; Rempel et al. 2002; Tateno and Goldstein 2004; Winter et al. 2002; Wohlschlager et al. 2011)
LSL	*Laetiporus sulphureus*	315 aa	β-trefoil chimeric (1W3A)	Hexamer	12	Lac LacNAc	na	nd	(Angulo et al. 2011; Mancheno et al. 2005; Mancheno et al. 2010; Tateno and Goldstein 2003)
PSL	*Polyporus squamosus*	286 aa	β-trefoil chimeric (3PHZ)	Homodimer	2	Neu5Ac-α2,6-Gal-β	na	nd	(Kadirvelraj et al. 2011; Mo et al. 2000; Tateno et al. 2004; Zhang et al. 2001)
PIC (cospin)	*Coprinopsis cinerea*	150 aa	β-trefoil (3N0K)	Monomer	na	S1 (trypsin)	na	Entomotoxic	(Avanzo Caglič et al. 2014; Sabotič et al. 2012)
Cnp (cnispin)	*Clitocybe nebularis*	146 aa	β-trefoil	Monomer	na	S1 (trypsin)	na	Entomotoxic	(Avanzo Caglič et al. 2014; Avanzo et al. 2009)
Macrocypin	*Macrolepiota procera*		β-trefoil (3H6Q)	Monomer	na		na	Entomotoxic	(Renko et al. 2010; Sabotič et al. 2009; Šmid et al. 2013)
Mcp1		169 aa				C1/C13			
Mcp3		167 aa				C1/C13			
Mcp4		167 aa				C1/S1			
Clitocypin Clt	*Clitocybe nebularis*	150 aa	β-trefoil (3H6R)	Monomer	na	C1/C13	na	Entomotoxic	(Brzin et al. 2000; Renko et al. 2010; Sabotič et al. 2007a; Sabotič et al. 2006; Sabotič et al. 2011; Šmid et al. 2015)

AAG Agrocybe aegerita galectin, *AAL Aleuria aurantia* lectin, *ABL Agaricus bisporus* lectin, *ACG Agrocybe cylindracea* galectin, *AFL Aspergillus fumigatus* lectin, AOL_1 *Aspergillus oryzae* lectin, AOL_2 *Arthrobotrys oligospora* lectin, *BEL Boletus edulis* lectin, *BEL β-trefoil Boletus edulis* lectin β-trefoil, *CCL2 Coprinopsis cinerea* lectin 2, *CGL Coprinopsis* galectin, *Clt clitocypin Clitocybe nebularis* cysteine protease inhibitor, *CNL Clitocybe nebularis* lectin, *Cnp cnispin*, *Clitocybe nebularis* serine protease inhibitor, *GSL* glycosphingolipid, *LbTec2 Laccaria bicolor* tectonin 2, *LSL Laetiporeus sulphureus* lectin, *Mcp Macrocypin*, *Macrolepiota procera* cysteine protease inhibitor, *MOA Marasmius oreades* agglutinin, *MpL Macrolepiota procera* lectin, *PIC* cospin, *PSL Polyporus squamosus* lectin, *PVL Psathyrella velutina* lectin, *RSA Rhizoctonia solani* agglutinin, *SRL Sclerotium* (*Athelia*) *rolfsii* lectin, *SSA Sclerotinia sclerotiorum* agglutinin, *TAP1 Sordaria macrospora* transcript associated with perithecial development, *XCL Xerocomus chrysenteron* lectin, *nd* not determined, *na* not applicable

[a] Minimal carbohydrate ligand for lectins and protease family for protease inhibitors

[b] Unpublished data

(91 % sequence similarity) and are approximately 32 % identical (43 % similar) to CGL2.

Galectins CGL1 and CGL2 are highly abundant in young fruiting bodies but hardly produced in the vegetative mycelium of *C. cinerea* (Boulianne et al. 2000; Plaza et al. 2014). Expression of the genes is, however, induced in the vegetative mycelium upon challenge of this tissue with the fungivorous nematode *Aphelenchus avenae* (Bleuler-Martinez et al. 2011). Similarly, AAG expression is high in fruiting bodies and absent from vegetative mycelium (Luan et al. 2010).

Minimal ligands of fungal galectins are Gal-β1,4-GlcNAc (LacNAc), Gal-β1,4-Glc (Lac), Gal-β1,3-GalNAc, and Gal-β1,4-Fuc (Butschi et al. 2010; Walser et al. 2004; Walti et al. 2008; Yagi et al. 1997; Yang et al. 2009). Substitutions at positions 2 and 3 of the galactose residues in these ligands can increase their affinity toward the galectins considerably. As examples, ACG shows a strong preference for NeuAc-α2,3-Lac (Ban et al. 2005) and one of the best ligands for CGL2 is Gal-α1,3-Lac (Walser et al. 2004).

Crystal structures of CGL2, CGL3, AAG, and ACG revealed a typical galectin fold composed of two antiparallel, six-stranded β-sheets that form a β-sandwich (Fig. 1) (Ban et al. 2005; Walser et al. 2004; Walti et al. 2008; Yang et al. 2009). Galectin CGL2 and galectin-like protein CGL3 from *C. cinerea* oligomerize into homotetramers, whereby all carbohydrate-binding sites are located on one side of the tetramer (Walser et al. 2004; Walti et al. 2008). This spatial arrangement of the carbohydrate-binding sites increases the avidity of the lectins to multivalent ligands and allows the clustering of different glycoconjugates displayed on cell surfaces (Boscher et al. 2011; Rabinovich et al. 2007). In contrast, ACG and AAG form homodimers (Ban et al. 2005; Yang et al. 2009).

Both CGL1 and CGL2 are toxic toward the bacterivorous nematode *Caenorhabditis elegans*, the mosquito *Aedes aegypti*, and the amoebozoon *Acanthamoeba castellanii* (Bleuler-Martinez et al. 2011). The toxicity toward *C. elegans* has been shown to be mediated by the binding of

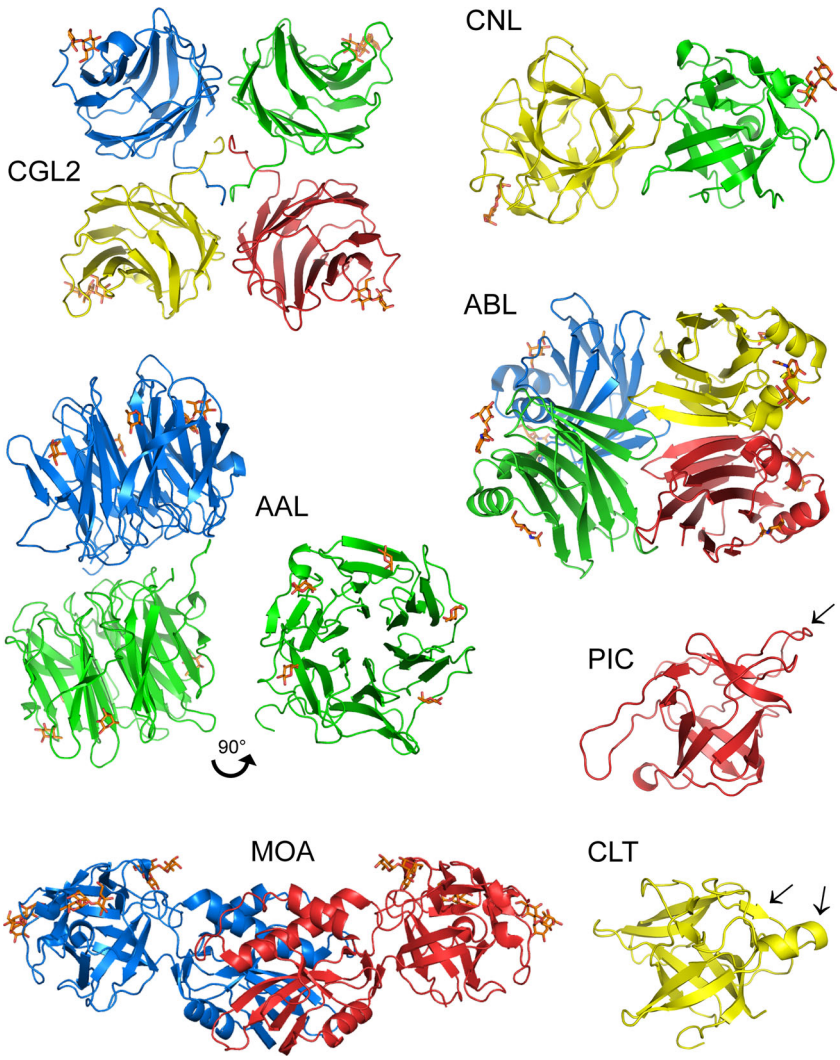

Fig. 1 Structures of fungal defense lectins and protease inhibitors. *Ribbon diagrams* are shown of homotetramer of galectin CGL2 (PDB code 2WKK), homodimer of β-trefoil-type lectin CNL (PDB code 3NBD), homodimer of β-propeller-type lectin AAL (PDB code 1OFZ) and its side view, homotetramer of actinoporin-type lectin ABL (PDB code 1Y2X), homodimer of chimerolectin MOA (PDB code 3EF2), serine protease inhibitor cospin (PIC) (PDB code 3N0K), and cysteine protease inhibitor clitocypin (CLT) (PDB code 3H6R). Monomers in dimers and tetramers are shown in *yellow*, *green*, *blue*, and *red*. Bound carbohydrate ligands are shown in *orange*. *Arrows* point to loops critical for protease inhibition

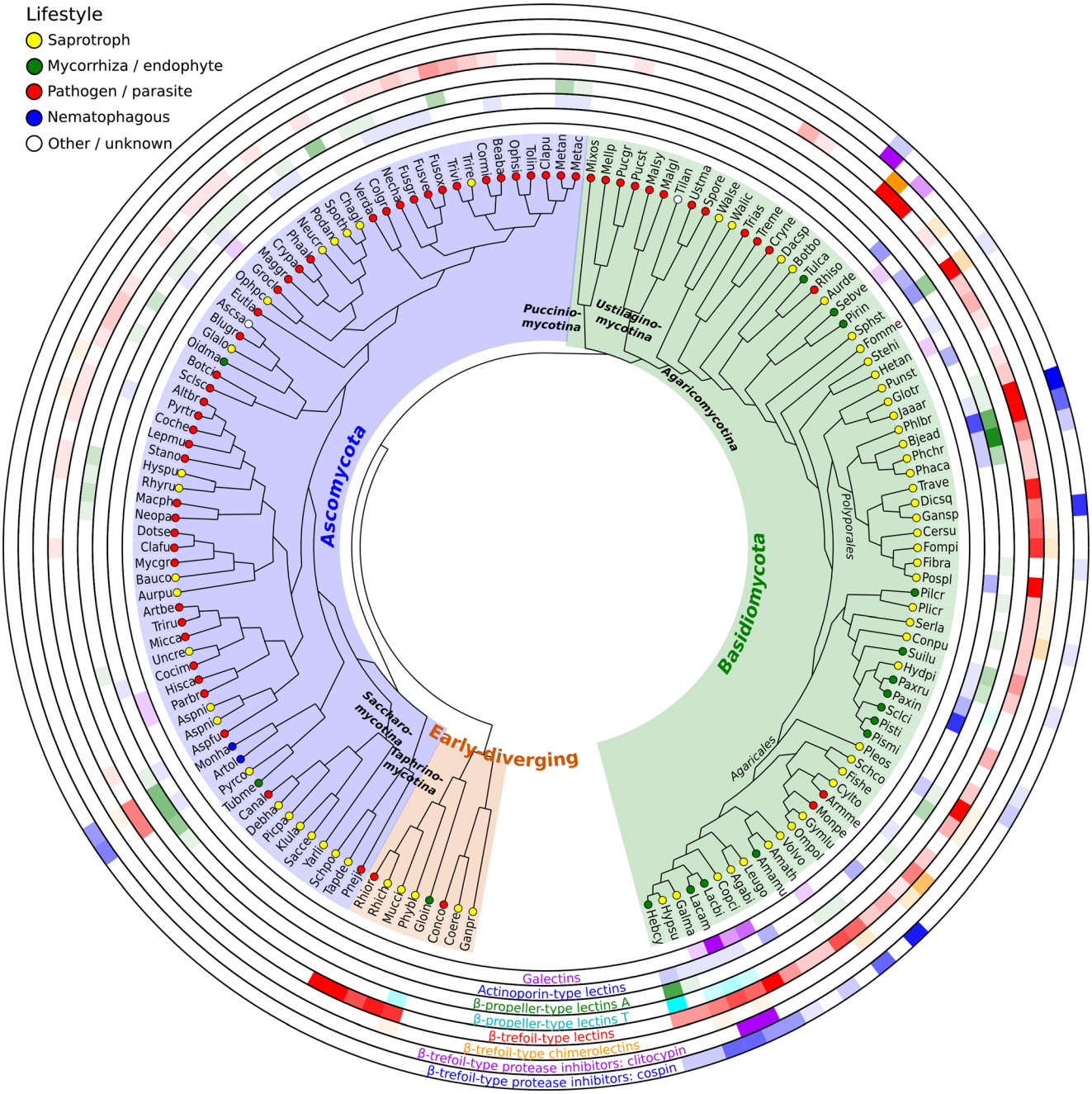

Fig. 2 Distribution of families of defense lectins and protease inhibitors in the fungal kingdom. A phylogenetic tree of the 145 fungi included in this study is depicted in the center. The full names of the organisms can be found in Table S1. The lifestyles of the individual fungi are indicated with a colored circle on the leaf node: *red* indicates a pathogen/parasite, *yellow* indicates a saprotroph, *green* indicates a mycorrhiza/endophyte, *blue* indicates a nematophagous fungus, and *white* indicates other/unknown. *Basidiomycota* are shaded in *green*, *Ascomycota* are shaded in *blue*, and "early diverging" fungi (non-dikarya) are shaded in *orange*. Selected subphyla (in bold) and orders are indicated. Each ring outside the phylogenetic tree is a heat map, representing the gene counts for each type of lectin and protease inhibitor. *White* indicates that this organism has no genes of that type, and the color is increasingly opaque when the organism has more genes of that type. The gene count that corresponds to fully opaque is 10, except in the case of the galectins for which it is 5. β-Propeller-type lectins are separated into AAL-like (A) and LbTec2-like (T) family members. β-Trefoil-type cysteine protease inhibitors include only clitocypin type as there were no macrocypin homologs found in any of the organisms in the study set. The genes were identified by the presence of a conserved domain (PFAM domain or a custom hidden Markov model) or by BLASTP, full details on the methodology can be found in Text S1. The exact gene counts can be found in Table S1 and Table S2

CGL2 to a Gal-β1,4-Fuc-α1,6 epitope on the proximal GlcNAc residue of N-glycan cores of glycoproteins of the nematode intestinal epithelium (Butschi et al. 2010). This epitope has also been detected on N-glycans of animal-parasitic

nematodes (Paschinger and Wilson 2015) and of platyhelminths (Paschinger et al. 2011).

Interestingly, phylogenetic analysis of the galectin family in fungi (Supplementary Table S2) indicates that some representatives may not be hololectins but chimeric lectins similar to the galectins from some invertebrates (Shi et al. 2014).

β-Trefoil-type lectins

One of the most prevalent hololectin families in mushrooms is constituted by proteins with sequence and structural similarity to the B-subunit of ricin, a protein toxin from the castor bean *Ricinus communis*, and is hence referred to as β-trefoil-type lectins (Cummings and Etzler 2009; Hazes 1996) (Fig. 2). These proteins adopt the so-called β-trefoil fold with pseudo-3-fold symmetry that usually harbors three potential, so-called canonical carbohydrate-binding sites. In addition to these canonical sites, the β-trefoil fold can also harbor non-canonical carbohydrate-binding sites (Schubert et al. 2012) and, as in case of protease inhibitors, binding sites for proteases (Žurga et al. 2015) (see below). The best characterized representatives of this family of mushroom lectins are *Rhizoctonia solani* agglutinin (RSA) and *Sclerotinia sclerotiorum* agglutinin (SSA) of the plant pathogens *R. solani* (basidiomycete) (Hamshou et al. 2013) and *S. sclerotiorum* (ascomycete) (Sulzenbacher et al. 2010), as well as CNL, CCL2, MpL, and BEL β-trefoil of the homobasidio(agarico)mycetes *Clitocybe nebularis* (Pohleven et al. 2009; Pohleven et al. 2012), *C. cinerea* (Schubert et al. 2012), *Macrolepiota procera* (Žurga et al. 2014), and *Boletus edulis* (Bovi et al. 2013) (Table 1). These proteins show high sequence variability, as they share only 7 to 16 % sequence identity (25 to 35 % similarity), the exception being CNL, MpL, and RSA that are 23 to 26 % identical (30 to 40 % similar).

All these proteins were isolated from sclerotia (RSA, SSA) or fruiting bodies (CNL, CCL2, MpL, BEL β-trefoil) of the originating fungi. Expression of RSA was found to be developmentally regulated, lectin expression being low in vegetative mycelium and the protein accumulating in adult sclerotia (Hamshou et al. 2007; Kellens and Peumans 1990). Based on the accumulation of protein during sclerotium formation and its depletion during mycelium germination, a storage function for RSA in *R. solani* has been proposed. CCL2 and its paralog CCL1 exhibit a pronounced fruiting body-specific expression with almost no expression in the vegetative mycelium of *C. cinerea* (Plaza et al. 2014; Schubert et al. 2012).

The carbohydrate-binding specificity of the different β-trefoil lectins varies (Table 1). Many of these lectins bind terminal Gal or GalNAc residues of oligosaccharides by their canonical carbohydrate-binding sites. In the case of CCL2, however, a single, non-canonical binding site binds with high affinity to Gal-β1,4-(Fuc-α1,3)GlcNAc and to GlcNAc-β1,4-(Fuc-α1,3)GlcNAc (Schubert et al. 2012). The latter epitope is called the anti-HRP epitope, since it is found in the core of plant N-glycans, e.g., on the horseradish peroxidase and glycoproteins of many invertebrates, and is a known allergen (Paschinger et al. 2009).

β-Trefoil-type lectins are composed of approximately 150 amino acid residues with acidic (CNL, MpL) or very basic (RSA) isoelectric points. The typical β-trefoil fold consists of α, β, and γ repeats built from 12 β-strands. These strands are connected by loops, arranged in pseudo-3-fold symmetry, forming a six-stranded β-barrel (Fig. 1). The canonical carbohydrate-binding sites are found on the α-, β-, and/or γ-repeats, with either none (CCL2), one (CNL and SSA), two (RSA), or all three (BEL β-trefoil) being functional. The architecture of the canonical carbohydrate-binding sites is very similar. β-Trefoil-type lectins bind β-galactosides in an orientation that differs from that for galectins. While galectins bind linear glycans in a groove parallel to the protein surface, β-trefoil-type lectins bind them in a perpendicular orientation, in which only the non-reducing end of the glycan interacts with the binding pocket. All these proteins assemble to homodimers but, interestingly, each protein uses a different interface for dimer formation (Bovi et al. 2013; Pohleven et al. 2012; Schubert et al. 2012; Skamnaki et al. 2013; Sulzenbacher et al. 2010; Žurga et al. 2014).

Many of the β-trefoil-type lectins exhibit entomotoxic activity (Table 1). The mechanism of action has been analyzed at the cellular level for RSA and SSA. The toxicity of RSA toward the cotton leafworm depends on its binding to Gal/GalNAc-containing glycans on the midgut epithelium (Hamshou et al. 2013). RSA is not taken up by the epithelial cells, and intoxicated epithelial cells show symptoms of apoptosis, possibly caused by lectin-mediated activation of the respective signaling pathways (Hamshou et al. 2012). In the case of RSA-mediated toxicity toward the red flour beetle *Tribolium castaneum*, the ability of RSA to pass through the perithrophic matrix to reach intestinal epithelial cells has been shown to be a prerequisite for toxicity (Walski et al. 2014). SSA is highly toxic toward the pea aphid *Acyrthosiphon pisum*. It binds to the insect midgut cells and, like RSA, is not internalized but causes death of midgut epithelial cells probably via a signal transduction pathway triggered by a glycoreceptor (Hamshou et al. 2010a). In contrast, CCL2 is not toxic for insects but exhibits strong toxicity toward *C. elegans*. This toxicity is mediated by binding to the anti-HRP epitope in the core of nematode N-glycans (Schubert et al. 2012). The absence of entomotoxicity may be due to spatial restriction of the anti-HRP epitope to the nervous system in insects (Paschinger et al. 2009). The mechanism of toxicity of these (and other) hololectins is not clear. The toxicity of CNL against *C. elegans* has been shown to depend on both glycan binding and dimer formation (Pohleven et al. 2012). Recently, CCL2 was shown to bind to the nematode

intestinal epithelium without being endocytosed (Stutz et al. 2015). This binding led to complete disintegration of the microvillar organization—interestingly without breaching the barrier function of the epithelium. Finally, nematotoxicity of CCL2 appears to depend on active feeding, since application of the lectin without a supply of bacterial food was not toxic to the worms. Nematotoxicity against *C. elegans* has also been demonstrated for MpL, but the in vivo ligand remains obscure in this case (Žurga et al. 2014).

β-Propeller-type lectins

Members of the β-propeller-type family of mushroom lectins include hololectin AAL from the orange peel mushroom *Aleuria aurantia* (Fujihashi et al. 2003; Wimmerova et al. 2003) and its homologs from various ascomycetous molds such as AOL_1 from *Aspergillus oryzae*, and AFL from *Aspergillus fumigatus* (Houser et al. 2015; Matsumura et al. 2008) as well as PVL and LbTec2 from the basidiomycetes *Psathyrella velutina* and *Laccaria bicolor* (Cioci et al. 2006; Wohlschlager et al. 2014). Basidiomycetous PVL and LbTec2 show very low sequence homology to each other and to ascomycetous AAL (below 15 % sequence identity and 30 to 45 sequence similarity), while AFL and AOL_1 are highly homologous to each other (82 % sequence identity and 90 % similarity) and 30 % identical (60 % similar) to AAL.

AAL, PVL, and LbTec2 have been isolated from fruiting bodies of the respective fungi. AAL was expressed in vegetative mycelium and in fruiting bodies (Ogawa et al. 1998). Expression of LbTec2 in vegetative mycelium has been shown to be upregulated in the presence of mycorrhizal helper bacteria (Deveau et al. 2015).

These lectins are characterized by highly repetitive, wheel-like structures made from β-strands which harbor multiple carbohydrate-binding sites. Their molecular weights range from 24 kDa (LbTec2) to 42 kDa (PVL). AAL (33.5 kDa) is folded into six propeller blades, each composed of four antiparallel β-sheets with an additional small antiparallel β-sheet that is involved in the dimerization of the protein (Fig. 1) (Fujihashi et al. 2003; Wimmerova et al. 2003). Each of the AAL protomers contains six potential binding sites for terminal fucose residues, but their affinities have been shown not to be equivalent. One binding site is not functional at all, while another has a much higher affinity than the others and was occupied by free fucose when the hololectin was isolated from fruiting bodies (Olausson et al. 2008). The primary sequence and fold of AFL are very similar to those of AAL, but, in contrast to AAL, all six binding sites, although not equivalent, are functional in that they bind fucose (Houser et al. 2015). The three homologous proteins: AAL, AOL_1 and AFL, differ slightly in their specificity for the connectivity of the bound fucose residues (Matsumura et al. 2008) and are structurally similar to fucose-binding hololectins from bacteria, including RSL from *Ralstonia solanacearum* (Sudakevitz et al. 2002). The primary sequence of hololectin LbTec2 is unrelated to that of AAL but is similar to those of proteins from filamentous bacteria (actinobacteria), slime moulds, and animals and is predicted to also adopt a six-bladed β-propeller fold with six carbohydrate-binding sites (Wohlschlager et al. 2014). LbTec2 binds to Sepharose and is specific for 2-O-Me-fucose and 3-O-Me-mannose residues. The affinity of LbTec2 for these monosaccharides is very low (millimolar range), but, according to a commonly accepted concept in the lectin field (Shinohara et al. 1997), when they are displayed on a surface, the avidity to these carbohydrates could be very high due to the oligomerization of the protein. Finally, PVL, whose primary sequence is not related to AAL or LbTec2, sharing 8 and 11 % sequence identity (33 % similarity), respectively, folds into a monomeric, seven-blade β-propeller with a total of six binding sites for terminal GlcNAc or Neu5Ac residues located at the interfaces between the blades (Audfray et al. 2015; Cioci et al. 2006; Ueda et al. 2002).

AAL is toxic toward nematodes, insects, and amoebozoa (Bleuler-Martinez et al. 2011) and also to the mucoromycete fungus *Mucor racemosus* (Amano et al. 2012). Both nematotoxicity and entomotoxicity have been shown to depend on binding of the hololectin to fucose-containing glycoconjugates in the target organisms. Based on the lack of toxicity resistance of various *C. elegans* strains with mutations in N-glycan and glycosphingolipid biosynthesis, fucosylated O-glycans have been hypothesized as most likely target glycan of AAL in this organism. LbTec2 was recently shown to be toxic to *C. elegans* and genetic evidence, in combination with glycome analysis, showed that the nematotoxicity is dependent on 2-O-Me-fucose and 3-O-Me-mannose in *C. elegans* N-glycans (Wohlschlager et al. 2014). According to a recent report on *C. elegans* N-glycan structure, LbTec2 most likely binds to 2-O-Me-Fuc-α1,2-Gal-β1,4 and 3-O-Me-Man-α1,3 on the core β-mannose of *C. elegans* N-glycans (Yan et al. 2015). To date, no nematotoxicity or entomotoxicity has been reported for PVL, but the lectin has been shown to bind truncated N-glycans on cancer cells (Audfray et al. 2015).

Actinoporin-type lectins

One of the best known and characterized mushroom lectin families is that of actinoporin-type hololectins, also referred to in the PFAM database (http://pfam.xfam.org/) as the fungal fruiting body lectin family (FB_lectin, PF07367). Members of this protein family show structural homology to actinoporins, a family of pore-forming proteins (cytolysins) originally isolated from sea anemones (Birck et al. 2004; Kristan et al. 2009). It was suggested that the archetypal actinoporin fold is used for specific binding to various molecules at the plasma membrane surface (Kristan et al. 2009). Characterized representatives of the actinoporin-type mushroom lectin family

include XCL, ABL (ABA), SRL, and BEL from the basidiomycetes *Xerocomus (Boletus) chrysenteron*, *Agaricus bisporus*, *Sclerotium (Athelia) rolfsii*, and *B. edulis* (Birck et al. 2004; Bovi et al. 2011; Carrizo et al. 2005; Leonidas et al. 2007) and AOL_2 and TAP1 from the ascomycetes *Arthrobotrys oligospora* and *Sordaria macrospora* (Nowrousian and Cebula 2005; Rosen et al. 1996b). These representatives of basidiomycetes share 53 to 82 % sequence identity (67 to 89 % similarity), while AOL_2 is approximately 45 % identical (62 % similar) to them and TAP1 only 34 % identical (50 % similar). Surprisingly, AOL_2 and TAP1 share only 26 % sequence identity (45 % similarity).

With the exception of AOL_2 which was isolated from nematode traps, all actinoporin-type lectins have been isolated from fruiting bodies or sclerotia, suggesting a developmental control of their synthesis. In accordance with this, the expression of TAP1 was demonstrated to be strongly upregulated during fruiting body formation (Nowrousian and Cebula 2005). Similarly, expression of SRL is much higher in sclerotia than in vegetative mycelium (Swamy et al. 2004). Using respective knockout mutants, TAP1 and AOL_2 were shown not to be essential for the formation of fruiting bodies (Nowrousian and Cebula 2005) or the function of nematode traps, respectively (Balogh et al. 2003).

The actinoporin-type lectins are small proteins of approximately 16 kDa with neutral to basic isoelectric points. They all bind specifically to N-acetyl-galactosamine (GalNAc). ABL is a dual specificity lectin harboring separate binding sites for Gal-β1,3-GalNAc (T- or Tn-antigen) and N-acetyl-glucosamine (GlcNAc) (Nakamura-Tsuruta et al. 2006). Similarly, structural studies on SRL revealed two carbohydrate-binding sites: a primary one for GalNAc and secondary one for GlcNAc (Leonidas et al. 2007). Based on sequence homology and functional studies, the GlcNAc-binding site is conserved in XCL, AOL_2, and BEL but not in TAP1 (Bleuler-Martinez et al. 2011). The protein structure is, similar to those of pore-forming actinoporins, composed of two β-sheets that consist of six and four β-strands connected by a helix-loop-helix motif. XCL, ABL, and BEL form tetramers in solution that have been described as dimers of dimers (Fig. 1). SRL was shown to be a dimer but can, from a structural point of view, form similar tetramers as other representatives (Birck et al. 2004; Bovi et al. 2011; Carrizo et al. 2005; Leonidas et al. 2007).

Several representatives of this lectin family have been shown to exhibit entomotoxic and nematotoxic activity (Table 1). XCL is toxic toward the hemipterans *A. pisum* and *Myzus persicae*, the dipterans *Drosophila melanogaster* and *A. aegypti*, as well as the nematode *C. elegans* (Bleuler-Martinez et al. 2011; Jaber et al. 2008; Trigueros et al. 2003). Both the entomotoxic and nematotoxic activity of XCL are dependent on carbohydrate binding, and genetic data suggested that XCL binds to terminal GlcNAc residues in nematode N-glycans (Jaber et al. 2008; Yan et al. 2012). TAP1 is toxic to *C. elegans*, *A. aegypti*, and the amoebozoon *A. castellanii* (Bleuler-Martinez et al. 2011). SRL is toxic toward the cotton leaf worm *Spodoptera litura* by binding to membrane proteins of the midgut epithelium, thus triggering caspase-dependent cell death (Vishwanathreddy et al. 2014). Thus, in vitro and in vivo experiments suggest that these proteins mediate their toxicity by binding, at the same time, to the Gal-β1,3-GalNAc epitope on mucin-type O-glycans and terminal GlcNAc on N-glycans. This binding mode would lead to clustering of glycoproteins on the intestinal epithelia of insects or nematodes via two types of protein-bound glycans (Chachadi et al. 2011; Yan et al. 2012). Interestingly, actinoporin-type lectins are also found in primitive plants, where they may also have a role in defense (Peumans et al. 2007).

β-Trefoil-type chimerolectins

Besides the β-trefoil-type hololectins described above, mushrooms also produce chimerolectins in which one or several β-trefoil-type lectin domains are fused to a domain with a different function. These proteins are analogous to bacterial and plant AB toxins including the previously mentioned plant toxin ricin. The best characterized examples of this lectin family are MOA and LSL from the saprophytic mushrooms *Marasmius oreades* (Cordara et al. 2011; Grahn et al. 2007; Grahn et al. 2009; Wohlschlager et al. 2011) and *Laetiporus sulphureus* (Mancheno et al. 2005; Mancheno et al. 2010; Tateno and Goldstein 2003). These proteins consist of a single N-terminal β-trefoil-type lectin domain fused to a cysteine protease (MOA) and to an aerolysin-type pore-forming domain (LSL). The numbers and specificities of the carbohydrate-binding sites of the β-trefoil domains differ in that all three canonical binding sites of MOA are functional and bind to glycans carrying terminal Gal-α1,3-Gal/GalNAc-β epitopes (Grahn et al. 2007; Grahn et al. 2009; Wohlschlager et al. 2011) while only two of the three canonical binding sites of LSL appear to be functional and specific for β-galactosides including lactose and LacNAc (Angulo et al. 2011; Mancheno et al. 2005). Homologs of both proteins from other dikaryotic fungi have been partially characterized (Chumkhunthod et al. 2006; Kadirvelraj et al. 2011; Plaza et al. 2014; Wohlschlager et al. 2011). The MOA homologs PSL and SCA (*Schizophyllum commune* agglutinin), from the saprophytic mushrooms *Polyporus squamosus* and *S. commune*, differ from MOA in that PSL harbors a lower number of functional carbohydrate-binding sites with a different specificity toward terminal Neu5Ac-α2,6-Gal-β epitopes but with the catalytic triad of the cysteine protease domain conserved (Kadirvelraj et al. 2011; Wohlschlager et al. 2011). SCA exhibits the carbohydrate-binding specificity of

MOA but not its catalytic activity (Wohlschlager et al. 2011). On the other hand, the homology of LSL to fruiting body-specific proteins of *C. cinerea* is restricted to the aerolysin domain (Plaza et al. 2014). SCA and the *C. cinerea* homolog of LSL are differentially expressed in fruiting bodies compared to the vegetative mycelium in *S. commune* and in *C. cinerea* (Ohm et al. 2010; Plaza et al. 2014).

MOA and the *C. cinerea* homolog of LSL have nematotoxic and the latter also entomotoxic activity (Plaza et al. 2014; Wohlschlager et al. 2011). LSL was shown to associate into hexamers in solution and crystals (Mancheno et al. 2005). By analogy to other aerolysin-like pore-forming toxins, it has been hypothesized that LSL undergoes a conformational change in its aerolysin domain on insertion into the target membrane, thus forming a pore (Mancheno et al. 2010). In contrast, MOA forms a dumbbell-shaped dimer (Fig. 1) in which the two β-trefoil domains represent the balls of the dumbbell and the dimerized cysteine protease domain the connecting bar (Grahn et al. 2007; Grahn et al. 2009). The latter domain undergoes a conformational change on binding divalent cations, which has been shown to be necessary for activation of the protease in vitro (Cordara et al. 2011; Wohlschlager et al. 2011). Using truncations and alterations of single residues responsible for carbohydrate-binding and protease activity of MOA, it was demonstrated that both functions are necessary for full nematotoxicity of this protein (Wohlschlager et al. 2011). Exploitation of *C. elegans* mutants defective in the biosynthesis of glycosphingolipids coupled with in vitro binding assays with glycosphingolipids isolated from *C. elegans* enabled Gal-α1,3-GalNAc-β-containing glycosphingolipid species to be pinpointed as target glycans of MOA in *C. elegans* (Wohlschlager et al. 2011). The pH and Ca^{2+} requirements of the cysteine protease activity suggest that the protein has to be internalized to be toxic, in a manner analogous to that of bacterial AB toxins (Wohlschlager et al. 2011). In the cases of both LSL and MOA, the β-trefoil domains of these chimerolectins are thought to mediate initial binding of the protein toxins to the plasma membrane.

Additional families of potential fungal defense lectins

In addition to the above-described families of fungal defense lectins, several families of potential fungal defense lectins have recently been described. From fruiting bodies of the basidiomycetes *Hygrophorus russula* and *Grifola frondosa*, two mannose-specific lectins, termed *H. russula* lectin (HRL) and *G. frondosa* lectin (GFL), were isolated and characterized (Nagata et al. 2005; Suzuki et al. 2012). The amino acid sequence of these proteins is related to that of Jacalin from plants, and some plant members of this lectin family have recently been shown to protect the producing plants from herbivorous insects (Al Atalah et al. 2014). Similarly, recent reports suggest that both ascomycetes and basidiomycetes contain cytoplasmic homologs of GNA (*Galanthus nivalis* agglutinin or snowdrop lectin), another family of mannose-binding entomotoxic plant lectins (Fouquaert et al. 2011; Shimokawa et al. 2012). Cyanovirin-N homologs (CVNH) constitute a family of fungal cytoplasmic lectins that comprises mannose-binding lectins from various ascomycetes including *Tuber borchii* and *Neurospora crassa* (Koharudin et al. 2008). Isolation and characterization of FVE (*Flammulina velutipes* fungal immunomodulatory protein) from fruiting bodies of *F. velutipes* has identified a family of immunoglobulin (Ig)-type cytoplasmic lectins present in many basidiomycetes (Paaventhan et al. 2003). From the basidiomycete *Pholiota squarrosa*, a very small cytoplasmic lectin, PhoSL (*P. squarrosa* lectin), specific for α 1,6-linked fucose on N-glycan cores, has been identified (Kobayashi et al. 2012). The lectin is homologous to *Rhizopus stolonifer* lectin (RSL), a lectin from the zygomycete *R. stolonifer* (Oda et al. 2003) and constitutes a novel family of cytoplasmic lectins. No nematotoxicity or entomotoxicity has so far been reported for any of these lectins.

Protease inhibitors

Proteolytic enzymes (also known as proteases, proteinases, or peptidases) are present in all organisms and play essential metabolic and regulatory roles in many biological processes. Due to the essential functions of proteases in life and death processes, anomalous proteolytic activities can be very harmful. Therefore, regulation of proteolytic activity is vital and takes place on several levels, from gene expression to post-translational modification and compartmentalization, and most importantly by their interaction with protease inhibitors (Lopez-Otin and Bond 2008; Rawlings et al. 2014).

Protease inhibitors are present in all kingdoms of life and can be broadly classified into those that inhibit peptidases of more than one catalytic type, those that inhibit families of peptidases of one catalytic class, and those that inhibit peptidases belonging to one family or a single peptidase. A detailed classification of protease inhibitors based on sequence homology is available in the MEROPS database (http://merops.sanger.ac.uk/inhibitors/). There are two general mechanisms by which protein inhibitors inhibit peptidases: irreversible "trapping" reaction, involving a conformational change of the inhibitor, and reversible tight-binding interactions, in which the inhibitor binds with high affinity to the peptidase active site. The detailed physiological roles of many protein protease inhibitors are still unknown. They are either involved in control of endogenous proteases or in defense mechanisms. Exogenous proteases targeted in defense are either virulence factors of pathogens and parasites or digestive proteases in grazers, predators, and parasites, that are involved in nutrient acquisition for growth and development or in evasion of host

defenses (Christeller 2005; Rawlings et al. 2014; Sabotič and Kos 2012).

The MEROPS database (release 9.12) identifies more than 650 protease inhibitors (Rawlings et al. 2014). They are classified into 78 families, 22 of which include members of fungal origin and only 7 families that include members from higher fungi. The latter are all inhibitors of serine and cysteine proteases. Protein inhibitors of metalloproteases have not been identified in fungi. Two protein aspartic protease inhibitors have been isolated from *Ganoderma lucidum* (Tian and Zhang 2005) and *Coriolus versicolor* (Zhang et al. 2012), but their sequences have not been determined. Families I51 (serine carboxypeptidase Y inhibitor), I32 (survivin-like caspase inhibitor), and I4 (serpin or α-1-peptidase inhibitor) include potential inhibitors of serine and cysteine proteases identified only as homologues in genomes. Inhibitory activity against serine proteases has been established in crude protein extracts of several species of mushrooms (Gzogyan et al. 2005; Vetter 2000; Zuchowski and Grzywnowicz 2006; Zuchowski et al. 2009), but only a few protease inhibitors from mushrooms have been biochemically and structurally characterized. One of them, the peptidase A inhibitor 1 from *Pleurotus ostreatus* (POIA1) has been classified into family I9, based on sequence homology to the propeptides of subtilisin-like proteases. In addition to its inhibitory activity, this inhibitor can also act as an intramolecular chaperone, assisting the folding of the cognate protease. Its biological role is probably that of controlling endogenous proteases (Kojima et al. 2005; Sasakawa et al. 2002). Most thoroughly characterized are the serine protease inhibitors of family I66 (mycospins) and cysteine protease inhibitors of families I48 (clitocypins) and I85 (macrocypins), which all exhibit entomotoxic activity.

Mycospins, fungal inhibitors of serine proteases

Mycospins are serine protease inhibitors from mushrooms that constitute family I66 in the MEROPS classification. This family includes three biochemically characterized members: cospin from *C. cinerea* (Sabotič et al. 2012), cnispin from *C. nebularis* (Avanzo Caglič et al. 2014; Avanzo et al. 2009), and LeSPI (*Lentinula edodes* serine protease inhibitor) from *L. edodes* (Odani et al. 1999).

Cospin is expressed abundantly in fruiting bodies of *C. cinerea* in contrast to vegetative mycelium where its expression is approximately 700-fold lower. In addition to cospin (PIC1), there are three more isoproteins in the *C. cinerea* genome sharing 38 to 95 % sequence identity with cospin (Sabotič et al. 2012). Cospin (CC1G_09480) is, together with its paralog (CC1G_09479), one of the most highly upregulated genes in *C. cinerea* young fruiting bodies (Plaza et al. 2014). Sequence variability at the protein level has also been shown for the natural isolates of cnispin from *C. nebularis* from which three proteins with similar biochemical properties and N-terminal amino acid sequences were isolated, indicating that cnispin could be encoded by a multigene family. There is 31 % sequence identity and 46 % sequence similarity between cnispin and cospin amino acid sequences. Comparison of expression levels of cnispin in *C. nebularis* fruiting body and in vegetative mycelium suggests that it is constitutively expressed in mycelium and fruiting body, although expression was higher in the cap of the fruiting body. It was also shown that cnispin is not secreted into the medium (Avanzo et al. 2009).

Mycospins are small proteins (16 to 18 kDa) with acidic isoelectric points that are resistant to exposure to extreme pH conditions. They have very similar inhibitory profiles, all exhibiting strong trypsin-specific inhibition. They inhibit chymotrypsin only weakly and other serine proteases not at all or very weakly. Cospin inhibits trypsin with an equilibrium constant for inhibition (*K*i) in the picomolar range and cnispin in the nanomolar range, while both inhibit chymotrypsin with *K*i in the micromolar range (Avanzo et al. 2009; Sabotič et al. 2012). The crystal structure of cospin reveals that these proteins are members of the β-trefoil fold protein family. This fold is composed of 12 β-strands connected by 11 loops of various lengths and composition. Surprisingly, the reactive site residues for trypsin inhibition in the two proteins differ, being Arg27 of the β2-β3 loop in cospin and Lys127 of the β11-β12 loop in cnispin. They are both classic inhibitors that bind to the protease active site in a substrate-like manner and form a tight and stable complex with trypsin. The difference between cospin and cnispin is that in vitro the former persists in complex with trypsin for over a month at 37 °C, while cnispin is completely degraded by trypsin in 24 h. It is suggested that the β2-β3 loop involved in the inhibition of trypsin by cospin is better optimized for trypsin inhibition, since only small conformational changes are needed for binding to trypsin and the loop is more stable than the β11-β12 loop of cnispin that presumably undergoes more substantial changes on binding to trypsin (Avanzo Caglič et al. 2014; Sabotič et al. 2012).

Cospin exhibits a strong entomotoxic activity against *D. melanogaster* that is mediated by specific inhibition of the fly's serine proteases based on the lack of toxicity and protease inhibition of the cospin R27N mutant (Sabotič et al. 2012). Natural isolates of cnispin also show entomotoxic activity against *D. melanogaster*, albeit lower than that for cospin (Avanzo et al. 2009), validating the entomotoxic potential of the I66 family. Furthermore, cospin showed no toxicity against the nematode *C. elegans* and amoebozoon *A. castellanii*. A biological role for cospin in the defense of fruiting bodies against Drosophilidae is further corroborated by the absence of trypsin-like protease genes in the *C. cinerea* genome and by the fact that serine proteases are the predominant digestive proteolytic enzymes in dipterans (Sabotič et al. 2012; Terra and Ferreira 2005). In addition to the defensive

role directed against exogenous serine proteases, there is some evidence that cnispin and cospin could have a dual role, also participating in the regulation of endogenous serine proteases, in fruiting body development and/or resource recycling (Avanzo et al. 2009; Sabotič et al. 2012).

Mycocypins, inhibitors of cysteine proteases

Two families of cysteine protease inhibitors—clitocypins from *C. nebularis* (family I48 in Merops) and macrocypins from *M. procera* (family I85 in Merops)—have been identified in mushrooms and are collectively called mycocypins.

Clitocypins are encoded by a small gene family in the *C. nebularis* genome with significant sequence variability. This variability however has no influence on the inhibitory activity of clitocypins. Macrocypins in *M. procera* show even higher amino acid sequence variability, their sequences being grouped into five groups (macrocypins 1–5) with 75–86 % sequence identity between groups and more than 90 % sequence identity within groups. The sequence variability is reflected in their inhibitory profiles, since different macrocypins exhibit different strengths of inhibition for proteases. Even though they have many biochemical properties in common, sequence identity between clitocypin and macrocypin amino acid sequences is low. They are approximately 23 % identical and 33 % similar (Sabotič et al. 2007a; Sabotič et al. 2006; Sabotič et al. 2009).

The expression pattern of clitocypin in *C. nebularis* fruiting bodies has revealed similar amounts of clitocypin protein throughout the fruiting body while, at the messenger RNA (mRNA) level, expression varies in different parts of fruiting bodies. Clitocypin is also expressed in the vegetative mycelium and shown not to be secreted into the medium (Sabotič et al. 2006). Macrocypins exhibit tissue-specific expression (Sabotič et al. 2011). In *M. procera*, the protein is present throughout the fruiting body, but the amount of protein is significantly higher in the veil fragments on the cap and in the ring (annulus). Again, little congruence was observed with mRNA and protein expression profiles. Regulation of mycocypin expression has been further analyzed in the model mushroom *C. cinerea*, using mycocypin promoters and a reporter gene. Clitocypin and macrocypin promoters were transcriptionally active in vegetative mycelium and in fruiting bodies of *C. cinerea*. The clitocypin promoter displayed an expression pattern similar to that of a constitutive promoter with uniform expression throughout the tissues. In contrast, the macrocypin promoter displayed tissue-specific expression during fruiting body development which was similar to the macrocypin expression pattern in *M. procera*. This difference in temporal and spatial expression indicates specific developmental or protective roles for individual mycocypins (Sabotič et al. 2011).

Mycocypins are small proteins (17–19 kDa) with similar isoelectric points (pH 4.8) and apparent stability against high temperature and extreme pH mediated by the ability to unfold reversibly. They are strong inhibitors of papain-like proteases with equilibrium constants for inhibition (*K*i) of papain in the low nanomolar range. Mycocypins also strongly inhibit cysteine cathepsins: Cathepsins L, V, and S are inhibited with *K*i values in the nanomolar range by both clitocypin and macrocypins, and cathepsin K is strongly inhibited by clitocypin and more weakly by macrocypins. Cathepsins B and H, that exhibit both endopeptidase and exopeptidase activity, are not or only very weakly inhibited by mycocypins. In addition to papain-like cysteine proteases (family C1), mycocypins also inhibit asparaginyl endopeptidase (AEP) also called legumain (family C13) while trypsin, but not AEP, is inhibited by macrocypin 4 (Renko et al. 2010; Sabotič et al. 2007a; Sabotič et al. 2009).

Mycocypins have a β-trefoil fold. A distinct motif for their binding to papain-like cysteine proteases was revealed by the three-dimensional structure of clitocypin in complex with cathepsin V. Two broad loops of mycocypins (β1-β2 and β3-β4) fill the active site cleft of the protease along its whole length, occluding the catalytic cysteine residue and preventing the approach of substrate molecules. The two loops are stabilized by numerous hydrogen bonds. A different site is involved in the inhibition of AEP or trypsin by mycocypins. Asparagine in the β5-β6 loops of clitocypin and macrocypins 1 and 3 mediates the inhibition of AEP. In macrocypin 4, the asparagine is replaced by a lysine, enabling inhibition of trypsin. Consideration of the crystal structures leads to the conclusion that the binding loops of clitocypin and macrocypins exhibit substantial conformational flexibility during binding into the active site of their target enzymes (Renko et al. 2010; Renko et al. 2012).

The sequence diversity, with sites showing positive evolutionary selection, the variations in spatial and temporal expression, the variations in inhibitory profiles, and scarcity of cysteine proteases in basidiomycetes, provides evidence that mycocypins' biological role is defense against pathogen infection and/or predation by insects or other pests. They would target exogenous cysteine proteases found in mycoviruses, nematodes, insects, mites, and slugs, all known antagonists of higher fungi (Brzin et al. 2000; Sabotič et al. 2007a; Sabotič et al. 2006; Sabotič and Kos 2012; Sabotič et al. 2007b). Entomotoxic activity of mycocypins was shown directly for the model coleopteran insect pest Colorado potato beetle. It was mediated by inhibition of specific digestive proteases, and evidence suggests that the negative effect of mycocypins on larval growth and development is mediated through multiple levels (Šmid et al. 2013; Šmid et al. 2015).

Distribution within the fungal kingdom

In the past decade, over 400 fungal genome sequences have become available, due in large part to the efforts of the US Department of Energy Joint Genome Institute (DOE JGI) in compliance with its missions in alternative energy, global carbon cycling, and biogeochemistry (Grigoriev et al. 2011). On the basis of their predicted proteomes, we selected a set of 145 genomes from the latest JGI database (http://genome.jgi-psf.org/programs/fungi/index.jsf) in order to examine the distribution of the above-described families of fungal defense proteins within the fungal kingdom and to draw conclusions about their evolution and physiological function. For these purposes, we calculated a phylogenetic tree and included available metadata on the lifestyle of the selected fungi. The presence or absence of fungal defense families was based either on BLASTP searches with single family representatives or on available consensus sequences using the PFAM database (see Supplementary Text for details). In addition, we performed a gene clustering analysis of the identified hits of a specific defense gene family (see Supplementary Text for details). The analysis relies inherently on the quality of the published gene predictions. The results of this analysis are summarized in Fig. 2 and Supplementary Tables S1, S2, and S3.

One of the main outcomes of the analysis is that both types of fungal defense proteins, lectins and protease inhibitors, occur more frequently in the phylum *Basidiomycota* than in the phylum *Ascomycota*. This may be partly due to the fact that most of these lectins and protease inhibitors were initially identified in basidiomycetes and they exhibit quite high sequence diversity. Thus, orthologs encoded in genomes of ascomycetes might not be detected using BLASTP searches because the sequence similarity is too low. Within *Basidiomycota*, these proteins appear to be restricted to the subphylum *Agaricomycotina* and to be almost absent from the other subphyla, *Pucciniomycotina* and *Ustilaginomycotina*. These results are in agreement with a previous, preliminary study (Bleuler-Martinez et al. 2011) and may be explained by the parasitic lifestyle of these subphyla, since the fungus may be sheltered from fungivores by the defense system of the host. Accordingly, within the phylum *Ascomycota*, the subphylum *Taphrinomycotina* (Pneji, Schpo, Tapde), which also harbors mainly parasitic fungi, also completely lacks fungal defense proteins. An alternative explanation for the lack of fungal defense proteins in these subphyla is, however, suggested by examination of the closely related subphylum *Saccharomycotina* (Yarli, Sacce, Klula, Picpa, Debha, Canal). Members of this subphylum propagate mainly as yeasts, i.e., as unicellular organisms. Since many members of the subphyla *Pucciniomycotina*, *Ustilaginomycotina*, and *Taphrinomycotina* are dimorphic, i.e., propagate as yeasts for a significant part of their lifestyle, the lack of fungal defense proteins may correlate with their unicellular morphology with the reasoning that a defense system against fungivores only makes sense for a multicellular organism. In accordance with both of the above reasonings, the pathogenic basidiomycetous yeast *Cryptococcus neoformans*, which belongs to *Agaricomycotina*, also lacks the described defense proteins. Along these lines, the apparent difference in occurrence of fungal defense proteins between the phyla *Ascomycota* and *Basidiomycota* might also be due to the fact that most analyzed basidiomycetous species are saprotrophs, whereas most ascomycetous species are (plant) pathogens (biotrophs). On the other hand, the genomes of some of the pathogenic members of the *Agaricomycotina* (Rhiso, Armme) code for a significant number of fungal defense proteins.

Among *Agaricomycotina*, mycorrhizal species appear to be particularly rich in fungal defense proteins. In fact, there was a good correlation of the presence of lectins homologous to LbTec2 (β-propeller-type lectin T) with this lifestyle which included also the early diverging, endomycorrhizal fungus *Rhizophagus irregularis* (*Glomus intraradices*, Gloin) (Supplementary Table S3). Eventually, these fungi take over part of the defense of the root system of the host plant against root-targeting herbivores during symbiosis. In accordance with this hypothesis, genes coding for defense proteins like LbTec2 are induced in the mycorrhizal state (Martin et al. 2008) and some fungivorous nematodes are believed to be able to feed also on plant roots (Yeates et al. 1993). Similarly, the occurrence of defense proteins in the two nematophagous ascomycetes, *Arthrobotrys oligospora* (Artol) and *Monacrosporium haptotylum* (Monha) compared to that of other ascomycetes, may be explained by an increased demand of these fungi for protection from fungivorous species among the prey nematodes.

Early diverging fungi generally appear to lack defense proteins but the subphylum *Mucoromycotina* and the related, sole representative of the phylum *Glomeromycota* among the selected fungi are surprisingly rich in β-trefoil-type defense lectins. This finding suggests that this family of defense proteins is ancient.

Analysis of individual fungal genomes has confirmed previous results that the genomes often encode several paralogs of a given defense protein and that the paralogous genes are often clustered, most probably as the result of gene duplications (Supplementary Table S2). Examples are the tandem repeats of galectin- and cospin-encoding genes in *C. cinerea*. The regulation of these genes and the specificity of the encoded proteins are similar (Butschi et al. 2010; Plaza et al. 2014; Sabotič et al. 2012), but there might be cases where either the regulation of the duplicated genes or the specificity of the encoded proteins is different. Defense gene duplications and diversifications may enable the composition of species-specific armories against predators and parasites. The scattered distribution within the fungal kingdom, the conservation in bacteria, and the lack of introns in the coding regions (Supplementary Table S2) may indicate that some of these

genes were acquired from bacteria by horizontal gene transfer (HGT). As an example, Moran et al. recently presented evidence suggesting acquisition of LSL by HGT from bacteria (Moran et al. 2012).

Potential applications

The described fungal defense lectins have been considered as potential pesticidal agents in crop protection. Strong entomotoxicity affecting development and survival of the economically important plant pests cotton leafworms *Spodoptera littoralis* and *S. litura* was shown for β-trefoil-type lectin RSA and actinoporin-type lectin SRL, respectively, both binding Gal/GalNAc-containing glycans (Hamshou et al. 2010b; Vishwanathreddy et al. 2014). Additionally, RSA exhibited toxicity toward the red flour beetle *T. castaneum*, an important pest of stored products (Walski et al. 2014). Furthermore, the effect of different fungal lectins against the important sap sucking crop pests, using pea aphid *A. pisum* as a model organism, has been analyzed. The β-trefoil lectin SSA exhibited strong toxicity in feeding assays (Hamshou et al. 2010a). Actinoporin-type lectin XCL also exhibited toxicity against aphids including *A. pisum* and green peach aphid (*M. persicae*) (Jaber et al. 2007; Jaber et al. 2006; Trigueros et al. 2003).

In addition to lectins, fungal protease inhibitors have been considered as potential insecticidal agents against herbivorous insects. Both families of mycocypins, clitocypin and macrocypins, affected larval growth and development of the major potato pest Colorado potato beetle (*Leptinotarsa decemlineata*). They acted by inhibiting specific digestive proteases without triggering adaptive responses in larval guts at transcriptional level (Šmid et al. 2013; Šmid et al. 2015).

Analogously to the application of entomotoxic fungal defense proteins against herbivorous insects, nematotoxic fungal defense proteins could be used for protecting crops from plant-parasitic nematodes, e.g., root knot nematode (*Meloidogyne incognita*), root lesion nematode (*Pratylenchus* spp.), or the pine wilt nematode (*Bursaphelenchus xylophilus*) (Jones et al. 2013). However, no toxicity of fungal defense proteins for plant-parasitic nematodes has, to our knowledge, been reported so far.

One of the issues of the use of fungal defense proteins in crop protection is the possible toxicity of the proteins for beneficial insects, humans, and livestock. As an example, XCL was endocytosed by various animal and human cell lines and caused inhibition of proliferation of some of these cell lines (Francis et al. 2003; Marty-Detraves et al. 2004). A second issue is the way the protein is applied to the crop plant. The most effective way would be to modify the crop plant genetically so that it expresses the fungal proteins in the tissue that is under attack by the herbivore. Currently, however, the use of genetically modified crops is limited due to strict regulations and public opposition. Thus, external application of proteins as deterrents, preventing contact between the herbivore and the plant, might be a more promising option.

Another potential application of nematotoxic fungal defense proteins, in particular lectins, is in veterinary and human medicine for fighting animal and human parasitic nematodes. A recent study reported a dose-dependent toxicity of CGL2, CCL2, AAL, and MOA toward larval and adult stages of the barbers pole worm, *Haemonchus contortus* in vitro, in which toxicity of the lectins correlated with their binding to the intestinal epithelium of this animal-parasite nematode (Heim et al. 2015). Successful in vivo applications of nematotoxic, carbohydrate-binding protein toxins of microbial origin against parasitic nematodes were demonstrated for the *Bacillus thuringiensis* toxin Cry5B against the hookworm *Ancylostoma ceylanicum* in hamsters and against the large roundworm *Ascaris suum* in pigs (Cappello et al. 2006; Hu et al. 2012; Urban et al. 2013). The nematotoxicity of all those carbohydrate-binding proteins proven to be effective against parasitic nematodes had previously been demonstrated in *C. elegans*, showing the power of this model organism for detection and characterization of nematotoxic proteins. As an alternative to the direct use of nematotoxic lectins as therapeutics of infestations with parasitic nematodes, these proteins could be used as leads for a vaccination strategy against these parasites. Nematotoxicity of many of the above-described fungal defense lectins, and also of bacterial Cry5B, has been shown to rely on the binding of specific, lipid- or protein-bound glycoepitopes on the intestinal epithelium of *C. elegans*. A combination of genetic, biochemical, and toxicity assays allowed the structure of the target glycoepitope of some of these lectins in *C. elegans* to be identified (Table 1). Since some of these epitopes are conserved in parasitic nematodes with respect to both structure and localization (Heim et al. 2015; Paschinger and Wilson 2015), they represent candidate hidden antigens for vaccination. Vaccination with hidden antigens is based on the idea that epitopes that are hidden from the animal immune system during a normal infection with a pathogen or infestation with a parasite have a higher likelihood of inducing a strong and protective antibody response (Munn 1997). In support of this idea, vaccinations with partially purified, intestinal hemoglobinases isolated from adult *H. contortus* significantly lowered the burden of sheep and avoided reinfections with this parasite (LeJambre et al. 2008). It is not clear at the moment whether this reported protection was mediated by carbohydrate or protein epitopes or a combination of both.

Fungal lectins are also being considered for treatment of cancers and microbial infections, based on their antitumor and immunomodulatory, antiproliferative and mitogenic, antiviral, and antimicrobial activities. Finally, fungal lectins have been considered widely as diagnostic tools based on their highly specific binding of glycoconjugates and on the altered

glycosylation profiles observed in various diseases. These potential medical applications of fungal defense lectins and protease inhibitors have been described in more detail in recent reviews (Erjavec et al. 2012; Hassan et al. 2015; Sabotič and Kos 2012; Xu et al. 2011).

Conclusions and perspectives

The abundance and diversity of toxic proteins in dikaryotic fungi is astonishing. On one hand, structurally similar proteins, for example β-trefoil-type lectins and β-trefoil-type protease inhibitors, perform different functions, and, on the other hand, structurally distinct lectins exert toxicity by binding to the same glycoepitopes. Some of these toxic proteins are directed toward very specific target organisms. Cospin, for example, appears to be toxic to *D. melanogaster* but not to other diptera, nematodes, amoebozoa, fungi, or bacteria (Sabotič et al. 2012; Sabotič, unpublished observations), whereas other toxic proteins provide a general protection against different antagonists (Bleuler-Martinez et al. 2011). LbTec2, for example, binds a glycan modification that has been observed in different phyla ranging from bacteria to plants and animals (Wohlschlager et al. 2014). Another level of diversity is found in the regulation of the biosynthesis of these fungal toxins. Most of these proteins were found to be expressed abundantly in fruiting bodies, as a form of constitutive defense of this reproductive structure. Biosynthesis of some of the proteins was demonstrated to be induced in the vegetative mycelium upon challenge with a fungivorous nematode (Bleuler-Martinez et al. 2011).

Searches of available fungal genomes for homologs of the already characterized lectins and protease inhibitors reveal that these proteins are widely present among ascomycetes and basidiomycetes. Based on the current pace of identification, of, e.g., novel fungal lectins, the proteins described in this review most probably represent only the tip of an iceberg and there is no doubt that more protein toxins from fungi still await to be identified. In order to identify novel families of fungal protein toxins, such identification should be performed not only on the basis of sequence similarity but rather on the basis of toxicity or biochemical activity (inhibition of proteases or binding to carbohydrates).

Fungal protein toxins have advantages over bacterial, animal, and most plant toxins for biotechnological applications since they are produced in the cytoplasm, which eases their expression in bacterial expression systems. They are, therefore, more readily available in recombinant form for applications in crop protection or veterinary and human medicine. Finally, fungal defense proteins are invaluable research tools.

Understanding the mechanism of action of these protein toxins will provide further insight into the general mechanism of these effectors. In the case of hololectins, the exact toxicity mechanism, beyond the dependence on carbohydrate binding, is still unclear. Differential expression of these effectors of a fungal innate defense system can be exploited as a readout to identify the signals and receptors that convey their induction upon challenge by the antagonist and upon developmental cues. Knowledge about these players would certainly be a big step forward in the understanding of the fungal innate defense system. At the moment, the limited availability of genetic tools, for example, for *Agaricomycotina*, reduces the pace of these investigations. Results obtained by such studies are relevant as they can be readily translated into the areas of plant and animal innate immunity. It has been shown that plants and, more recently, animals employ lectins as effector molecules in innate immunity against pathogens and parasites. Thus, learning tricks from fungi could provide an edge in combating plant pests as well as animal and human pathogens and parasites.

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