

Spatial and Temporal Expression of Laccase in *Coprinopsis Cinerea* Using Galectin Promoters

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Galectins are β -galactoside binding lectins defined by a conserved sequence. In mammals, there is a family of galectins that interact with glycoproteins in both extracellular and intracellular milieu and regulate various biological phenomena including cell growth, cell differentiation, cell adhesion, and apoptosis. Outside of the animal kingdom, genes for galectins are known in *Arabidopsis* and a few basidiomycetes. In fungi, galectins have been studied in detail in *Coprinopsis cinerea*. The *C. cinerea* galectins Cgl1 and Cgl2 are specific to the fruiting body. When nutrients are exhausted, Cgl2 is expressed under dark conditions in the mycelium at places of fruiting body initiation and primary hyphal knot formation. Cgl2 expression continues within the initial stages of fruiting body development till primordia formation is completed. In contrast, expression of Cgl1 starts later with the light-induced formation of secondary hyphal knots. Cgl1 expression also continues during primordia development to end at the stage of meiosis. In the genome, there is a gene for a third galectin (*cgl3*) but nothing is yet known about its expression. In this study, we establish the *C. cinerea* laccase gene *lcc1* as a reporter gene to study spatial and temporal regulation of galectin gene promoters during fruiting body development.

1. Introduction

1.1. *Mushroom lectins*

Lectins have originally been detected and defined from plant origin as agglutinins of erythrocytes. Subsequently, lectins have been isolated from most king-

doms and the lectin definition has become more generalized as a protein other than enzymes and antibodies that binds tightly but reversibly to a specific sugar or sugars. Lectins comprise several different protein classes defined by sugar-binding specificities and sequence similarities (Goldstein et al. 1980, Ambrosi et al. 2005). Lectins are most often found extra cellular with suggested functions in binding to the ECM (extra cellular matrix), cell-to-cell adhesion and defense of pathogens (Kilpatrick 2002). Other lectins have fundamental intracellular roles such as in glycoprotein processing (Schrag et al. 2003), in regulation of cell cycle and apoptosis, in nuclear pre-mRNA splicing (Liu et al. 2002, Wang et al. 2004) and possibly in nitrogen storage (Law 2000).

The study of lectins in fungi started in 1907 with toxicological investigations on hemolytic agglutinins from edible fruiting bodies and from the fly agaric (Ford 1907, 1910). Since then, a large number of lectins have been purified and characterized from vegetative mycelia and, mostly, from fruiting bodies of basidiomycetes (Guillot and Konska 1997, Wang et al. 1998). Lectins have been implicated in growth and morphogenesis of mushrooms (Richard et al. 1994, Walser et al. 2003, Swamy et al. 2004, Wösten and Wesels 2005), may function in storage (Kellens and Peumans 1990) and in symbiotic and parasitic relations including mycorrhiza (Guillot et al. 1994) and lichen associations (Elifio et al. 2000, Lehr et al. 2000), plant-pathogenic interaction (Rudiger 1998), insect defense (Birck et al. 2004), virus defense (Sun et al. 2003) and mycoparasitism (Inbar and Chet 1992, 1994). One aspect in research concentrates on the medicinal and pharmacological potential of lectins from mushrooms. Lectins from basidiomycetes have been demonstrated to have anticancer activities, mitogenic activities and immunomodulatory activities (for examples see Wang et al. 2000, 2003, Lee et al. 2003, Ngai et al. 2003, Ho et al. 2004, Kawamura et al. 2004, Sze et al. 2004).

Currently, the best understood fungal lectins are the two fruiting body-specific galectins Cgl1 and Cgl2 of *Coprinopsis cinerea* (Cooper et al. 1997, Boulianne et al. 2000, Walser et al. 2004, 2005).

1.2. *Galectins.*

Galectins are a large family of β -galactoside binding lectins that are characterized by conserved amino acids in the carbohydrate recognition domain

(CRD; Fig. 1). Originally, galectins were isolated from various phyla of the animal kingdom (mammals, birds, amphibians, fish, nematodes and sponges) but galectins or at least genes for galectins are now also known from plants (*Arabidopsis*) and basidiomycetes (Fig. 2). In humans, several different galectins are known with widespread functions. Each galectin exhibits a specific pattern of expression in various cells and tissues, and expression is often closely regulated during development. In the extracellular compartment, galectins are thought to act by cross-linking β -galactoside containing glycoconjugates, resulting in modulation of cell adhesion and cell signaling. Within cells, galectins have been shown to regulate cell cycle, cell growth and apoptosis and to act in pre-mRNA splicing (Leffler 1997, Cooper 2002, Wang et al. 2004). Understanding the roles of galectins in basic biological processes is vital for possible applications of galectins in diagnosis and therapy of cancer, autoimmunity and transplant-related disease (Hughes 2001).

Whilst highly similar to each other (21 to 86% identity, 38 to 92% similarity), fungal galectins have only 7 to 19% amino acid identity and 15 to 21% similarity to human galectins (Table 1). In similarity cluster analysis, they form a separate branch from the animal galectins (Fig. 3). Nevertheless, fungal galectins might be very useful for medical purposes but still have to be tested for any medical application. In support of this idea, the β -galactoside binding lectins ABL from fruiting bodies of *Agaricus bisporus* and XCL from fruiting bodies of *Xerocomus chrysenteron* have been shown to have antiproliferative effects on human epithelial cancer cells, without having any apparent cytotoxicity (Marty-Detravas et al. 2004, Carrizo et al. 2005). These lectins

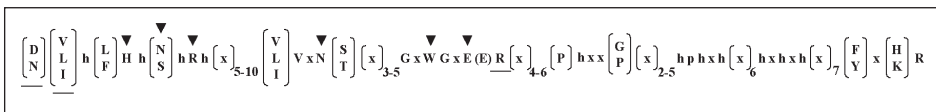


Figure 1. Conserved sequence elements within the carbohydrate recognition domain of the galectin family. Arrows mark residues with invariant bonding to carbohydrate ligands. Underlined positions indicate residues that coordinate ligands in extended binding site (e.g. acetamido group of N-acetyllactosamine or substituted β -galactosides). Furthermore, several positions of hydrophobic amino acids (h) are conserved that possibly help in stabilizing the galectins fold. x: any amino acid. After Barondes et al. (1994a,b).

FULL LENGTH CONTRIBUTIONS

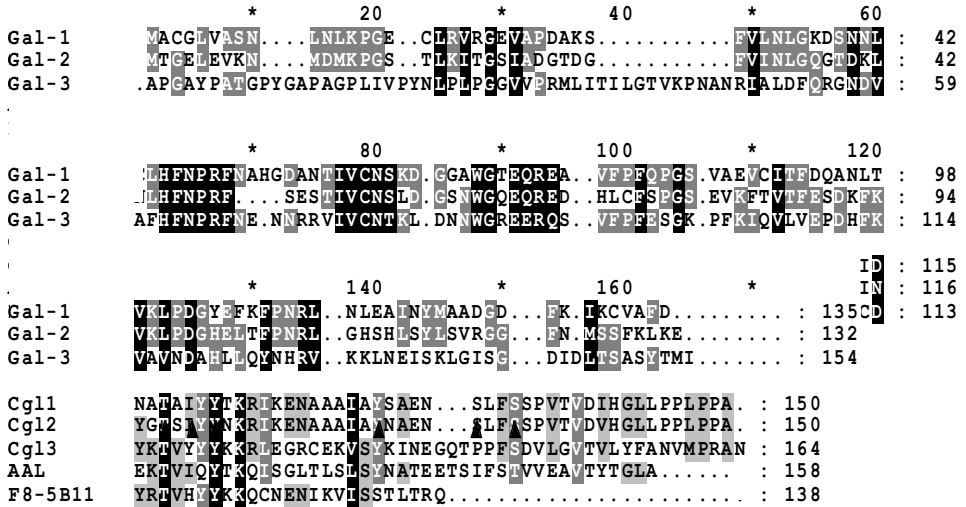


Figure 2. Sequence comparison of human and fungal galectins. Sequences of human and of fungal proteins were aligned separately from each other in order to better maintain the impression of sequence similarities between the fungal proteins. Arrows mark residues with invariant bonding to carbohydrate ligands (see Fig. 1). Protein and gene accession numbers in the NCBI GenBank: Cg11 of *Coprinopsis cinerea* (Q06100), Cg12 of *C. cinerea* (Q9P4R8), AAL of *Agrocybe aegerita* (Q6WY08), F8-5B11 of *Heterobasidium annosum* (BM346916), human Gal-1 (NM_002305.2), human Gal-2 (NM_006498.2), human Gal-3 (NM_002306.1). Cg13 was deduced from contig 1.28 (position 715943 to 715452) from the *C. cinerea* sequence (http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/). Note that the sequence of AAL has been deduced from a partial cDNA and that Gal-3 is N-terminal truncated by 96 amino acids.

Table 1
The amino acid identity and similarity between fungal and human galectins

Galectin	Cgl1	Cgl2	Cgl3	AAL	F8-5B11	Gal-1	Gal-2	Gal-3
Cgl1	100%	86%	32%	27%	34%	15%	17%	8%
	100%	92%	50%	44%	46%	31%	30%	15%
Cgl2		100%	32%	27%	32%	16%	19%	8%
		100%	47%	43%	44%	32%	32%	16%
Cgl3			100%	29%	31%	13%	14%	7%
			100%	44%	48%	24%	28%	16%
AAL				100%	21%	14%	16%	8%
				100%	38%	27%	29%	17%
F8-5B11					100%	15%	15%	7%
					100%	25%	25%	16%
Gal-1						100%	41%	11%
						100%	59%	23%
Gal-2							100%	13%
							100%	21%
Gal-3								100%
								100%

and the related β -galactoside binding lectin PCL F1 from *Pleurotus cornucopiae* fruiting bodies and a lectin expressed during mycorrhiza in *Paxillus involutus* however distinguish from the galectins by their specific carbohydrate binding sites (Fig. 4). The proteins define a new class of lectins, the actinoporin-related family of fungal lectins (Birck et al. 2004). Moreover, a third class of fungal β -galactoside binding lectins have recently been detected in fruiting bodies of the mushroom *Laetiporus sulphureus* (Tateno and Goldstein 2003) that is neither related to galectins nor to the family of actinoporin-related lectins (not shown). Lectins of the actinoporin-related family and the *L. sulphureus* lectins (LSLa, LSLb and LSLc) are pore-forming proteins and seem to act as toxins (Tateno and Goldstein 2003, Trigueros et al. 2003, Birck et al. 2004).

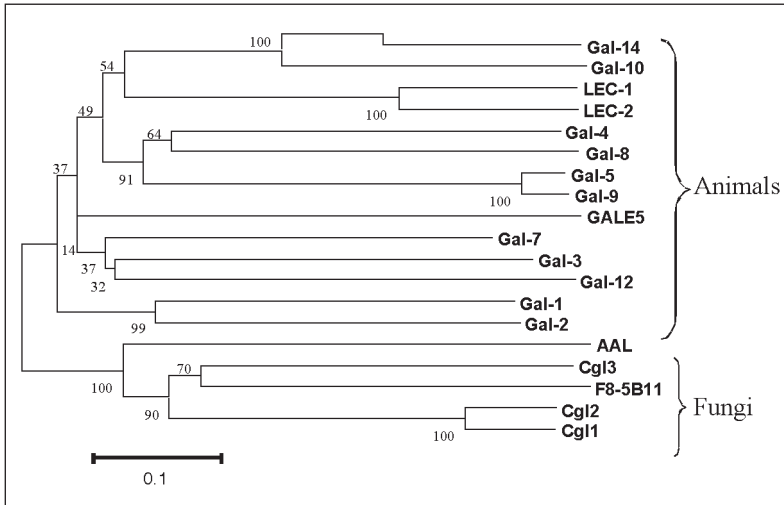


Figure 3. Phylogenetic tree of galectins from humans (GenBank accession numbers: Gal-1: NM_002305.2, Gal-2: NM_006498.2, Gal-3: NM_002306.1, Gal-4: NM_006149.2, Gal-5: AAH73889.1, Gal-7: NM_002307.1, Gal-8: NM_201545, Gal-9: NM_002308.2, Gal-10: Q05315, Gal-12: AAG40864.1, Gal-13: NM_013268.2, Gal-14: Q8TCE9), the roundworm *Caenorhabditis elegans* (LEC-1: NP_496801.2, LEC-2: NP_496165.2), the mosquito *Anopheles gambiae* (GALE 5: XP_309359.2) and fungi. For origin of fungal proteins and accession numbers see legend of Fig. 2.

In basidiomycetes, galectins are known in *C. cinerea* and in *Agrocybe aegerita* (Boulianne et al. 2000, Yagi et al. 2001; Fig. 2). Furthermore, EST sequences suggest galectins to also occur in *Heterobasidion annosum* (Walser et al. 2003; Fig. 2, 3). Expression of the galectin genes *cgl1* and *cgl2* in *C. cinerea* correlates with fruiting body development (Charlton et al. 1992, Boulianne et al. 2000). Within Petri-dishes on complete medium, the genes are expressed in the outer zone of the culture in the youngest aerial mycelium, once the fungus covers the whole plate. Western blot and RT-PCR analyses showed very little or no expression of galectins in younger, actively growing cultures. With the onset of fruiting body development, Cgl2 was detected in fruiting zones and, in low amounts, also in non-fruiting zones. Formation of primary hyphal knots (lose compact structures occurring at the early stages of fruiting body development; Walser et al. 2003, Kües et al. 2004) was correlated with galectin gene transcription. *cgl1* transcripts and Cgl1 protein ap-

peared during later stages of development, starting with the stage of light-induced secondary hyphal knots (in which cap and stipe tissues differentiate; Walser et al. 2003, Kies et al. 2004). Expression continues throughout primordia development and declines at early meiosis at the stage of prophase I (Charlton et al. 1992, Boulianne et al. 2000).

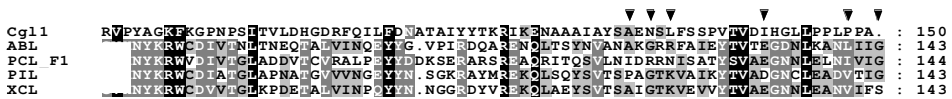


Figure 4. Comparison of fungal β -galactoside binding lectins from various basidiomycetes. Cg11 is a galectin from *Coprinopsis cinerea*. All other lectins belong to a recently defined family of fungal lectins that have structural similarities to actinoporins (Birck et al. 2004). Arrows above the sequences mark residues in galectin Cg11 with invariant bonding to carbohydrate ligands (see Fig. 1). Regions with possible residues for carbohydrate binding in the actinoporin-related family of fungal lectins are underlined (Birck et al. 2004). Protein and gene accession numbers in the NCBI GenBank: Cg11 of *C. cinerea* (Q06100), ABL of *Agaricus bisporus* (Q00022), PCL F1 of *Pleurotus cornucopiae* (BAB63922.1), PIL of *Paxillus involutus* (AAT91249.1), XCL of *Xerocomus chrysenteron* (AAL73236.1).

Galectins in *C. cinerea* are secreted and found in cell walls and the extracellular matrix of mushroom tissues. Cellular localization of galectins showed a marked accumulation of the proteins in the veil, the outer cap and stipe tissues of the primordium. These tissues are subjected to strong tensions during rapid stipe elongation and cap opening in the process of fruiting body maturation. Therefore, a function in hyphal-hyphal aggregation and tissue formation has been proposed for the galectins (Boulianne et al. 2002, Walser et al. 2005). In support of this argument, the outer cap and stipe tissues also contain galectin ligands. Possible ligands are also detected in the hymenium, the outer spore-bearing cell layer of the gills not expressing the known galectins. Currently, it is not clear whether β -galactoside binding lectins other than Cg11 and Cg12 are expressed in these tissues. At least one candidate exist in *C. cinerea* (see results). Remarkably, the galectin ligands are of glycolipid nature (Walser et al. 2004, 2005). The β -galactoside binding lectin SRL from sclerotial bodies (mycelial aggregates serving in dormancy) of *Sclerotium rolfii* has also been described to bind glycosphingolipids (Swamy et al. 2004).

1.3. *Laccase as reporter for tissue-specific expression of galectins genes.*

One way to further understand the role of β -galactoside binding lectins and their ligands in mushroom development is to look more deeply into temporal and spatial expression of their genes. To this end, for *C. cinerea* we are developing a reporter system based on enzymatic laccase activities.

Laccases are phenoloxidases that belong to the multi-copper oxidase (Mco) family. These enzymes are versatile redox-enzymes that oxidize various phenolic compounds and aromatic amines (Messerschmidt 1998). The colorless compound ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) is an artificial substrate for laccases. Upon oxidation by a laccase, ABTS gives rise to a colored product that can be used in photometric tests as well as in plate tests to assess enzyme activities. Using the *C. cinerea lcc1* gene as reporter, we previously have established a laccase reporter assay to test activity of various homologous and heterologous constitutive promoters in *C. cinerea* (Kilaru et al. 2005 and submitted). It is now our goal to test whether gene *lcc1* can also serve in studying developmental regulated promoters. As first promoters, the regulatory sequences of the fruiting body specific galectin genes *cg11* and *cg12* will be analyzed.

2. Material And Methods

pYSK2 (Kilaru et al. 2005) is a yeast shuttle-vector that contains the yeast 2 μ m *ori* and *URA3* selection marker, the ColE1 *ori* (*ori Ec*) and *amp^R* from *E. coli*, the phage f1(+) *ori*, the *C. cinerea* genes *pab1* (for *para*-aminobenzoic acid synthesis; James et al. 2002) and *lcc4* (for laccase Lcc4; Hoegger et al. 2004). This plasmid was used in *in vivo*-recombination (Raymond et al. 1999) in *Saccharomyces cerevisiae* strain RH 1385 (Mösch et al. 1990) to replace *lcc4* sequences with gene *lcc1* under control of galectin gene promoters. Promoter sequences were amplified with chimeric primers from genomic DNA of *C. cinerea* homokaryon AmutBmut (Granado et al. 1997). Gene *lcc1* was obtained from plasmid pESK1 (Kilaru et al. submitted). The constructs were isolated from yeast, transformed for plasmid amplification into *Escherichia coli* strain XL1-Blue (Stratagene) and isolated from the bacterium by standard protocols (Sambrook et al. 2001). Constructs were used in trans-

formation of *C. cinerea* homokaryon strain AmutBmut (*A43mut*, *B43mut*, *pab1-1*). Plasmid pPAB1-2 with the *pab1* wildtype gene (James et al. 2002) was used as a control in transformation. 0.5 mM ABTS was added to regeneration agar and YMG/T complete medium to detect laccase activity. Media recipes, growth and fruiting conditions are given by Granada et al. (1997). tblastn searches were performed with the *C. cinerea* genomic sequence (http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/).

3. Results And Discussion

3.1. Promoter comparison

Genes *cg11* and *cg12* are highly similar (87% sequence identity in the coding regions, 65% sequence identity in the promoter regions and 63% sequence identity in the terminator regions) and are found in tandem arrangement in a distance of 1366 bp (Boulianne et al. 2000; Fig. 5). Deduced from alignments with the *cg11* promoter region and with the *cg12* terminator region, 495 bp of the *cg11-cg12* interim region present the *cg11* terminator sequence and 871 bp the *cg12* promoter region (Fig. 6). The corresponding promoter (*cg11*) and terminator (*cg12*) sequences are 841 bp and 522 bp long, respectively.

Bertossa et al. (2004) found minimal *cg12* promoter activity to reside within 627 bp. The authors defined a number of potential promoter elements for *cg12*, of which at least direct repeats of the sequence TGGAAAG (a CRE-like binding sequence(s) and a Sp1-like motif seem to participate in promoter regulation. A sequence resembling mating-type protein binding sites of other fungi (*hsg*-like motif) was found to be not essential.

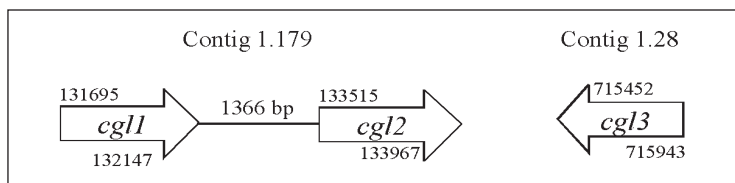


Figure 5. Galectin genes: localization in the genome of *Coprinopsis cinerea*. Sequences were identified by tBlast searches of the *C. cinerea* Okayama 7 genome (http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/) with Cg11 from strain AmutBmut. Identical results hits were obtained in tblastn searches with Cg12 and Cg13.

When comparing the *cg11* and *cg12* promoter sequences (Fig. 6), large regions of high similarity are obvious. Interestingly in these regions, few potential regulatory elements were defined. In contrast, regions with identified or postulated elements are more dissimilar and, often, elements are unique to the *cg12* promoter. Most of the non-conserved elements locate in a 120 bp sequence that mediates induction of *cg12* expression in the dark (Bertossa et al. 2004; Fig. 6). *cg11* and *cg12* are differentially regulated by light and dark signals. *cg12* is dark induced, whilst *cg11* is light induced (Boulianne et al. 2000) and may need other, yet to be defined regulatory elements.

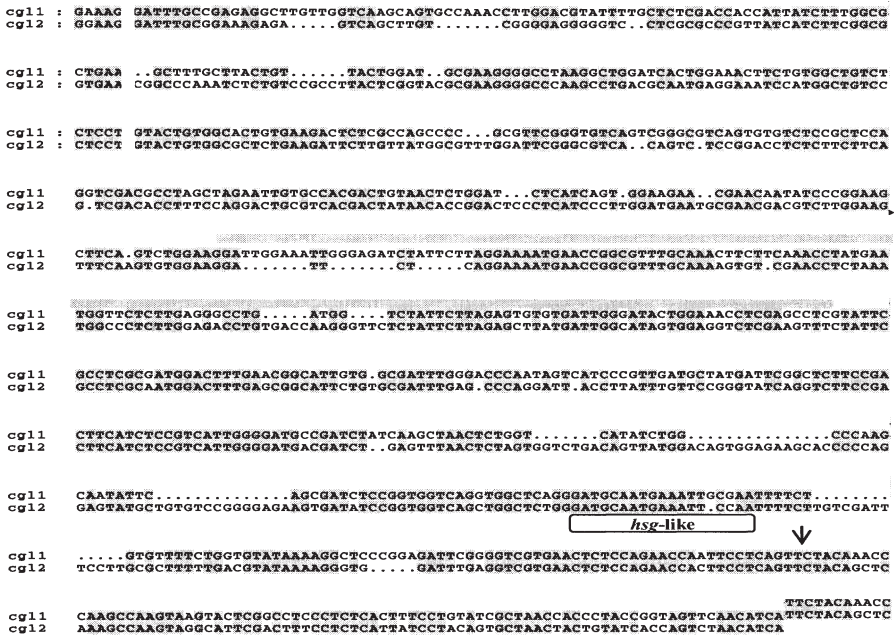


Figure 6. Comparison of the *cg11* and *cg12* promoter sequences. A black triangle indicates the maximal extension of the *cg12* promoter – upstream sequences belong to the *cg11* terminator region. The minimal *cg12* promoter sufficient to confer regulated expression is marked by an open triangle. The 120 bp sequence needed for dark induction of the *cg12* promoter is indicated by a grey bar. Within the *cg12* promoter sequence, two identical, equal-spaced sequence stretches are outlined by open boxes. Boxes underneath the *cg12* promoter sequence indicate motifs resembling known binding sites of eukaryotic transcription factors (CRE, SP1, *hsg*-like). Direct repeats are marked by open arrows, indirect repeats by inverse oriented black arrows. A (non-essential) TATA box is marked by a strong black bar [further details on promoter elements are given in Bertossa et al. (2004)]. A potential transcription start site (Charlton et al. 1992) is marked by a vertical arrow. A black line indicates an intron in the 5'-untranslated region of *cg11* and *cg12* transcripts (Boulianne et al. 2000).

3.2. Reporter constructs.

In order to study the temporal and spatial expression of galectins within mycelial cultures and different primordia tissues and for future definition of individual promoter elements, we developed a reporter system using the enzymatic activities of *C. cinerea* laccase Lcc1 as a marker.

By *in vivo*-recombination in yeast, we subcloned the promoters of the galectin genes *cg11* and *cg12* in front of laccase gene *lcc1* (see Fig. 7). The resulting *cg11* and *cg12* promoter constructs were called pYNS2 and pYSK36, respectively. These constructs and as a control pPAB1-2 were transformed into *C. cinerea* homokaryon AmutBmut. 77 different transformants were obtained for construct pYNS2, 23 different transformants for pYSK36 and 176 different transformants for pPAB1-2. On regeneration agar containing uncolored ABTS, none of the transformants caused green staining of the agar which is indicative for oxidation of ABTS by laccase activity. Some clones were cultured on YMG/T plates with ABTS. It appears that positive *lcc1* transformants with the *cg12* promoter produce laccase at the edges of the plates when cultures are kept in dark. In contrast, transformants of the *cg11* promoter seem to produce laccase at the outer edge of the cultures in a day/night rhythm under fruiting conditions. Later during incubation, laccase activity is seen within all cultures including those of pPAB1-2 transformants. Each time, laccase activity starts from the inoculum in the middle of the plates. However, there appear to be differences in quality (intensity of staining) and timings, raising possibility that both promoters are active at a senescent mycelial stage (not shown). Former work revealed that Cgl1 and Cgl2 are expressed specifically at outer colony edges at places of fruiting body initiation and within fruiting structures (Boulianne et al. 2000, Bertossa et al. 2004). Our preliminary analysis of transformants with *lcc1* reporter constructs seem to confirm the results of *cg11* and *cg12* expression at the initiation of fruiting body development. Ongoing studies target at tissue specific localization of laccase activity during different stages of fruiting body development.

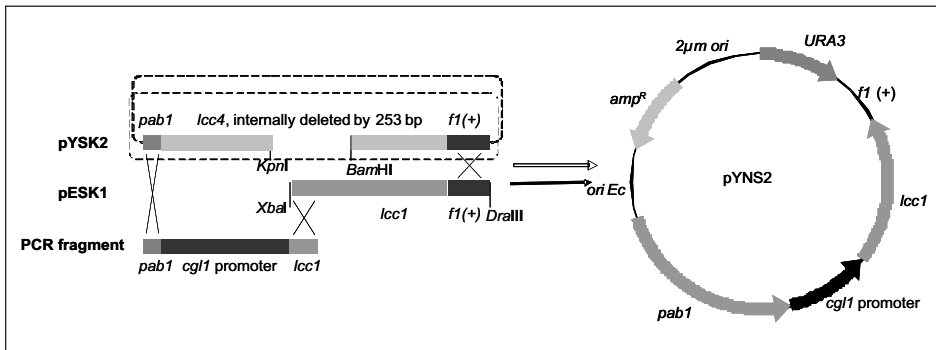


Figure 7. For *in vivo*-recombination in *Saccharomyces cerevisiae*, the yeast-*Escherichia coli* shuttle vector pYSK2 (Kilaru et al. 2005; only simplified map) was digested with *Bam*HI and *Kpn*I that cut within the *C. cinerea* gene *lcc4*. The linearized and purified vector was mixed with i. a 3.0 kb *Xba*I-*Dra*III fragment from plasmid pESK1 (Kilaru et al. submitted) containing the *lcc1* gene of *C. cinerea* monokaryon AT8 and ii. a 3.0 kb DNA fragment obtained by PCR from homokaryon AmutBmut genomic DNA with the chimeric *pab1-cgl1* and *cgl1-lcc1* primers. Upon yeast transformation, positive clones were identified by colony-PCR using the chimeric primers for DNA amplification. Following plasmid amplification in *E. coli*, the identity of the construct was conformed by restriction enzyme analysis (for explanation of other elements on the construct see Materials and Methods). To obtain pYSK36 with *lcc1* under control of the *cgl2* promoter, an analogous strategy with chimeric *pab1-cgl2* and *cgl2-lcc1* primers was followed.

3.3. Genes for β -galactoside binding lectins in sequenced genomes of basidiomycetes

In addition to *cgl1* and *cgl2*, within the genome of *C. cinerea* there is a gene for a third galectin (Fig. 5), Cgl3 (Fig. 2, 3) found by tblastn searches with Cgl1 and Cgl2. Gene *cgl3* is less similar to the other *C. cinerea* galectin genes (55/55% sequence identity in the coding region, 46/47% sequence identity in the promoter region and 48/48% sequence identity in the terminator region compared to *cgl1/cgl2* sequences) and it is found at another chromosomal location (Fig. 5). Temporal and spatial regulation of *cgl3* expression might be tested in future by the *lcc1* reporter gene system.

We also searched the *C. cinerea* genome with lectins ABL from *A. bisporus*, PCL F1 from *P. cornucopiae*, PIL from *P. inovolutus*, XCL from *X. chrysenteron* and LSLa, LSLb and LSLc from *L. sulphureus* but without hitting a gene. Apparently, *C. cinerea* does not produce lectins belonging to these two other families of β -galactoside binding lectins.

Tblastn searches of the established genomes of *Phanerochaete chrysosporium* (Martínez et al. 2004), *Cryptococcus neoformans* (http://www.broad.mit.edu/annotation/fungi/cryptococcus_neoformans/) and *Ustilago maydis* (http://www.broad.mit.edu/annotation/fungi/ustilago_maydis/) with the *C. cinerea* galectins suggests that these species have no galectins. Searches with the other fungal β -galactoside binding lectins also gave no positive result. This does not exclude that there are other types of β -galactoside binding lectins, both in the analyzed fungi and/or in other basidiomycetes.

4. Conclusions

Various types of β -galactoside binding lectins are by now described within the basidiomycetes. In most instances, they are implicated with developmental processes. However, species differ in the scenario of lectins they are equipped with. In *C. cinerea*, there are genes for three different galectins. Using laccase Lcc1 activity as reporter, we now can follow the temporal and spatial regulation of expression of all galectin genes under *in vivo*-conditions. The laccase reporter system provides both qualitative and quantitative information.

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