Pamplona 3-6 June 2005

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VI

Genetics and Cellular Biology of Basidiomycetes

Edited by
ANTONIO G. PISABARRO and LUCÍA RAMÍREZ

VI Meeting on Genetics and Cellular Biology of Basidiomycetes

(GCBB-VI)

VI Meeting on Genetics and Cellular Biology of Basidiomycetes (GCBB-VI)

Antonio G. Pisabarro
and Lucía Ramírez
(editors)

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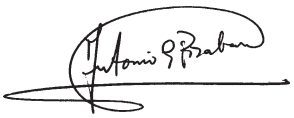
Foreword

This volume summarizes the scientific communications presented at the 6th Meeting on Genetics and Cellular Biology of Basidiomycetes (GCBB-VI) held in Pamplona (Spain) from June 3rd to 6th, 2005. GCBB-VI continues the tradition of putting together scientist working with basidiomycetes around the world. Our interest, as organizers, was to strength the communication between groups working on basic and applied research both in the field of edible mushrooms and in that of other industrial applications of these microorganisms.

The scientific program included sessions focused on genetics and breeding coordinated by Lucy Ramírez and Rick Kerrigan; Genome Analysis chaired by Allen Gathman; Cellular and Molecular Biology, coordinated by Regina Kahmann and Erika Kothe, Industrial Applications moderated by Giovanni Sannia and Kerry Burton; Plant and Animal Pathogens coordinated by José Pérez-Martín; and Biodiversity coordinated by Philippe Callac. A complete version of the scientific program can be found at the end en of this Volume.

In the coffee talks the need of a more active community of scientist working on basidiomycetes was a recurrent topic. After this meeting took place, several proposals for the complete sequencing of basidiomycete genomes are going to be presented for evaluation by groups participating in GCBB-VI. We look forward for GCBB-VII where some of these sequences would be likely available for speeding up our field of research.

I would like to that the Editorial Office of the Public University of Navarra for covering the costs of publishing this volume, and for the general support of this institution to GCBB-VI



Antonio G. Pisabarro
Cair of Microbiology
Organizing Commetee.
Pamplona December 20th, 2005

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Contents

Section 2
Lecture Abstracts

Section 3
Poster Abstracts

Welcome

From the 3rd to the 6th of June of 2005 a congress called *"Genetics and Cellular Biology of Basidiomycetes VI"* took place in the School of Nursing of the Public University of Navarre. This way Pamplona hosted an important scientific event whose previous meeting took place in Montreal (Canada).

I had the honor to chair, in representation of Prof. Dr. Pedro Burillo López, his honor the President of the University, the opening ceremony, sharing the presidency with Prof. Dr. Gerardo Pisabarro de Lucas and the Town Councilor D. Juan José Unzué Gaztelu in representation of the Mayor of Pamplona, her honor Yolanda Barcina Angulo.

It is an honor for the Public University of Navarre to have hosted such an important scientific event as it is the *"Genetics and Cellular Biology of Basidiomycetes VI"*. In which researches from 13 countries have taken part and in which papers, oral interventions and various posters of great relevance and scientific interest were presented. This meeting has not only be an excellent occasion for the experience exchange among specialist but also has contribute to the international projection of the Public University of Navarre and to show the hard work of its Professors and researchers.

I would like to finish expressing my gratitude to Profs. Drs. Antonio G. Pisabarro and Lucia Ramírez, directors of the Genetics and Microbiology Research Group of the Public University of Navarre, for their work in organizing this Congress; to Profs. Drs. Juan Francisco Martín (León University)

and Reyes G. Roncero (Córdoba University), for scientific revision of the book; to the lecturers and speakers, for their magnificent contributions; and, last but not least, to all those present in the Congress, whose contributions have grounded its quality.

Prof. Dr. José Luis Iriarte Ángel
Vice-President for European Convergence
and Institutional Relations
Public University of Navarre

Section 1

Full Length contributions

Genetic Breeding of Edible Mushrooms: from the Genome to the Production of New Varieties of *Pleurotus ostreatus*

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The breeding of new varieties of industrially cultivated edible mushrooms must proceed in the framework defined by the breeding objectives, the biological characteristics of the material and the legal and cultural constraints imposed to the breeding technology to be used. This last aspect is of the greatest importance in the case of a food that is considered in European countries as high quality and closer to nature than other industrially produced foods. This fact prevents the use of genetic-engineering based technologies for breeding, as the consumers would hardly accept genetically modified mushrooms. Consequently, mushroom breeding should be based on time-consuming processes of classic breeding. Molecular biology, however, can offer to the breeders useful tools for speeding up the selection process, for evaluating the new bred lines and, last but not least, to identify and eventually protect legally the outcome of their breeding programs.

1. Introduction

The breeding of new varieties of industrially cultivated edible mushrooms using genetic-engineering based technologies would be hardly acceptable by the final consumers in the European Union. Consequently, mushroom breeding should be based on low efficient and time-consuming classic-breeding

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approaches. Moreover, the lack of mechanisms to protect legally the new bred varieties has limited the research efforts in mushroom breeding programs. Molecular biology, however, can facilitate the selection of elite parentals, the evaluation of the new-bred lines and the determination of molecular signatures useful for identifying the breeding outcomes and for their legal protection (Ramírez et al., 2001). This review summarizes the process that we have followed to produce new varieties of industrially cultivated, edible basidiomycetes using molecular marker-assisted breeding techniques, and discuss some of the results obtained using *Pleurotus ostreatus* (oyster mushroom) (Jacq.: Fr.) Kumm. (*Pleurotaceae*) (Moncalvo et al., 2002) as model organism.

Besides its cultivation for food production, the interest in *P. ostreatus* biochemistry and molecular biology is growing because of its nutritional (Mattila et al., 2001) and health-stimulating (Hossain et al., 2003) properties, its use in paper pulp bleaching (Sigoillot et al., 2005), in recycling agricultural wastes (Aggelis et al., 2003), and in bioremediation protocols (D'Annibale et al., 2005) among other biotechnological applications (Cohen et al., 2002). Moreover, the relatively simplicity of *P. ostreatus*' life cycle, the long experience in its industrial cultivation, its easy manipulation under laboratory conditions, and the availability of transformation protocols (Kim et al., 1999; Honda et al., 2000; Irie et al., 2001a; Irie et al., 2001b; Sunagawa and Magae, 2002), make this fungus a suitable model organism for studying molecular-based genetic breeding strategies in basidiomycetes (Ramírez et al., 2000a; Ramírez et al., 2000b).

2. Mating factors

The strategy for genetic breeding depends upon the mating systems of the target species. *P. ostreatus* is a bifactorial tetrapolar fungus (Raper, 1966). A dikaryotic individual produces four different classes of meiotic spores as result of the segregation of two unlinked mating factors. Spores belonging to one of these classes are only compatible with those belonging to one of the other three. The lack of identifiable mating phenotypes in the spores and in the monokaryotic mycelia makes difficult the selection of parentals for directed crosses. In order to facilitate determining the mating type of monokaryons,

Larraya *et al*. (Larraya et al., 1999a; Larraya et al., 2001) used the Bulk-Segregant Analysis method previously described by Michelmore et al. (Michelmore et al., 1991) to identify RAPD and RFLP markers genetically linked to the two mating factors (MatA and MatB). In his study, they scored six unrelated *P. ostreatus* strains and found 11 different MatA and 14 different MatB types. Moreover, new non-parental MatB types were detected that resulted from genetic recombination of two genetically linked loci (*matB*α and *matB*β) controlling this factor, the linkage distance between them varying in different strains between 0.6 (*MatB7-MatB8*) and 15.8 (*MatB1-MatB2*) cM. In an older study, Eugenio and Anderson (Eugenio and Anderson, 1968) found 17 different MatA and 20 different MatB types in a survey of 22 monokaryons, and reported the appearance of new types in the two mating factors. In our hands, however, this only happens within the MatB factor suggesting that MatA is either controlled by a single gene (*matA*) or by two genes linked below the resolution power of our analysis, that are inherited as a single one. More recently, James et al. have described the sequence of the *matA* and *matB* loci in the tropical oyster mushroom *P. djamor* (James et al., 2004), and have found that the *A* mating-type locus in this species consists of only pair of completely linked genes.

3. Genetic linkage maps of *P. ostreatus*

For developing a molecular marker-based breeding program, it is convenient to know the number and structure of the chromosomes present in the species to be bred. In the case of basidiomycetes, the small size of their chromosomes and the occurrence of intranuclear mitosis have hampered classical cytogenetic studies. This small size, however, makes fungal chromosomes amenable to be studied using size-fractionating techniques such as Pulse Field Gel Electrophoresis (PFGE). Our group, in collaboration with the Mushroom Experimental Station in Horst (The Netherlands), separated the two nuclei present in *P. ostreatus* dikaryotic strain N001 by protoplasting and resolved the chromosomes present in each nucleus using PFGE (Larraya et al., 1999b). This study revealed that *P. ostreatus* basic number is n = 11 and that the size of the individual chromosomes rang from 1.45 to 4.7 Mbp. Moreover, the comparison of the homologous chromosomes present in the

two nuclei present in the dikaryon revealed prominent (i.e. larger than 10%) length polymorphisms (CLPs) in chromosomes II, VI and VII. This fact, however, did not affect the total amount of DNA per haploid genome that was estimated in 35.3 and 34.7 Mbp for the two N001 nuclei. CLPs have been reported in a diversity of fungal species (Zolan, 1995; Muraguchi et al., 2003).

The construction of genetic linkage maps based on molecular markers is facilitated in higher fungi by the availability of monokaryotic mycelia produced by germination of haploid spores. In this material, the products of meiotic recombination are directly testable and the linkage distances between markers can be easily measured. Moreover, the separation of chromosomes by PFGE and the information provided by the molecular karyotype facilitates the construction of the maps by anchoring marker clusters to specific chromosomes. The use of monokaryons for genetic mapping, however, is limited by the reduced umber of different phenotypic markers that can be studied and because characters expressed in the dikaryons cannot be directly testable. In order to solve this second drawback, it is necessary to construct dikaryotic populations by mating each one of the individuals of the monokaryotic population to a compatible tester. By this way, all the variation found in this type of dikaryotic population is the result of the genetic variation in the nuclei present in the monokaryotic population as the second nucleus is common in all the individuals. In order to construct the genetic linkage map of *P. ostreatus* N001, we have produced a segregating population formed by 80 monokaryons derived haploid spores. The theoretical linkage resolution power of this population is 1.25 cM (that is: one recombinant in the population of 80 haploid individuals).

3.1. *Scaffold genetic linkage map*

We have constructed two different upgraded versions of the linkage map: a scaffold map based on molecular markers (Figure 1), and two maps identifying the positions of quantitative trait loci (QTLs) on the scaffold map. In a first study, Larraya et al. (Larraya et al., 2000) scored the segregation of 178 RAPD, 23 RFLP markers, eight functional genes and the two mating factors. This study identified 130 map positions non-uniformly scattered across

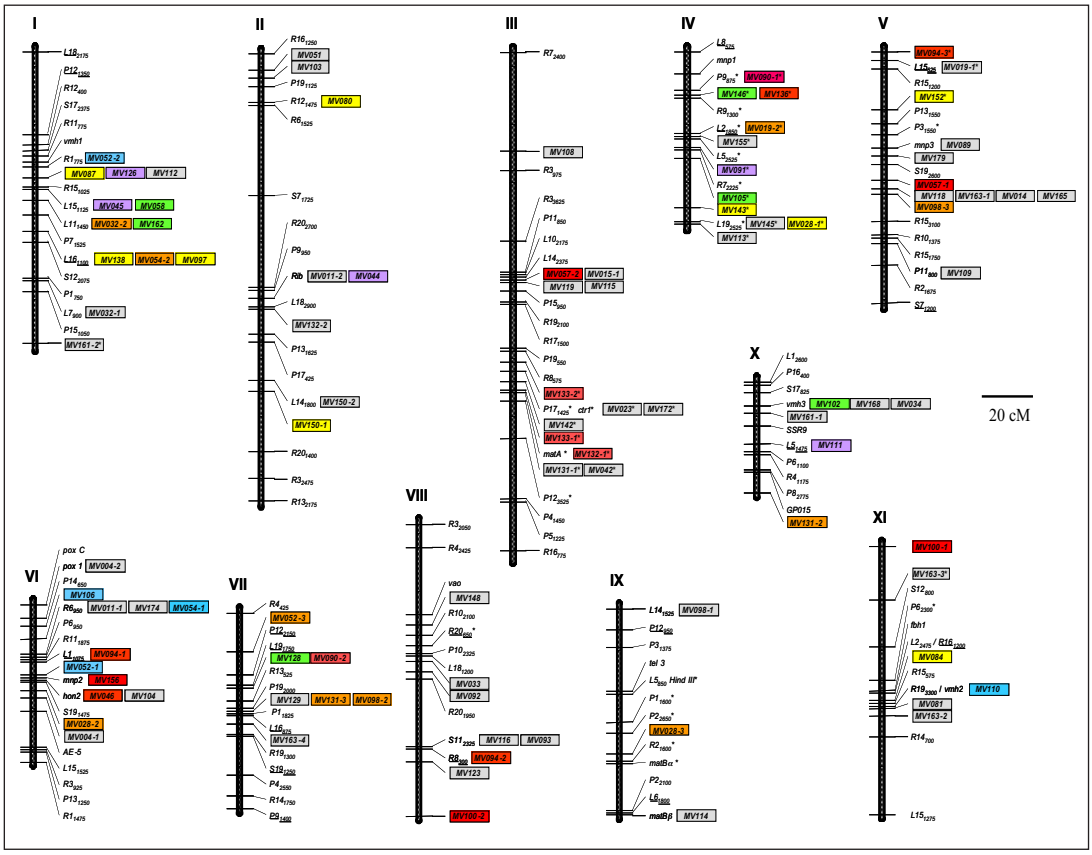
the 11 chromosomes and the total linkage map size was 1000 cM. In a more recent study Park et al. (Park et al., 2006) added to that map 82 functional genes and 12 PCR-based markers that have increased the total number of map positions to 187 and the total map size to 1061 cM (Table 1). Finally, the linkage groups ends are being currently bounded by the mapping of the telomeric sequences (Pérez et al., 2006) that, in this fungus, have the same sequence than the human telomeres.

Table 1
***P. ostreatus* genetic linkage map statistics**

Chrom. No.	*Size (Mb)*	*Length (cM)*	*Kbp / cM*	*Crossover events*	*No. of genes mapped*	*No. of map positions*
I	4.7	107	43.9	0.98	17	18
II	4.4	164	26.8	1.71	6	19
III	4.6	183	25.1	1.75	14	24
IV	3.6	63	57.1	0.59	12	14
V	3.5	92	38.0	0.81	12	18
VI	3.1	58	53.4	0.76	17	19
VII	3.2	72	44.4	0.74	4	16
VIII	3.0	107	28.0	0.84	9	15
IX	2.1	76	27.6	0.74	3	19
X	1.8	41	43.9	0.34	6	12
XI	1.5	98	15.3	0.59	8	13
Total	35.5	1061	36.3 (13.0)	0.90 (0.44)	108	187

Genetic linkage maps are usually interpreted assuming that wide genetic linkage distances reflect wide physical distances in the chromosome; however, the correlation between physical and linkage distances can be distorted by the occurrence of sequences that promote or difficult meiotic recombination (Lichten and Goldman, 1995; Petes, 2001). Regions of repetitive DNA, containing transposons and heterochromatic regions have been associated to recombination frequency bias (Gerton et al., 2000).

Figure 1



Some examples of influence of the sequence structure on the recombination frequency have been recently described in the literature: in an analysis of the genetic structure of the *matB* locus in *S. commune*, Fowler et al. (Fowler et al., 2004) found that the 8 kb region separating the *Bα3* and *Bβ2* loci contains 19 different short sequences with imperfect repeats as well as a 1 kb segment where the GC content was highly biased, and that the 5 kb region separating the loci *Bβ3* and *Bβ3* contains 17 short imperfect direct repeats similar in length and number to those of the *Bα3-Bβ2* complex. The ratio of physical to genetic distance (up to 1 kb/cM) suggests that this region forms a recombination hotspot. On the contrary, Espeso et al. (Espeso et al., 2005)

have found that recombination frequencies are greatly reduced near the centromeres in two *Aspergillus nidulans* chromosomes altering the physical to genetic distance ratio at these locations.

The uneven distribution of map positions across the *P. ostreatus* linkage map argues in support of the occurrence of recombination-prone genome regions that appear as long empty linkage regions separating marker clusters. Assuming that the RAPD markers, as a whole, are stochastically distributed across the genome sequence, long linkage distances between correlative markers could very well be the result of increased recombination rather than indicate long physical distance. If we consider the DNA elements promoting recombination, little is known about the presence of transposable elements in *P. ostreatus* genome, although their presence has been described in other basidiomycetes (Gaskell et al., 1995; Sonnenberg et al., 1999; Wostemeyer and Kreibich, 2002); and the analysis of a 255 kb genomic sequence allows to estimate that 5.7% of the sequence corresponds to repetitive elements (Palma et al., 2006). This figure, that does not include the rDNA, fits with that of repetitive elements in other higher fungi (Wostemeyer and Kreibich, 2002).

The estimation of the number of crossover events per chromosome per cell (0.90 ± 0.42) reveals that basidia in which no crossovers occur in one or some of the chromosomes are frequent in *P. ostreatus* (Larraya et al., 2000), and poses the question about the mechanisms ensuring an appropriate distribution of the homologous chromosomes to the meiotic products. Besides that, recombination between chromosomes with prominent length polymorphisms questions about the conservation of the genome structure and karyotype stability (Wostemeyer and Kreibich, 2002). A preliminary study of the 880 chromosomes present in the segregating mapping population revealed that 11.4% of the chromosomes were not involved in recombination and that the recombination behavior differs in different chromosomes (Castellón et al., 2006).

3.2. *QTL map*

We have mapped two different groups of QTLs to the genetic linkage scaffold map described above: (1) QTLs controlling mycelial growth rate and (2) QTLs controlling industrial yield and productivity.

The genetic control of mycelial growth rate is complex and depends on several loci. In a first study, Larraya *et al.* (Larraya et al., 2001) found that

monokaryons carrying *matA2* allele grew significantly (P = 0.01) faster than their *matA1* sibs. Moreover, among the *matA2* individuals, those carrying the *matBα1* allele grew faster than those carrying the *matBα2* (P = 0.04). These differences were not observed when comparing different *matBβ* alleles or when the comparisons were made in a *matA1* genetic background. Consequently, the mating type influences the monokaryotic vegetative growth rate in synthetic culture medium. The presence of the *matA1* allele seems to act as a growth rate-limiting factor; *matA2*, on the contrary, releases this limitation and the *matBα* locus acts as a new limiting control. James et al. (James et al., 2004) have found hypothetical genes responsible for cellular energy metabolism in close sequence vicinity to the *matA* gene in *P. djamor*. These genes could explain the differences in growth rate associated to the mating factors that we have found in *P. ostreatus*. However, a direct effect of the mating factor on the growth rate or the occurrence of a selection for balanced gene combinations could not be discarded as responsible for this behavior.

For systematic search of loci controlling growth rate, we studied the correlation between the presence of discrete genome regions (defined by two consecutive map markers) and the vegetative growth rate in monokaryons and dikaryons, cultured on synthetic culture medium (Eger medium, SEM) or on straw (Larraya et al., 2002). For studying the variation in the monokaryotic growth rate, the collection of 80 segregating monokaryons was used. For the corresponding study in dikaryons, four populations were constructed by mating each one of the 80 sib monokaryons to four a different monokaryon unrelated to N001. The study identified two major QTLs controlling monokaryotic growth rate on SEM, three controlling monokaryotic growth rate on straw, and eight major QTLs controlling dikaryotic growth rate on SEM (four of which were detected in two dikaryotic different populations). Three QTL clusters controlling monokaryotic and dikaryotic growth rate were found on chromosomes IV, VII and IX. Chromosome I, on the other hand, bore the two main dikaryotic growth rate QTLs both in terms of individual contribution to the character (R^2 value 25.90% and 11.81%) and robustness of the determination (P = $2x10^{-5}$ and $3.8x10^{-3}$, respectively).

The evaluation of the quantitative effect of the QTLs can distinguish between the effect of different QTLs and the effect of different haplotypes in a given QTL. The effect of different QTLs on the variation of the growth

rate seems to be additive since the sum of the individual effects of different QTLs roughly equals the proportion of the total variation of the character that can be genetically explained at the sensitivity level used in this analysis (see R^2 values in Table 2, as an example). In a study on the interaction between specific QTLs in homologous chromosomes, we have constructed dikaryons which were completely homozygous or completely heterozygous for chromosome VIII (which contains a cluster of QTLs controlling monokaryotic and dikaryotic growth rate) and we have measured the variation in the dikaryotic growth rate as a function of the chromosomal constitution. The preliminary results suggest that the action of the QTLs tested is dominant (i.e., the presence of a "fast" chromosome is enough to produce a fast growing dikaryon) rather than additive (i.e.: dikaryons heterozygous for chromosome VIII present growth rate values intermediate between those of the two types of chromosome VIII homozygotes) (Castellón et al., 2006).

Table 2
QTLs for mycelial growth rate on SEM

Name	*Linkage group*	*Position*	*Probability*	*Absolute effect*	R^2	*Total* R^2
Qmgre1	IV	$L5_{2525}$ + 1.4	0.017	3.830	9.90	
Qmgre2	VIII	$P10_{2325}$	0.00011	5.030	20.27	38.46
Qdgre1	IV	$R7_{2225}$ + 3.8	0.00001	2.240	23.17	
Qdgre2	VIII	$R10_{2100}$ + 3.8	0.011	1.030	11.31	
Qdgre3	XI	*fbh1*	0.035	1.041	8.18	41.19

Besides monokaryotic and dikaryotic growth rate, we mapped quantitative traits related to industrial production (and its components) and quality (Larraya et al., 2003) using the compatible monokaryon PC21 as provider of the common nucleus. The experiment was carried out at two different incubation temperatures (15 and 21 ºC), and the parameters scored were yield, flush precocity, first flush yield, fruit body average weight, clean fruit-body weight, stipe weight loss, fruit-body fleshness (texture) and fruit-body color. Most of the parameters displayed a normal distribution that allowed the mapping. Table 3 shows a summary of the mapping results. The analysis revealed that the (1) QTLs controlling production could explain a large por-

tion of the variation of the character. Particularly, nearly the half of the variation in precocity and yield at 21 ºC could be explained by the corresponding QTLs. (2) For all the production characters studied, a strong QTL effect could be mapped to the central region of chromosome VII. This QTL could explain roughly half of the variation of the main production traits. Moreover, this QTL maps to a position where a QTL signal for dikaryotic growth had been detected. (3) The QTLs controlling quality traits explained a more reduced portion of the character variation than the QTLs for production do and are scattered across different chromosomes. Moreover, no quality QTLs have been mapped to chromosome VII.

The control of quantitative traits complicates even more if the interactions between the different loci are considered. As an example, 17 and 39 different significant (P< 0.005) digenic interactions were observed between loci controlling growth rate and various quality and production traits (respectively) and other genome regions. Consequently, the molecular bases explaining these results are far from being completely understood.

Table 3
Summary of QTL effect for production and quality

Trait	*QTL chromosome VII R^2*	*Probability*	*Other Linkage groups*	*Total R^2*
P-21	31.83	$<10^{-5}$	I, VI, VIII	47.96
P-15	36.30	$<10^{-5}$	–	
Y-21	45.47	$<10^{-5}$	IV	47.69
Y-15	32.07	$<10^{-5}$	–	
FFY-21	48.36	$<10^{-5}$	IV	49.26
FFY-15	28.77	$<10^{-5}$	VI	34.28
CFW	13.83	$< 4X10^{-4}$	III	18.13
SWL	–	–	I, II	14.27
FLE	–	–	II, V, VI	26.87
COL	–	–	IV, X	17.31

Precocity (P), Yield (Y), flush, first flush yield (FFY), clean fruit-body weight (CFW), cold-storage weight loss (SWL), fruit-body fleshness (texture) (FLE), and fruit-body color. 21 and 15 indicate the incubation temperature (ºC) of the experiment.

Table 4
Summarizes the most relevant genes (quantitative or qualitative) mapped to the *P. ostreatus* chromosomes.

Chrom. No.	*Quantitative trait*	*Gene or function*
I	dgre*, P_{21}, SWL	–
II	dgre, SWL*	rDNA
III	CFW, FLE*	MatA
IV	mgre, mgrs*, dgre, Y_{21}, FFY_{21}, NUM, COL*	Lignocellulolytic enzymes
V	CWL, FLE	–
VI	dgre, P_{21}, FFY_{15}, CWL, FLE*	Lignocellulolytic enzymes
VII	dgre, P_{21}*, P_{15}*, Y_{21}*, Y_{15}*, FFY_{21}*, FFY_{15}*, NUM*, CFW*	–
VIII	mgre*, mgrs, dgre, P21, CWL*	–
IX	–	MatB
X	COL	–
XI	mgrs, dgre	Hydrophobins

Legend dor the QTLs: Growth rate: mgre, monokaryotic on SEM; mgrs, monokaryotic on straw; dgre, dikaryotic on SEM. Industrial Production: P_{21}, precocity at 21 °C; P_{15}, precocity at 15 °C; Y_{21}, yield at 21 °C; Y_{15}, yield at 15 °C; FFY_{21}, first flush yield at 21 °C; FFY_{15}, first flush yield at 15 °C; NUM, number of mushrooms; CFW, clean fruit body weight. Industrial Quality: SWL, stipe weight loss; CWL, storage weight loss; FLE, texture; COL, color. The asterisk indicates the principal QTL for each class.

4. *P. ostreatus* genes

The information about the *P. ostreatus* genome structure provided by the karyotype and the linkage maps is complemented with that corresponding to the structure of genes in this species. We harvested this knowledge from two main sources: (1) genes isolated or identified by direct cloning or during EST screenings, and (2) genes identified or predicted in long stretches of genomic DNA. Currently (Nov. 2005) there are more than 2100 *P. ostreatus* sequences deposited in the genetic databases. Out of them, less than the 10% correspond to core nucleotides and the rest to ESTs. If we estimate the total number of *P. ostreatus* genes as 12.000 (see below), the sequences deposited represent 17% of the expected genes and the sequences corresponding to core nucleotides represent 1.2% of the expected.

4.1. *EST analysis*

In order to contribute to identify new genes expressed differentially during vegetative and reproductive growth in *P. ostreatus*, to study their expression in other developmental stages, and to increase the density of the existing genetic linkage map of this fungus, we collected a number of ESTs isolated from mature lamellae, mapped the corresponding genes and analyzed their expression in different developmental phases (Park et al., 2006). The total number of lamellae-expressed genes identified and mapped was 82. The manual annotation of the genes based on BlastN and BlastX similarities revealed that 56.1% of the genes identified in this study were highly similar to databases entries whereas 34.1% of them corresponded to entirely new sequences. This value fits with those previously reported for other developmental studies carried in basidiomycetes (Ospina-Giraldo et al., 2000; Lacourt et al., 2002; Lee et al., 2002; Guettler et al., 2003; Posada-Buitrago and Frederick, 2005).

The mean GC content of the EST collection was 53.35% (s.d. 4.72). This value is slightly higher than the estimated for the general genome (50.53%) suggesting a higher GC content in the coding regions in comparison with the non-coding ones. In the white-rot fungus *Phanerochaete chrysosporium* strain *RP78*, the GC content of the coding regions (59%) was also higher than the overall value (57%) (Martinez et al., 2004). GC values were, however, higher in *P. chrysosporium* than in *P. ostreatus*.

Table 5 and Figure 2 summarize the genes expressed in the lamellae. In *P. ostreatus*, 30.8% of the genes identified in this study as expressed in the lamellae were also expressed in vegetative mycelia (pattern A), this number raises to 67.9% in the case of the subpopulation of genes expressed in fruit bodies, whereas Lee et al. (Lee et al., 2002) found that only 5.3% of the genes were expressed simultaneously in both developmental stages, and Ospina-Giraldo et al. found that 12% of the genes were simultaneously detected in primordia and basidiome *Agaricus bisporus* samples (Ospina-Giraldo et al., 2000). These authors conclude that gene expression must be quite different quantitatively and qualitatively during fruit body formation. We consider that the statistical sampling process involved in cDNA cloning can be responsible for their results, and that Northern analysis is more accurate in detecting gene expression. Consequently, our results suggest that the number of genes differentially expressed in the two developmental stages is smaller

than that previously reported. These expression results partially support the conclusion put forward by Zanzinge et al. (Zantinge et al., 1979) indicating that RNA sequences isolated from S*chizophyllum commune* fruiting and non-fruiting mycelia were identical for at least 90%.

Table 5
Genes expressed in the lamellae

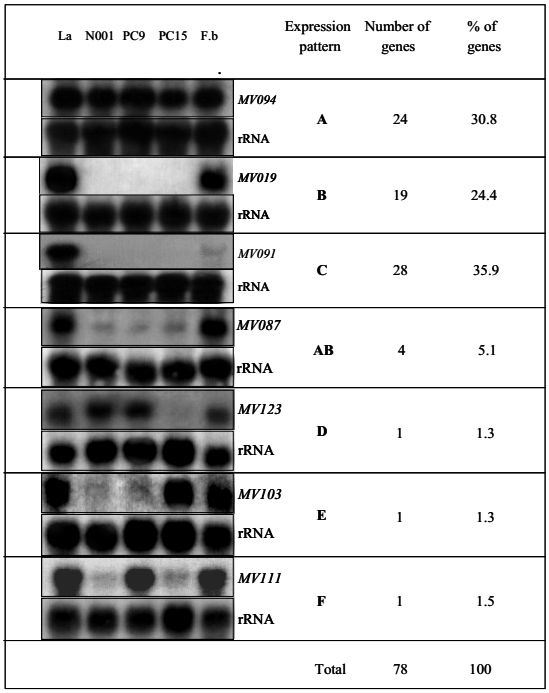
Expression pattern	*Genes with assigned function*	*Hypothetical proteins*	*New genes*	***Total***
A	17 (70.8)	3 (12.5)	4 (16.7)	24 (30.8)
B	5 (26.3)	4 (21.1)	10 (52.6)	19 (24.4)
C	8 (28.6)	8 (28.6)	12 (42.9)	28 (35.9)
AB	3 (75.0)	-	1 (25.0)	4 (5.1)
D	-	-	1	1 (1.3)
E+F	1	-	1	2 (2.6)
Total	34 (43.6)	15 (20.7)	29 (35.4)	78

The expression patterns correspond to those described in Figure 2

Besides the major expression patterns, the expression of two genes (*mv111* and *mv123*) was detected in fruit body and in one of the two *P. ostreatus* N001 parental protoclones, whereas it was not detected in the dikaryotic mycelium and in the second parental protoclone. This expression pattern suggests the existence of diffusible gene expression repressors active during the vegetative growth and inactive after the growth phase change (Figure 3).

Figure 2

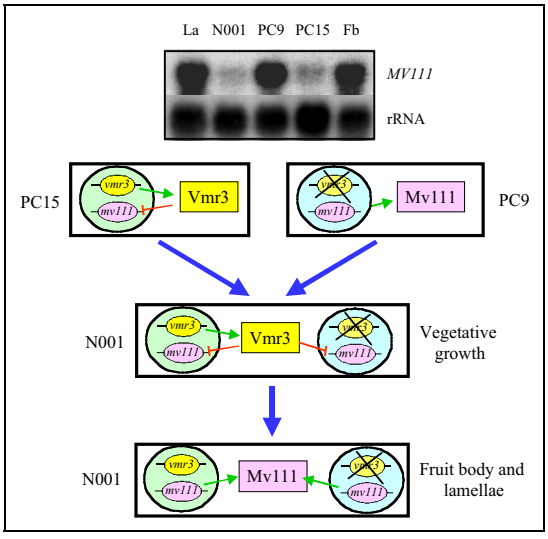
Expression patterns of lamellae-expressed genes in *P. ostreatus*



Expression pattern	Number of genes	% of genes
A	24	30.8
B	19	24.4
C	28	35.9
AB	4	5.1
D	1	1.3
E	1	1.3
F	1	1.5
Total	78	100

Figure 3

Hypothetical model for the repression of the expression of *mv011* by a diffusible factor produced by PC15



The genes identified in this study provide tags for the study of housekeeping, fruit body specific and lamellae specific promoters. Other genes studied in our laboratory were found to be expressed in vegetative mycelium and repressed in fruiting bodies had been previously found [hydrophobin genes *vmh1* and *vmh2* (Peñas et al., 2002)] or expressed in fruit body but not in lamellae [hydrophobin *fbh1* (Peñas et al., 1998) and copper transporter *ctr1* (Peñas et al., 2005)]. Leaving aside more bizarre expression patterns theoretically possible (for instance, genes expressed in vegetative mycelium and lamellae but repressed in fruit body), altogether, the main promoter types expected in this type of fungus are available for sequence comparative studies using these genes as tags.

4.2. *Genomic sequence*

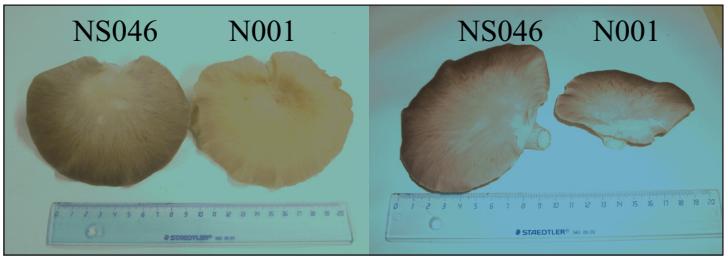
We have cloned and sequenced a 255 kb genomic region of *P. ostreatus* chromosome VII where the major QTL cluster for mushroom production maps to. This sequence is the longest genomic stretch sequenced in *P. ostreatus* up to date. A preliminary manual annotation based on BlastX and BlastN similarities identified 61 putative genes; however, if we take into account that nearly one third of the *P. ostreatus* genes correspond to sequences without counterpart in the databases, the expected gene number in this region raises to nearly 90. By extrapolation of the number of genes expected in this region, and assuming an even distribution of genes in this genome, we can expect that the complete *P. ostreatus* genome would consist in around 12.000 genes. The region sequenced included two short direct, two short inverted, two long direct and two long inverted repeated sequences that account for nearly 11.6 kb (4.5% of the region). The search for microsatellite sequences identified 588 elements involving 3.1 kb (1.22% of the sequence). Taken together, these two types of repetitive sequences add up to 5.7% of the sequence (Palma et al., 2006).

5. Selection of parentals and construction of new bred lines

The information derived from the genetic and molecular studies described above was used to select parentals for constructing new dikaryotic strains

with improved traits. Basically, the parentals for the new hybrid lines were monokaryons derived from N001 and selected on the basis of their performance on those characters controlled by the QTLs described. Different types of hybrids were constructed: outbred dikaryons produced by mating of monokaryons belonging to the mapping population to compatible monokaryons derived from other *P. ostreatus* strains unrelated to N001, and inbred dikaryons constructed by mating of compatible elite monokaryons present in the mating population. *P. ostreatus* commercial strain HK35, and the model strain used in our laboratory N001, were the controls for the breeding field trials that were carried out under the production conditions used by commercial producers in Spain. The parameters scored in these experiments were yield (Y), precocity (P), clean fruit body weight (CFW), and stipe weight lost (SWL). Five repetitions of each strain and an at random design were used in the field trials which were tested in autumn winter, spring and summer time. The performance of the hybrids was tested in successive trials: hybrids scoring high in a trial run into the next scaled-up one. At the end of the process two new strains scoring better than the parentals for CFW, SWL and P were produced. Figure 4 shows a comparison of the selected new strain 046 in comparison with the parental N001

Figure 4
NS046, a new *P. ostreatus* inbred strain produced by Molecular marker assisted selection of parentals



6. Acnowledgements

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Anticancer Activity of Polysaccharides Produced by *Pleurotus ostreatus* in Submerged Culture

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It has been known for many years that some compounds produced by edible mushrooms encompass anticancer activities. Most of production methods were based on cultivation of mushroom in solid medium. In the present study *Pleurotus ostreatus* mycelia were grown in submerged culture. The cultivation of fungal cells in submerged culture resulted in higher growth rate with better control of production process. The bioactive polysaccharides (both intracellular and extracellular) were extracted from culture by solvent repeated precipitation. The polysaccharide structure was determined by examining NMR, IR spectra and the primary structure of the polysaccharide was mainly glucan. The ^{13}C NMR spectral pattern indicated the polysaccharides are highly branched with mainly 1→3 and 1→6 linkage. The results of *in vitro* anti cancer studies demonstrate that this type of polysaccharides possesses anticancer activity against human oesophageal cancer cell line. Moreover, in the course of *in vitro* studies, mushroom polysaccharides showed anti-tumour activity and also considered to be biological response modifier because of their mechanism of action through stimulation of the immune system. The polysaccharide activity is especially beneficial in clinics when used as an adjuvant with chemotherapy to decrease its side effect. This work describes production process of anti cancer compound(s) by mushrooms and suitable for pharmaceutical industries.

1. Introduction

Mushrooms have been eaten and appreciated for their flavour, economical and ecological values, and medicinal properties for many years. Mushrooms

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are abundant sources of a wide range of useful natural products with biological activities (Lorenzen and Anke, 1998; Wasser, 2002; Mao and Zhong, 2004). Much interest in biotechnological methods for the production of microbial polysaccharides has been generated for applications in pharmaceuticals industries (Kuo *et al*., 1996, Liu *et al.,* 1997). However, most of polysaccharides with various physiological activities frequently originated from fungi especially mushrooms. Moreover, mushroom has some other medical applications for the treatment of diseases such as hypoglycaemia (Yuan *et al.,* 1998; Kiho *et al.,* 1994; Yang *et al.,* 2002). In spite of most researches deal with polysaccharides extracted from the fruiting bodies, other has studied extracellular polysaccharides (EPS). The EPS term is used to describe polysaccharides found outside the cell or which are free within the surrounding medium. The production of EPS from mushroom in submerged culture is interesting because of several advantages over the conventional method using fruit body extraction. In submerged culture, the growth rate of fungal cell is higher and the product requires only relatively simple purification steps (Cavazzoni and Adami, 1992; Jong and Birmingham, 1992). Moreover, some wild mushrooms are not able to be cultivated in traditional way and able only to grow in submerged culture using enriched culture medium under controlled cultivation conditions. Recent studies of EPS production from mushrooms demonstrated their significant anticancer activity (Kim *et al.,* 2001; Ng 1998).

Pleurotus ostreatus is an edible mushroom belonging to the family Basidiomycetes. This fungi is one of the five main edible fungi cultivated worldwide and display easy adaptation to different growth conditions in submerged cultures (Marquez-Rocha *et al.,* 1999; Bae *et al.,* 2000; Rosado *et al.,* 2003).

In this context, the current study reports some results for cell growth and EPS production kinetics during cultivation of *P. ostreatus* in stirred tank bioreactor. The produced polysacchariede was extracted and completely identified. Further studies of its anticancer activity were also done.

2. Materials and Methods

2.1. *Microorganism and cultivation conditions*

Basidiomycetes fungus *Pleurotus ostreatus* NRRL 366 was kindly provided by the agriculture research service Peoria, USA. The strain was maintained and

reactivated monthly in Petri dishes containing a sterile solid potato dextrose agar medium (PDA, Oxoid, UK). Cells were incubated at 28°C for 14 days and stored in a refrigerator at 5°C.

2.2. *Medium for cell growth and EPS production in bioreactor*

The medium used in bioreactor experiments was composed of [g l^{-1}]: glucose, 20.0; KH_2PO_4, 0.46; K_2HPO_4, 1.0; $MgSO_4 \times 7H_2O$, 0.5; peptone, 2.0 and yeast extract, 2.0. Glucose was sterilized separately and added to the cultivation medium before inoculation; pH was adjusted to 5.5 after sterilization.

2.3. *Inoculum preparation and bioreactor cultivation*

Pleurotus ostreatus was initially grown on PDA medium in a petri dish for 14 days at 28°C. The surface mycelia with spores were harvested in sterile saline solution and used as inoculum. Cells were gently homogenized to prevent the formation of large aggregates in submerged culture. Inoculum for bioreactor was in form of 50 ml (mycelium-spore) suspension with optical density (O.D.) of 1.0 at 600 nm Cultivation was carried out in a 3 L stirred tank bioreactor Bioflow III (New Brunswick Scientific Co., New Brunswick, NJ, USA) with a working volume of 2,0 L. Agitation was performed using a three 4-bladed rushton turbine impellers ($d_{i(impeller\ diameter)}$ = 65 mm; $d_{t(tank\ diameter)}$ = 135 mm, $d_i d_t^{-1}$ = 0.48) at 400 rpm. Aeration was performed by filtered sterile air [1 v/v×m]. Dissolved oxygen concentrations were analyzed by polarographic electrode (Ingold, Germany). Foam was suppressed, when necessary, by the addition of silicon antifoam reagent (Fluka, Switzerland).

2.4. *Analysis*

2.4.1. Sample preparation and cell dry weight determination

During cultivation in bioreactor, aliquots (in form of 10-15 ml) of the culture were taken from the bioreactor vessel through a sampling system. Samples

were collected in pre-weighed centrifugation tube of 15 ml (Falcon, USA), centrifuged at 4°C with 5000 rpm for 20 min. Supernatant was frozen at -20 °C for sugar and EPS determination. The cell pellets were washed twice by distilled water, centrifuged again and dried in oven at 60°C for determination of cell dry weight.

2.4.2. Determination of glucose

Glucose was determined in the fermentation media by enzymatic method using a glucose determination kit (Glucose kit Cat. No. 4611, Biocon Diagnostic GmbH, Burbach, Germany).

2.4.3. Extraction of polysaccharide from mycelial culture

After sample centrifugation, the resulting culture filtrate was mixed with equal volume of absolute ethanol, stirred vigorously and kept overnight at 4°C. The precipitate exo-biopolymer was centrifuged at 10.000g for 20 min. discarding the supernatant (Bae *et al.* 2000). The precipitate of pure EPS was washed separately with ethanol, acetone and ethyl ether then lyophilized.

2.4.4. IR spectrometry

Finely ground solid polysaccharide samples compressed with potassium bromide (KBr) into the form of a thin tablet then placed directly into the sample beam of the spectrometer and the absorption of the spectrum was measured.

2.4.5. ^{13}C NMR spectrometry

The experiment was done according to the method described by Barrett-Bee *et al.* (1982). The polysaccharide sample 50 mg was dissolved in one ml of concentrated dimethyl sulphoxide (DMSO) by ultrasonic for 10 minutes. The solution was introduced into a procession ground tube then subjected to measurement.

2.5. *Cytotoxicity Determination*

2.5.1. Cell culture

Oesophageal cancer cell line was routinely maintained at 37°C, 5% CO_2. Cells were cultured in DMEM supplemented with 10% fetal calf serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin.

2.5.2. Crystal violet assay

Initial screening of mycelial polysaccharide for cytotoxic activity was carried out by means of crystal violet staining of treated cells (Saotome *et al*., 1989). Oesophageal cancer cells were plated (1,500 cell/well) in 96 well tissue culture plate. After 24 hour incubation, polysaccharide was added in different concentrations (50-1000μg ml^{-1}). Following 48 hours incubation, observation of cell number and morphology were made and the plates were then processed for staining. Media were discarded; the plates allowed to dray and 100 μl absolute Methanol was applied to each well for 10 minutes. Methanol was discarded and replaced with staining solution for 20 minutes. Plates were rinsed with water and 100 μl of water was added to each well for 1 hour. The water was discarded and replaced with further 100 μl water. Plate was read at 595 nm on micro-plate reader.

2.5.3. Detection of immune stimulation activity of Mushroom ploysaccharides

Peripheral blood mononuclear cells (PBMC) were isolated from healthy individual by Ficoll-Hypaque (Sigma, St. Louis, MO, USA) gradient centrifugation. The purified cells was cultured at 1.0×10^6 cell ml^{-1} in RPMI 1640 medium (Cambrex, Verviers, Belgium) supplemented with 25mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES) (Sigma), 4mM L-glutamine (Cambrex), 100 U of penicillin and 100 μg streptomycin (Cambrex) and 10% FBS (GEBCO, BRL, USA). PBMC were stimulated with 200, 100, 80, 40, and 20 μg ml^{-1}, of each of *P. ostreatus* mycelia exo-polysaccharids. All samples were assayed in triplicates. A positive control culture was included, where PBMC was stimulated with 2 μg ml^{-1} Phytohemagglu-

tinin-L (PHA, Sigma). Proliferation was determined after incubation for 3 days at 37°C, 5% CO2, and 95% humidity, by addition of 201 of BrdU labelling reagent (Roche, Penzerg, Germany) in the last 2 hours of the culture. The labelled cultures were harvested and the BrdU uptake was determined using Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche) following the manufacturer instructions. Data were presented as stimulation index (SI), where proliferation is considered positive if SI is ≥ 2.

3. Results and Discussion

3.1. *Cell growth and EPS production in stirred tank bioreactor*

Cultivations were carried out in 3 L stirred tank bioreactor to investigate the kinetics of cell growth and EPS production. As shown in figure (1), cells grew exponentially during the first 216 h with growth rate of 0.009 [h^{-1}] and reached a cell mass of 4.7 g l^{-1}. After this time, cell mass kept more or less constant for the rest of cultivation time. During this phase, the glucose consumption rate was 0.054 [g l^{-1} h^{-1}] and reduced to 0.021 [g l^{-1} h^{-1}] as the cells entered the stationary growth phase. Thus, glucose was not the growth limiting substrate in this process. On the other hand, EPS production started after 48 h and produced with rate of 0.0063 [g l^{-1} h^{-1}], reaching the maximal value of about 1.6 g l^{-1} after 312 h. Thus, the production of EPS was not stopped as the cells entered the stationary phase. The data of dissolved oxygen [DO] clearly demonstrate also that during the exponential growth phase there was significant drop in DO value and increased again at the early stationary phase.

During exponential growth phase, the pH decreased gradually and reached about 3.5 after 144 h and increased with very low rate reaching only 3.8 at the end of cultivation. This drop in pH is assumed to be responsible for growth limitation in culture since neither carbon nor oxygen limitations were observed in this culture. The decrease in pH of culture medium during *Pleurotus* growth might be probably due to production of organic acids as suggested by Rajarathnam *et al.* (1992).

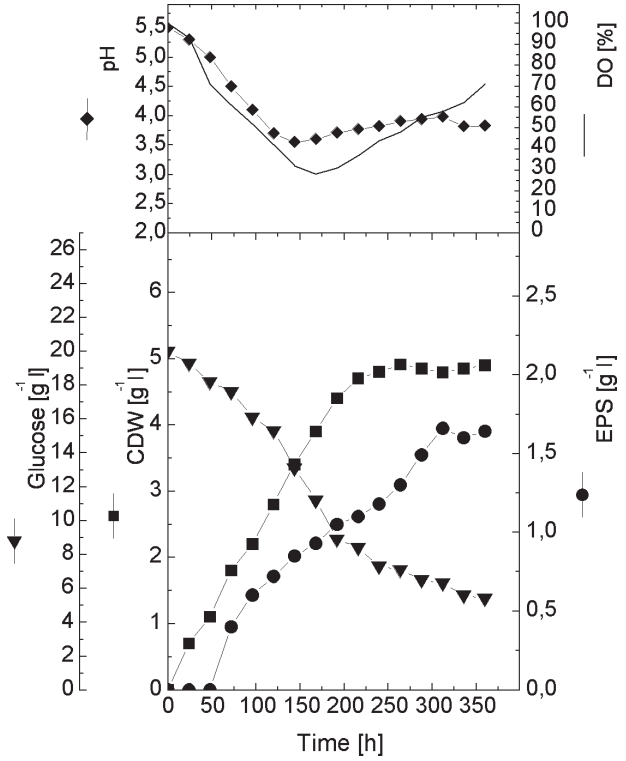

Figure 1. Cell growth and EPS production during submerged cultivation of *P. ostreatus* in 3 L stirred tank bioreactor.

3.2. *Structure of isolated polysaccharide*

The infrared spectrum of mycelial polysaccharide in Figure 2 indicates the presence of hydroxyl group which lies in the region between 3200-3600 cm while the band at 2900 is characteristic to C-H group as well, the IR spectrum shows finger print at 860 cm which lies in the anomeric region indicating the presence of a beta glucosidic bond. On the other hand, ^{13}C NMR spectrum of mycelial polysaccharide exhibited signals at different resonance which indicated that the basic structure of the isolated polysaccharide is glucan with different linkages mainly 1-3, 1-6 (Figure 3).

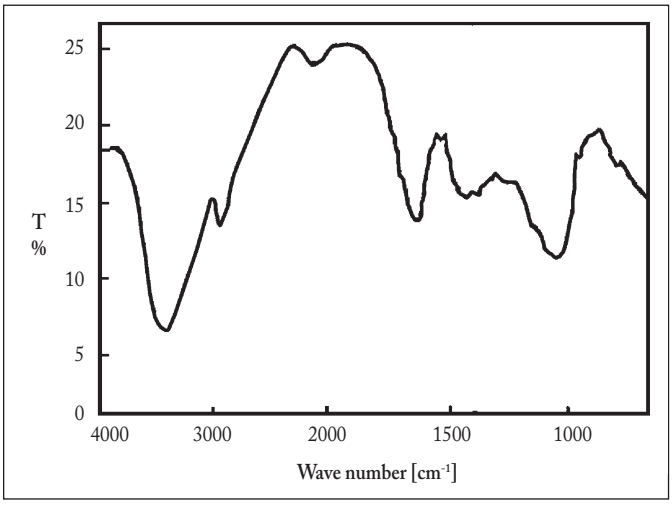

Figure 2. IR spectra of EPS produced by *P. ostreatus* in submerged culture.

The NMR spectral features of mycelial polysaccharide of *P.ostreatus* are very similar to polysaccharides isolated from different types of mushrooms such as *Lentinus edodes, Grifola frondosa* and *Ganoderma tsugae* (Mizuno *et al.,* 1992; Zhuang *et al.,* 1992; Zhang *et al.,* 1994). Although, there are significant differences in the relative intensities of peaks, reflecting the differences in the extent of branching.

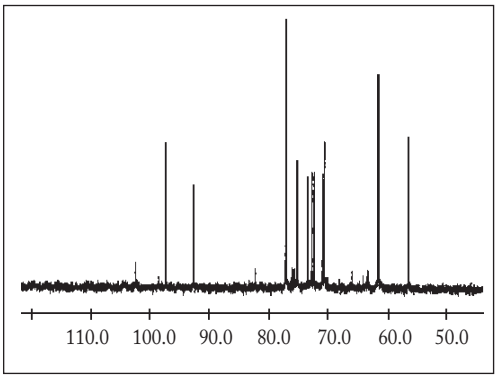

Figure 3. NMR spectra of EPS produced by *P. ostreatus* in submerged culture

3.3. *Antitumour activity of mycelial polysaccharide*

Polysaccharide isolated from mycelial culture broth was tested at different concentration and the results of crystal violet assay are presented as absorbance at 595 nm of treated and untreated cells (figure 4). In principle, an active compound could cause a decrease in cell number and hence ab-

sorbance with increasing drug concentration. The results indicate that the polysaccharide at 250, 500 and 1000 μg ml^{-1} have a direct cytotoxic effect against oesophageal cancer cell line. While doses of 50 and 100 μg ml^{-1} showed to have no cytotoxic effect on the cancer cell line. These data agrees with the data previously reported indicating that antitumour mushrom polysaccharides such as lentinan and schizophyllan showed no direct cyotoxicity to tumour cell lines *in vitro* (Aoyagi *et al.,* 1994). On the other hand, polysaccharide krestin and polysaccharopeptide PSP isolated from *Coriolus versicolor* mushroom had direct cytotoxicity to a wide range of tumor cell lines (Tsukagoshi *et al.,* 1984 and Yang *et al.,* 1992). We realize that that the polysaccharide did not fully enter into solution and sometimes even could increased absorbance reading because the precipitate contributed to the absorbance for this reason all cells were carefully examined microscopically prior to staining to determine the presence of precipitates and also to estimate cell density.

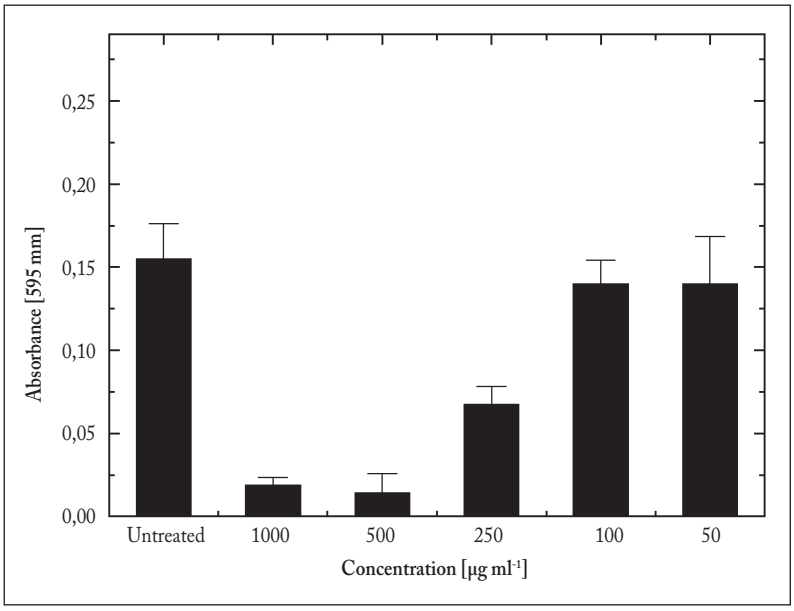

Figure 4. Cytotoxic activity of EPS of mushroom cells on Oseophygus cancer cell line.

3.4. *Immunostimulatory effect of EPS*

Several studies indicate that some natural mushroom (basidiomycete) products showed to have immuno-potentiating properties which generate considerable interest as possible pharmacological tumoricidal activity (Fujimiya *et al.,* 1998). Also it was reported that the (1→3)-branched (1→6)-ß –glucan, termed D-Fraction, extracted from the fruit body of the maitake mushroom (*Grifola frondosa*) can enhance the activity of immunocompetent cells such as macrophages, helper T cells, and cytotoxic T cells to attack tumour cells (Kodama *et al.,* 2002). These findings support our data which is presented in figure 5 and showed that EPS of the mushroom strain *P. ostreatus* can stimulate proliferative response of normal PBMC in a dose independent manner.

4. Conclusion

The results in this work clearly indicated that mycelial exo-polysaccharide (EPS) isolated from *P. ostreatus* culture broth seems to have anticancer activity that is host mediated and cytocidal. Further *in vivo* experiments will be carried out to ascertain the anti-tumour effect. Although the mode of cytotoxicity of the polysaccharides is still unknown, attempts will be made to examine and investigate the direct inhibition of RNA, DNA and protein synthesis as well as induction of specific functions of the immune system including macrophages, T-cells and natural killer cells. The confirmation of the curative effect of polysaccharides to cancer would be of great interest both to the nutraceutical industry and to the medical field. These facts gives a basis to opinion of many researchers in the field that sustainable development of mushroom and their products in the 21st century can become a non –green revolution in natural products medicine.

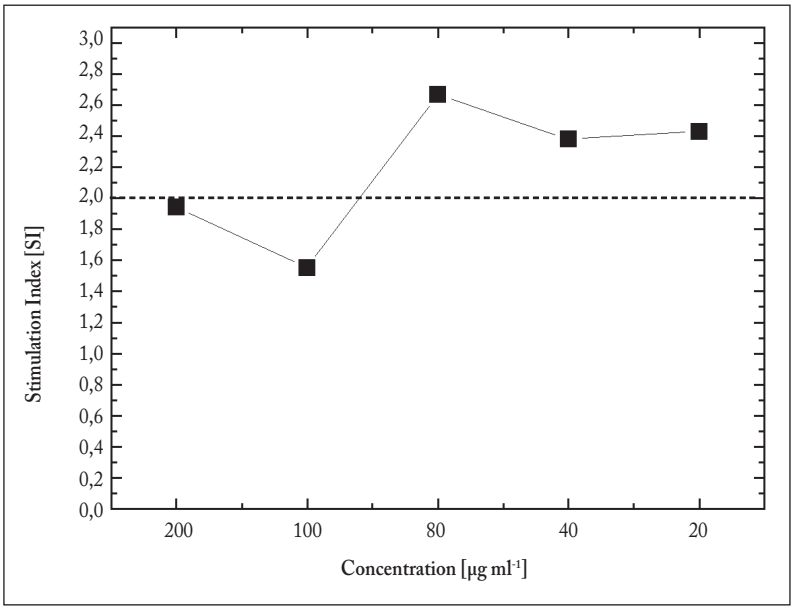

Figure 5. Stimulatory effect of different concentration of *P. ostreatus* EPS on normal PBMC. Data were presented as stimulation index (SI), where proliferation is considered positive if SI is ≥ 2.

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In situ RNA-RNA Hybridization: A Useful Method For Analysis of the Distribution of Transcripts of Various Genes in *Lentinula edodes* Fruiting Bodies

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The in situ RNA-RNA hybridization was renewed understanding that it is a useful method for analysis of the distribution of transcripts of various genes in fruiting bodies of Lentinula edodes. By using this method, we obtained the following results. Large amounts of the transcripts of ribonucleotide reductase small subunit gene (Le.rnr2) and UMP-CMP kinase gene (uck1), which is a target of developmental regulator PRIB, are present in both hymenium and outer region of trama in the hymenophore (gill tissue). The hymenium is the part for production of basidiospores and the outer region of trama is the region branching out into subhymenium (on the top of which hymenium is formed). The Le.ras transcript is present mostly in outer region of trama and in trama cells, while the transcript of trimeric G-protein αsubunit gene (Le.ga1) is mostly in hymenium. The transcript of *mfbC* gene, which is the target of PRIB and probably encodes the protein interacting with a putative translation initiation factor 5A (eIF5A), is present in outer region of trama. The transcript of *hyd1* (hydrophobin 1 gene), whose expressed product is considered to be involved in the formation in the extracellular matrix of lined air channels with a hydrophobic membrane, is present everywhere in the mycelial tissues of developing fruiting bodies except for the top parts of pileus (cap) and for prehymenophore. The region surrounding prehymenophore contains a high level of the transcript. These results suggest that *Le.rnr2* and *uck1* genes play a role mainly in the nucleotide biosynthesis essential for production of basidiospores and for divergence of trama cells into subhymenium cells. The *Le.ras* and *mfbC* play a role in divergence of mycelial cells and the *Le.ga1* plays a role in spore-production. The hydrophobin-mediated air channels may be formed almost all the parts of developing fruiting bodies.

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1. Introduction

To elucidate the molecular mechanism of the fruiting-body formation of homobasidiomycete *Lentinula edodes*, we previously isolated various genes that regulate development and analyzed their properties and functions using genetical, biochemical and cell-biological methods. To investigate the transcriptional expression of isolated genes during formation of fruiting body and in parts of fruiting body, usually total RNA was isolated from the corresponding mycelial cells and analyzed by Northern blotting. Here we present the data demonstrating that in situ RNA-RNA hybridization is a useful method to investigate the details of distribution of transcripts of isolated genes in parts of mature fruiting body such as hymenophore and stipe and in immature small fruiting body.

2. Method: In situ RNA-RNA hybridization

Parts of mature fruiting bodies and the whole immature small fruiting bodies were fixed with 4% paraformaldehyde in PBS at 4 ºC for 4 h and they were cut into 10-μm ultrathin longitudinal cryosections according to the method of Bochenek and Hirsch (1990). The cDNA fragments encoding the conserved regions of proteins were cloned into the *Eco*RI and *Hin*dIII sites of pSPT18 vector (Roche Diagnostics) and then, sense and antisense RNA probes were prepared by *in vitro* transcription with digoxigenine-UTP (Roche Diagnostics). Hybridization work and immunological detection of the hybridized probe were as described previously (Kaneko and Shishido 2001).

3. Results

3.1. *Ribonucleotide reductase small subunit gene* (Le.rnr2) *and UMP-CMP kinase gene* (uck1)

In the nucleotide metabolism, UDP, CDP, ADP and GDP are reduced to

the corresponding deoxyribonucleoside diphosphates (dNDP) to serve as precursors for syntheses of dNTPs. These reductions are catalyzed by ribonucleotide reductase (RNR) which consists of a heterodimer (α2ß2) that contains two non-identical homodimers: α2 is large subunit and ß2 is small subunit (Mathews et al. 1987). RNR plays a regulatory role in maintaining a balanced pool of all four deoxyribonucleoties (Reichard 1988). The small subunit gene (*Le.rnr2*) was cloned by Kaneko and Shishido (2001) from *L. edodes*. UMP-CMP kinase gene (*uck1*) of *L. edodes* (Kaneko et al. 1998) is a target of developmental regulator PRIB (565 amino acids (aa)) protein with a "Zn(II)2Cys6 zinc cluster" DNA-binding motif (Endo et al. 1994). The recombinant UMP-CMP kinase protein catalyzes the phosphoryl transfer from ATP to UMP and CMP efficiently and also to AMP and dCMP with lower efficiencies (Kaneko et al. 1998).

A Northern-blot analysis was carried out to investigate the expression of the *Le.rnr2* and *uck1* genes during fruiting-body formation of *L. edodes*. The intensity of hybridization bands gradually increased in proportion to enlargement/growing of fruiting bodies and the most intense signals were detected in the fruiting-body maturation stages (Kaneko et al. 1998, Kaneko and Shishido 2001). The *Le.rnr2* and *uck1* genes were shown to be actively transcribed in hymenophore of mature fruiting body (Kaneko et al. 1998, Kaneko and Shishido 2001).

The hymenophore is a complicated gill tissue consisting of trama, subhymenium and hymenium on which a large number of basidia and basidiospores are formed (Fig. 1). The trama cells diverge to form subhymeni-

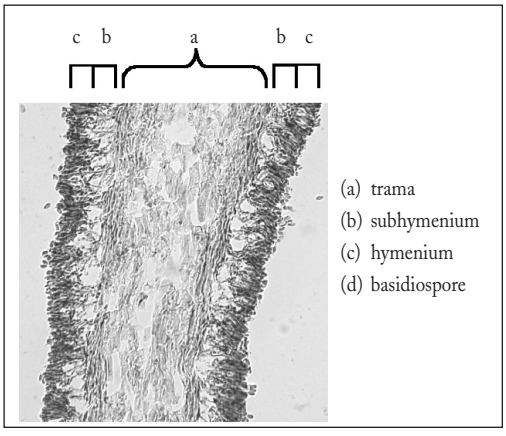


Figure 1. Hymenophore (gill tissue) of mature fruiting body of *L.edodes*

um on the top of which hymenium is formed.

In situ RNA-RNA hybridization was carried out to investigate the expression the *Le.rnr2* and *uck1* genes in the parts of hymenophore of fruiting body. The *Le.rnr2* and *uck1* antisense strand probes showed a specific distribution of the transcripts in the hymenophores of mature fruiting body (Fig. 2A and 2C), whereas their sense strand probes gave no signal (Fig. 2B and 2D). The intense signals of the *Le.rnr2* and *uck1* transcripts were detected in both the hymenium and the outer region of trama (the region branching out into subhymenium). The majority of trama cells and subhymenium cells gave weaker signals. These results imply that *Le.rnr2* and *uck1* genes play a role mainly in the nucleotide biosynthesis essential both for production of basidiospores and for divergence of trama cells into subhymenium cells in the hymenophore. During both the production of basidiospores and the divergence of mycelial cells in hymenophore biosyntheses of nucleic acids, carbohydrates, lipids etc. must be significantly active. Deoxyribonucleoside diphosphates and ribonucleoside diphosphates synthesized by ribonucleotide reductase or ribonucleoside monophosphate kinase all are required for syntheses of these biomolecules. Of course both genes are considered to be also involved in the nucleotide biosynthesis essential for growth of mycelial cells in other part(s) of fruiting body.

3.2. *Small monomeric Ras protein gene* (Le.ras) *and heterotrimeric G-protein a-subunit gene* (Le.ga1)

A Northern-blot analysis was carried out to investigate the expression of the *Le.ga1* gene during fruiting-body formation of *L. edodes*. Previous our study showed that the *Le.ras* is transcribed at similar levels in the course of fruiting-body formation (Hori et al. 1991). The *Le.ga1* is most actively transcribed in the fruiting body of maturation stage and is also transcribed in the preprimordial vegetative mycelia (Tanaka et al. 2005). Levels of the transcripts of *Le.ras* and *Le.ga1* were analyzed in parts of fruiting body of *L. edodes*. The hymenophore and stipe of mature fruiting body contained markedly higher levels of the transcripts of both genes when compared with the hymenophore-depleted pileus (Tanaka et al. 2005).

To investigate the expression of the *Le.ras* and *Le.ga1* genes in parts of the

hymenophore and stipe, in situ RNA-RNA hybridization was done. An intense signal of the *Le.ras* transcript was detected especially in the outer region of trama and relatively weak signal was observed in the trama cells (Fig. 2E). Differently from the case of hymenophore, the *Le.ras* transcript is pres-

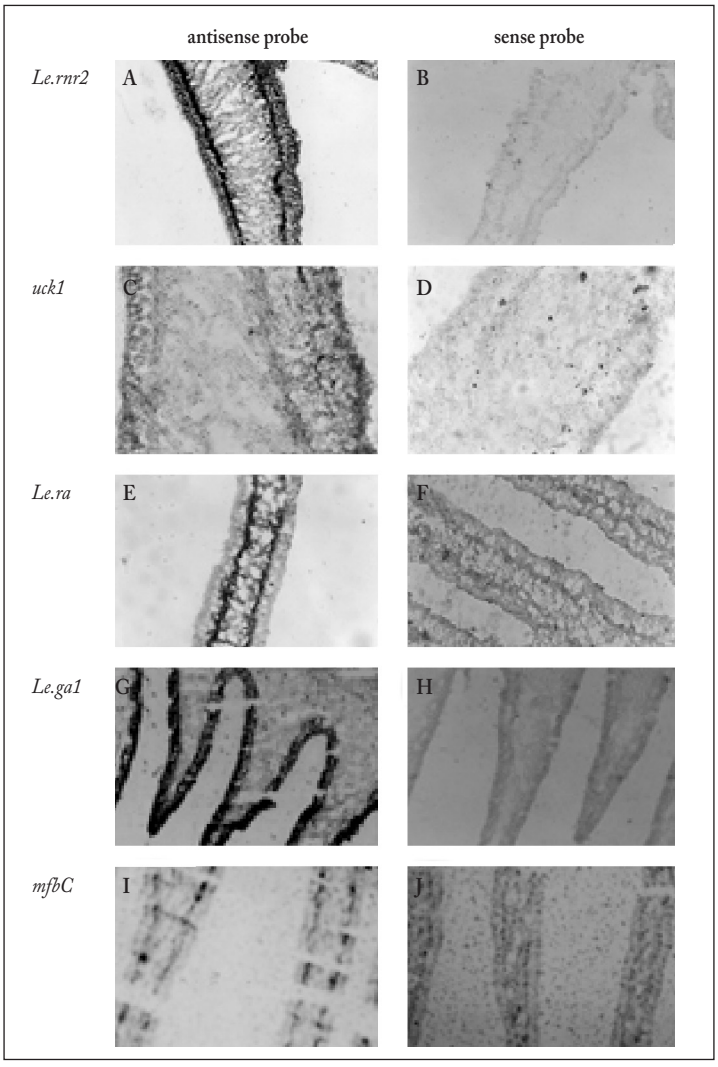


Figure 2. Expression of various genes in hymenophore of *L.edodes*

ent diffusibly in the stipe (data not shown). On the other hand, the *Le.ga1* transcript is present mostly in hymenium and subhymenium (Fig. 2G). The distribution pattern of the *Le.ga1* transcript in stipe was similar to that of *Le.ras* (data not shown). These results seem to suggest the possibility that *Le.ga1* is involved in production of basidiospores and *Le.ras* gene is involved in formation of inner part of hymenophore and in divergence of trama cells into subhymenium cells.

3.3. *Developmentally regulated* mfbC *gene*

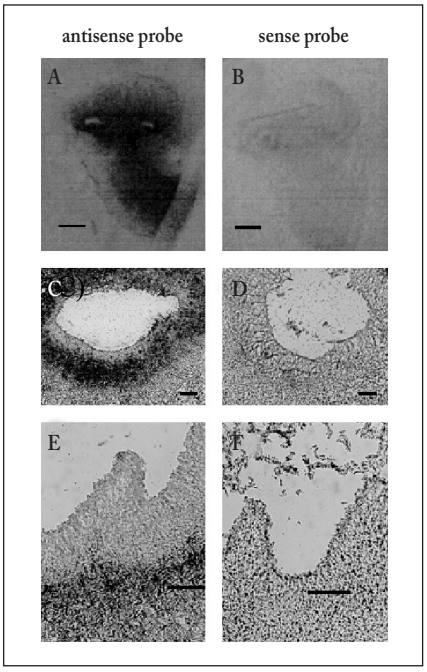
mfbC gene was cloned by Miyazaki et al. (2004) as a target of developmental regulator PRIB protein. As mentioned above, the *uck1* is also target of PRIB. The *mfbC* is a homologue of *S. cerevisiae YJR070C* /Lia (Thompson et al. 2003) and probably encodes the protein interacting with a putative translation initiation factor 5A (eIF5A), which is essential for cell viability and evolutionarily conserved (Thompson et al. 2003). Although the function of eIF5A is still obscure, there is the possibility that this factor might have a role in the translation of a specific subset of mRNAs.

Northern-blot analysis showed that all parts of the fruiting body contain *mfbC* transcript. But hymenophores and stipe contained higher levels of the transcript than the hymenophore-depleted pileus (Miyazaki et al. 2004). In situ RNA-RNA hybridization was carried out to investigate the expression of the *mfbC* gene in the parts of hymenophore, stipe, and hymenophore-depleted pileus (Miyazaki et al. 2004). Although the *mfbC* transcript appeared to distribute diffusively in the stipe and hymenophore-depleted pileus, this transcript showed a region-specific distribution in hymenophore (gill tissue). High levels of the *mfbC* transcript were detected in the outer region of the trama, the region branching out into the subhymenium (Fig. 2I). These results suggest that the *mfbC* gene is involved in the divergence of trama cells into subhymenium cells, but not in the production of basidiospores. To clarify the physiological function of the *mfbC* gene, however, more detailed ge-

netical and biochemical approaches are necessary.

3.4. *Hydrophobin 1 gene* (Le.hyd1)

Hydrophobins are moderately hydrophobic small proteins (100-150 amino acid residues) containing eight cysteine residues in a conserved pattern (Wessels 2000). These compounds appear to be unique to mycelial fungi such as basidiomycetes and ascomycetes, where they probably act in morphogenesis and pathogenesis (Wessels 2000). Three hydrophobin genes *SC1, SC4, and SC6* of *Schizophyllum commune* are expressed in dikaryons only, at the time of fruiting-body formation (Van Wetter 2000). Immunoelectron microscopy showed that SC4, the most abundant hydrophobin of the three



gene products, is secreted into the mucilage that surrounds hyphae of the plectenchyma (fungal tissues) of the fruiting bodies, lining air channels within them with a hydrophobic membrane (Van Wetter 2000).

Figure 3. Distribution of the *Le.hyd1* transcript in immature small fruiting bodies of L.edodes (A and B) and distribution of the le.hyd1 transcript in mycelial tissues around the prehymenophore of immature fruiting bodies (C-F). Bar, 200 μm

We isolated two hydrophobin genes, *Le.hyd1* and *Le.hyd2*, from *L. edodes* dikaryotic strain (Nishizawa et al. 2002). Northern blotting showed that immature small fruiting bodies that had just developed from primordia (pileus and stipe do not yet develop) contained the highest level of *Le.hyd1* transcript. Enlarged immature fruiting bodies also contained high levels of the transcript, but vegetatively growing mycelia or primordia and mature fruiting bodies contained no or a little *Le.hyd1* transcript. In the case of *Le.hyd2*, its transcript level was high in dikaryotic vegetative mycelial tissues.

Results of in situ RNA-RNA hybridization showed the presence of the *Le.hyd1* transcripts everywhere in the mycelial tissues of developing fruiting bodies except for the top parts of the pileus (cap) and for the prehymenophore (Fig. 3A). A high level of the transcript was detected in the parts surrounding the prehymenophore (Fig. 3C and E). As mentioned earlier, hydrophobins seem to be involved in the formation in the extracellular matrix of lined air channels with a hydrophobic membrane. These channels may help to provide gas exchange during respiration in mycelial tissues of developing fruiting bodies. Our results suggested that hydrophobin-mediated air channels may be formed all over the mycelial tissues of developing fruiting bodies except for the top parts of the pileus (cap) and for the prehymenophore. In particular, the parts surrounding the prehymenophore may come to have a large number of air channels.

4. Acknowledgements

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Rat Cytochrome P_{450}-Mediated Transformation of Dichlorodibenzo-P-Dioxins by Recombinant White-Rot Basidiomycete *Coriolus hirsutus*

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Rat cytochrome P450, CYP1A1, has been reported to play an important role in the metabolism of mono-trichlorodibenzo-*p*-dioxins (M-TriCDDs). To breed lignin (and M-TetraCDDs)-degrading basidiomycete *Coriolus hirsutus* strains producing rat CYP1A1, an expression cassette [*C. hirsutus gpd* promoter-*C. hirsutus gpd* 5'-portion (224-bp of 1st exon-8th base of 4th exon)-rat *cyp1a1* cDNA-*Lentinula edodes priA* terminator] was constructed and inserted into pUCR1 carrying the *C. hirsutus arg1* gene. The resulting recombinant plasmid, MIp5-(cyp1a1+arg1) was introduced into protoplasts of *C. hirsutus* monokaryotic strain OJ1078 (Arg^-, Leu^-), obtaining three good Arg^+ transformants. These transformants [ChTF5-2(CYP1A1), ChTF5-4(CYP1A1), and ChTF5-6(CYP1A1)] were estimated to carry nine, six, and seven copies of the expression cassette on their chromosomes, respectively. Immunoblot analysis revealed that the three transformants produce similar amounts of rat CYP1A1 enzyme. ChTF5-2(CYP1A1), ChTF5-4(CYP1A1), ChTF5-6(CYP1A1) and recipient OJ1078 were cultivated in a liquid medium containing 2,7/2,8 (at a ratio of 1:1)-dichlorodibenzo-*p*-dioxins (2,7/2,8-DCDDs) and the amount of intra- and extracellular 2,7/2,8-DCDDs remaining was measured. The results showed that all three transformants efficiently transform 2,7/2,8-DCDDs through the action of the recombinant rat CYP1A1 enzyme.

1. Introduction

Chlorinated dibenzo-*p*-dioxins (CDDs) have been of public concern for two decades because of their toxicity in animal tests (Safe 1990; Schecter et al.

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1987). Extracellular lignin-degrading enzymes such as lignin peroxidase (LiP) and manganese peroxidase (MnP) produced by white-rot basidiomycete fungi have been reported to be involved in transformation of various CDDs (and various chlorophenols etc.) (Armenante et al. 1994; Bumpus et al. 1985; Joshi and Gold 1993; Joshi and Gold 1994; Reddy and Gold 2000; Takada et al. 1996; Valli et al. 1992). Through various metabolic pathways, the white-rot basidiomycete fungi convert the chlorinated aromatic compounds to CO_2 and H_2O. We have recently produced monokaryotic strains of the white-rot basidiomycete *Coriolus hirsutus* with high MnP or LiP activity (Yamazaki et al. 2004a; Yamazaki et al. 2004b). The culture supernatants of these strains showed higher transformation activities of 2,7-dichlorodibenzo-*p*-dioxin (2,7-DCDD) and pentachlorophenol.

To date, the metabolism of various CDDs has been studied *in vivo* using experimental animals (Hu and Bunce 1999a; Poiger et al. 1982; Rose et al. 1976; Tulp and Hutzinger 1978; Van den Berg et al. 1994; Wroblewski and Olson 1985). The insertion of a single oxygen atom into the dioxin molecule to form an epoxide by cytochrome P450 (CYP) is considered to be the initial reaction in the metabolism of various CDDs. Hu and Bunce (1999b) suggested that mammalian CYP1A1 and CYP1A2 play an important role in the metabolism of mono-tri (M-Tri)CDDs. In vivo studies suggested that the CYP-dependent metabolism includes multiple reactions such as hydroxylation at an unsubstituted position, hydroxylation with migration of a chloride substituent, hydroxylation with elimination of a chloride substituent, and opening of the dioxin ring (Sakaki et al. 2002). All of these reactions appear to be reactions aimed at detoxifying M-TriCDDs. Thus, CYPs seem very likely to be key enzymes for metabolism of M-TriCDDs in mammals. So far this CYP-catalyzed metabolism of M-TriCDDs has been not reported in basidiomycete (and ascomycete) fungi, even though the CYPs of white-rot basidiomycete *Phlebia lindtneri* have been implicated in the catalysis of a simple mono-hydroxylation of non-chlorinated DD; the *in vivo* mono-hydroxylation has been shown to be inhibited by CYP inhibitors (Mori and Kondo 2002), and the CYPs of the white-rot basidiomycete *Lentinula edodes* (Akiyama et al. 2002, 2004), *Phanerochaete chrysosporium* (Van den Brink et al. 1998) and *Pleurotus pulmonarius* (Maspahy et al. 1999) have been shown to catalyze the conversions of benzo(a)pyrene and 7-ethoxycoumarin. In this paper, we attempted to breed *C. hirsutus* strains that produce mammalian

CYP and metabolize chlorinated dioxin molecules efficiently within mycelial cells. If such strains could be successfully bred, and used together with strains producing large amounts of extracellular LiP and MnP, various CDDs may be metabolized more efficiently. The rat *cyp1a1* cDNA encoding the CYP1A1 enzyme that transforms 2,7-DCDD, 2,8-DCDD, and 2,3,7-TriCDD (Murakami et al. 1990) was used for breeding of *C. hirsutus*. We constructed a chromosome-integrating recombinant plasmid carrying an expression cassette for rat CYP1Al and introduced it into protoplasts of monokaryotic strain of *C. hirsutus*. Here, we report successful production of strains that produce the rat CYP1A1 enzyme and transform 2,7/2,8(at a ratio of 1:1)-DCDDs (hereafter referred to simply as 2,7/2,8-DCDDs).

2. Results

2.1. *Transformation of C. hirsutus Arg⁻ Leu⁻ auxotrophic monokaryotic strain OJ1078 with MIp5-(cyp1a1+arg1)*

MIp5-(cyp1a1+arg1) contains the rat CYP1A1-expression cassette (*C. hirsutus gpd* promoter-*C. hirsutus gpd* 5'-portion-rat *cyp1a1* cDNA-*L. edodes priA* terminator) and the selection marker *C. hirsutus arg1* gene (Tsukamoto et al. 2003), as shown in Fig. 1.

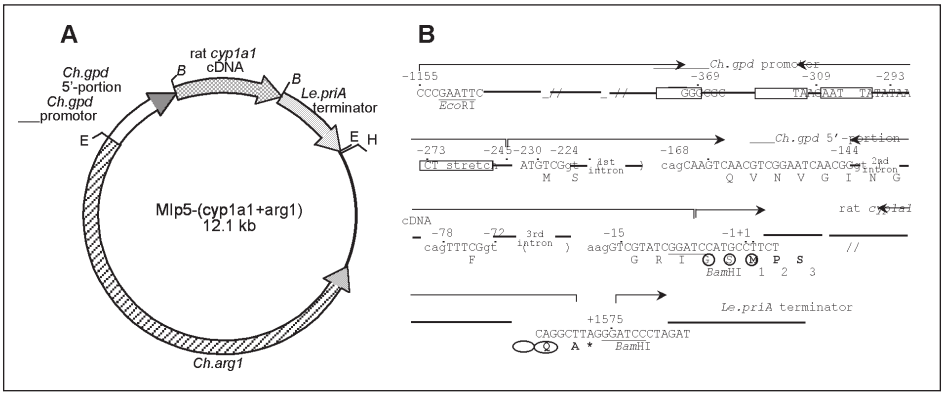

Figure 1. Structure of the rat CYP1A1 (524 amino acid residues)-expressing recombinant plasmid MIp5-(cyp1a1+arg1) (A), the structural features of the *C. hirsutus gpd (Ch.gpd)* promoter, the *Ch.gpd 5'*-portion (224-bp sequences of 1st exon-8th baseof 4th exon), and the nucleotide sequences at the fusion junction between the *Ch.gpd 5'*-portion, rat *cyp1a1* cDNA and the *L. edodes priA (Le.priA)* terminator (B)

This recombinant plasmid was introduced into protoplasts of *C. hirsutus* monokaryotic strain OJ1078 (Arg^-, Leu^-), obtaining 20 Arg^+ transformants. Of these, three Arg^+ transformants showed almost the same growth rates as that of recipient strain OJ1078. Total DNA from the three transformants was digested with *Eco*RI and subjected to Southern-blot analysis using a mixed probe [^{32}P-labelled 1,575-bp rat *cyp1a1* cDNA and 1,059-bp *C. hirsutus ras* (*Ch.ras*) gene; Yamazaki et al. 2004]. Our previous study has shown that *C. hirsutus* genome contains a single copy of the *ras* gene, and an *Eco*RI-digest of chromosomal DNA gives a *ras* signal at a position corresponding to 2.8 kb (Yamazaki et al. 2004b). As shown in Fig. 2, *Eco*RI-digests of three Arg^+ transformants, namely ChTF5-2(CYP1A1) (lane 2), ChTF5-4(CYP1A1)(lane 3), and ChTF5-6(CYP1A1)(lane 4), gave an intense signal at 3.9 kb, corresponding to the size of the rat CYP1A1-expression cassette (in addition to the 2.8-kb *ras* signal). An *Eco*RI-digest of the control OJ1078 (lane 1) showed only the *ras* signal. To estimate the copy number of the rat CYP1A1-expression cassette in the chromosomes of ChTF5-2(CYP1A1), ChTF5-4(CYP1A1), and ChTF5-6(CYP1A1), the radioactivity of the 3.9-kb rat *cyp1a1* band was compared with that of the 2.8-kb *ras* band. The specific radioactivities of the two probes of rat *cyp1a1* cDNA and *Ch.ras* gene were similar. The data suggested that ChTF5-2(CYP1A1), ChTF5-4(CYP1A1), and ChTF5-6(CYP1A1) carry nine, six, and seven copies of the rat CYP1A1-expression cassette on their chromosomes, respectively.

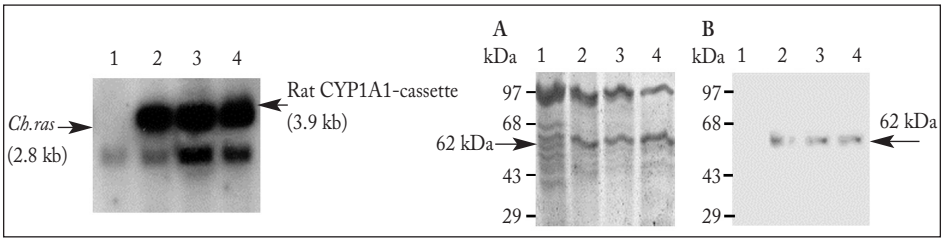

Figure 2 (Left). Southern-blot analysis of the *Eco*RI-digests of total DNAs prepared from the three rat CYP1A1-producing transformants and the recipient strain of *C. hirsutus*. Lanes: 1, the recipient OJ1078; 2, ChTF5-2(CYP1A1); 3, ChTF5-4(CYP1A1); 4, ChTF5-6(CYP1A1).

Figure 3 (Right). Immunoblot analysis of the microsomal proteins extracted from the three rat CYP1A1-producing transformants and the recipient strain of *C. hirsutus*. (A) SYPRO Ruby staining of the microsomal proteins after SDS-PAGE. (B) The microsomal proteins separated on SDS-PAGE were transferred to a PVDF membrane. The blot was incubated with anti-rat CYP1A1 antibody and the signal was made visible by chemilluminescence. Lanes: 1, the recipient OJ1078; 2, ChTF5-2(CYP1A1); 3, ChTF5-4(CYP1A1); 4, ChTF5-6(CYP1A1).

2.2. *Production of the rat* cyp1a1 *cDNA product, CYP1A1 by recombinant* C. hirsutus *strains*

The production and localization of rat CYP1A1 protein in ChTF5-2(CYP1A1), ChTF5-4(CYP1A1), and ChTF5-6(CYP1A1) were analyzed. In fungi (as in other eukaryotes) the majority of P450 protein has been reported to be present in microsomes [endoplasmic reticulum (ER); Akiyama et al. 2004; Oeda et al. 1985; Van den Brink 1998]. The total protein contained in the microsomal fractions of ChTF5-2(CYP1A1), ChTF5-4(CYP1A1), ChTF-5-6(CYP1A1), and OJ1078 was separated by SDS-PAGE (Fig. 3A) and transferred to a PVDF membrane. The protein-transferred membrane was analyzed by immunoblotting using anti-rat CYP1A1 antibody. As shown in Fig. 3B, a single signal was detected at the predicted position (62 kDa) for the microsomal protein-blots of ChTF5-2(CYP1A1) (lane 2), ChTF5-4(CYP1A1) (lane 3), and ChTF5-6(CYP1A1)(lane 4), and their intensities were similar. No signal was detected in the case of OJ1078 (lane 1). These results showed that similar amounts of the rat CYP1A1 protein were produced in ChTF5-2(CYP1A1), ChTF5-4(CYP1A1) and ChTF5-6(CYP1A1), and transferred to microsomes (ER).

2.3. *Transformation of 2,7/2,8-DCDDs by the rat CYP1A1-producing* C. hirsutus *strains*

We used 2,7/2,8-DCDDs for the experiment. To examine the transformation activity of 2,7/2,8-DCDDs, ChTF5-2(CYP1A1), ChTF5-4(CYP1A1), ChTF5-6(CYP1A1), and OJ1078 were cultivated in 10 ml MYGC medium containing 10 μg 2,7/2,8-DCDDs in an L-shaped tube at 30 ˚C for 5 days with shaking. We chose the 5-day cultivation from the following reason. *C. hirsutus* monokaryotic strain produces only limited amounts of lignin-degrading enzymes (LiP and MnP), which also transform 2,7/2,8-DCDDs (Yamazaki et al. 2004a; Yamazaki et al. 2004b), thus allowing rat CYP1A1-catalyzed transformation of 2,7/2,8-DCDDs to be easily assessed. Hexane extracts of whole cell cultures were subjected to gas chromatography (GC) and the total amount of 2,7/2,8-DCDDs remaining, both inside the mycelial cells and in the culture medium, was determined (Fig. 4).

2,7-DCDD and 2,8-DCDD give a single peak in GC. First, the recovery (%) by hexane extraction of 2,7/2,8-DCDDs from the whole cell culture was analyzed. The standard amount (10 μg) of 2,7/2,8-DCDDs was added to 10 ml of a 5-day preculture of OJ1078, and extracted with hexane immediately. The peak of 2,7/2,8-DCDDs recovered (Fig. 4B) was compared with that of 10 μg 2,7/2,8-DCDDs subjected directly to GC (Fig. 4A), indicating that 93% of the 2,7/2,8-DCDDs added to the preculture was recovered. The peaks of the 5 day-cultivations of ChTF5-2(CYP1A1) (Fig. 4D), ChTF5-4(CYP1A1) (Fig. 4E), and ChTF5-6(CYP1A1) (Fig. 4F) showed that the three strains transformed 2,7/2,8-DCDDs much more efficiently than OJ1078 (Fig. 4C). The relative transformation of 2,7/2,8-DCDDs (%) was calculated by the peak of Fig. 4B being taken as 100%. ChTF5-2(CYP1A1), ChTF5-4(CYP1A1), and ChTF5-6(CYP1A1) were indicated to transform 71.7%, 69.8%, and 69.4% of 2,7/2,8-DCDDs respectively, while recipient OJ1078 was shown to transform only 11.8% of 2,7/2,8-DCDDs (Fig. 5). The control Arg^+ transformant obtained by introduction of *C. hirsutus arg1*-carrying pUCR1 alone showed a level of transformation of 2,7/2,8-DCDDs similar to that of OJ1078 (data not shown). The results strongly suggest that, at 5-days of cultivation, about 58.5 (70.3-11.8)% of 2,7- and 2,8-DCDD molecules added to the culture medium were transported into the mycelial cells and transformed by the rat CYP1A1 enzyme within them.

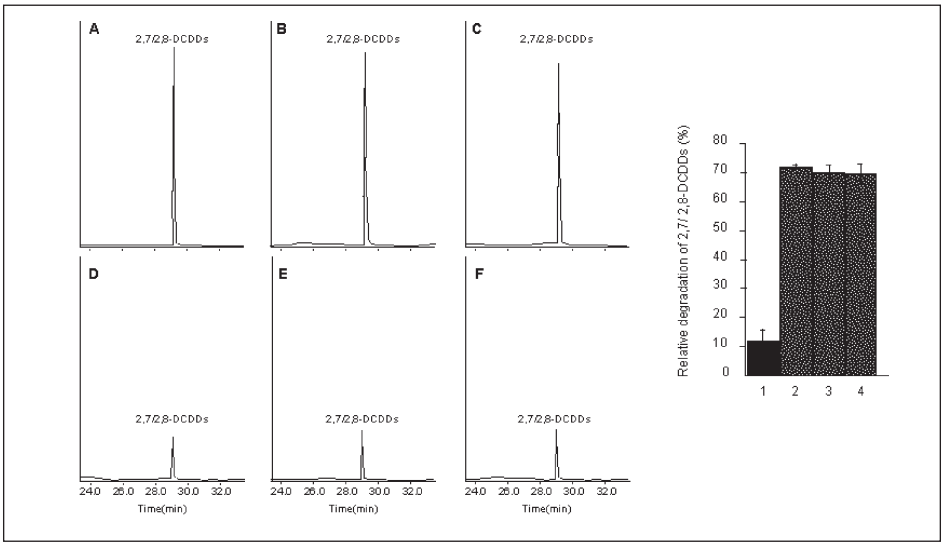

Figure 4 (Left) Gas chromatograms showing an efficient transformation of 2,7/2,8-DCDDs during cultivations of the three rat CYP1A1-producing transformants and the recipient strain of *C. hirsutus*. Ten μg each of 2,7/2,8-DCDDs was added to the culture media of ChTF5-2(CYP1A1) (D), ChTF5-4(CYP1A1)(E), ChTF-5-6(CYP1A1) (F), and the recipient OJ1078 (C) before the start of cultivations. After 5 days, the whole cell cultures were extracted with hexane and the resulting extracts were analyzed by gas chromotography (GC). The hexane extract obtained immediately after addition of 10 μg of 2,7/2,8-DCDDs to the 5-day preculture of OJ1078 (B), and 10 μg of the 2,7/2,8-DCDDs alone (A) were also analyzed.

Figure 5 (Right). Transformation of 2,7/2,8-DCDDs during cultivations of the three rat CYP1A1-producing transformants and the recipient strain of *C. hirsutus*. The remaining amounts of 2,7/2,8-DCDDs after 5-day cultivations of ChTF5-2(CYP1A1) (lane 2), ChTF5-4(CYP1A1) (lane 3), ChTF5-6(CYP1A1) (lane 4), and the recipient OJ1078 (lane 1) were estimated by analyzing the peaks of gas chromatograms shown in Fig. 4B-F. The amount of 2,7/2,8-DCDDs shown in Fig. 4B was taken as 100%. Error bars indicate the standard deviations of three replicates

We next examined the level of transformation of 2,7/2,8-DCDDs in a prolonged cultivation. ChTF5-2(CYP1A1), ChTF5-4(CYP1A1), ChTF5-6(CYP1A1) and OJ1078 were cultivated at 30 ˚C for 16 days, when the production of LiP and MnP reaches a maximum level (Yamazaki et al. 2004a; Yamazaki et al. 2004b). Although a constant recovery (%) of 2,7/2,8-DCDDs from the whole cell culture was not obtained, probably owing to the much larger mass of mycelial cells, roughly about 85-90% of 2,7/2,8-DCDDs was considered to be transformed by ChTF5-2(CYP1A1), ChTF5-4(CYP1A1), and ChTF5-6(CYP1A1), while about 30-35% was presumably transformed by OJ1078 (data not shown).

3. Discusion

Our result suggests that, after 5 days cultivation, only a fraction (11.8%) of 2,7- and 2,8-DCDD molecules were transformed by the extracellular lignin-degrading enzymes LiP and MnP, and that the majority (58.5%) of the 2,7- and 2,8-DCDD molecules, having escaped transformation by LiP and MnP, were transported into mycelial cells and transformed by the rat CYP1A1 enzyme within them. Although their transformation activities towards 2,7/2,8-DCDDs have not yet been examined, the CYPs of the white-rot basidiomycete *P. lindtneri* have been implied to transform non-chlorinated DD into a mono-hydroxylated form (Mori and Kondo 2002). Even if *C. hirsutus* OJ1078 produces CYPs that catalyze the transformation of chlorinated DD as well as non-chlorinated DD, their contribution to transformation of 2,7/2,8-DCDDs may not be significant; the relative transformation of 2,7/2,8-DCDDs (%) by such endogenous CYPs is clearly less than 11.8%.

Although varying slightly depending on the amount of 2,7/2,8-DCDDs added to culture medium, the degree of transformation of 2,7/2,8-DCDDs by the rat CYP1A1-producing *C. hirsutus* strains were very similar (approx. 70%). The activity of cytochrome P450 enzyme is regulated by NADPH-P450 reductase. Thus, it is possible that the amount of NADPH-P450 reductase produced in the recombinant *C. hirsutus* cells is insufficient compared with the amount of P450 enzyme, thereby regulating the latter at a constant lower level. To confirm this, introduction of a *C. hirsutus* NADPH-P450 reductase-expression cassette into the rat CYP1A1-producing *C. hirsutus* strains will be required. As mentioned in the Introduction, a much more efficient transformation (degradation) of TriCDDs as well as 2,7/2,8-DCDDs is thought to be achieved by co-cultivation of the *C. hirsutus* strains producing rat CYP1A1 and those producing large amounts of LiP and MnP.

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Wild Strains of *Agaricus bisporus*: a Source of Tolerance to Dry Bubble Disease

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The button mushroom *Agaricus bisporus* is susceptible to various pests and diseases. Dry bubble, caused by the Hyphomycete *Verticillium fungicola*, is currently the more serious disease and is worldwide in distribution. Cultivars are susceptible and the pathogen develops resistance towards the very few fungicides admitted at mushroom farms. Breeding for resistance is necessary and wild strains of *A. bisporus* are putative sources of tolerance to *V. fungicola*. We present results on the susceptibility of some wild strains of the INRA-CTC collection and the PPO MRU collection. Besides the severity of the disease, the strains were also compared for their ability to develop each of the symptoms induced by the pathogen: spotted mushrooms, stipe blow-out and spheroid masses (the bubbles) which are the typical symptom of the disease.

***Agaricus bisporus* 2100, cultivated in numerous French mushroom farms, was used to assess the aggressiveness of various isolates of *V. fungicola* var. *fungicola*, the variety responsible for the disease in Europe at present. Significant variability in aggressiveness was observed. Isolate VCTC, which induced severe symptoms on *A. bisporus* 2100 (30-40% of diseased mushrooms), revealed interesting tolerance (10-18% of diseased mushrooms) among five wild *A. bisporus* strains and hybrids between wild strains. A cross test was performed with two cultivars and seven wild stains of *A. bisporus* contaminated with five *V. fungicola* isolates, two of var. *fungicola* and three of var. *aleophilum*, the latter identified as responsible for the disease in USA and Canada. The wild strains screened in this experiment were far more tolerant than the cultivars, exhibiting 3-9% of diseased mushrooms compared to 20-22%. All the strains were more susceptible to the pathogens of var. *aleophilum* than to those of var. *fungicola*.**

These experiments showed that very tolerant material exists in collection and can be used as parents to breed for resistance. The greater susceptibility of *A. bisporus* to *V. fungicola* var. a*leophilum* must be taken into consideration in breeding programmes, this variety being present in North America and being isolated in Europe in the past.

Genetics and Cellular Biology of Basidiomycetes-VI. A.G. Pisabarro and L. Ramírez (eds.)
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1. Introduction

The INRA and the PPO MRU collections of *Agaricus bisporus* both consist of wild strains of a large genetic diversity and a great variability in phenotypic traits such as colour (white to dark brown) and morphology. The PPO MRU collection is derived from the collection of the Agaricus Resource Program (ARP program, Kerrigan, 1996). At the time when chemicals become more and more restricted breeding for *A. bisporus* strains that are resistant to the major diseases, especially dry bubble, is of prime importance. Resistant (or very tolerant) wild strains need to be selected and introduced in crosses. This paper describes the choice of isolates of *Verticillium fungicola* allowing the identification of strains potentially tolerant to any isolate of the pathogen, and shows the possibility to find some tolerant *A. bisporus* strains during the screening of collections.

2. Material and Method

2.1. *Agaricus bisporus*

Commercial strains 2100 (Amycel, France), A15 (Sylvan, the Netherlands), U1 (Horst, NL), wild strains W1, W2, W3, W4, W5, W7, W8 (PPO MRU collection), WA, WB (INRA collection) and hybrids HW1, HW3, HW4 (obtained from wild strains, INRA collection) were screened for susceptibility.

2.2. *Verticillium fungicola*

Isolates of *V. fungicola* var. *fungicola* collected from various geographical origins and of different dates of collection, and var. *aleophilum* isolates are representative of the clonal population of *V. fungicola* responsible for recent outbreak in Pennsylvania (Collopy *et al*. 2001) (Table 1).

Table 1
Isolates of *Verticillium fungicola* screened for aggressiveness

Isolate variety	*Isolate code*	*Date*	*Geographic origin*	*Source*
fungicola	VCF	1996	Chancelade, France	INRA-Bx
fungicola	VCTC	1997	St Paterne, France	INRA-Bx
fungicola	VF	1987	St Paterne, France	INRA-Bx
fungicola	VK	1994	Nancy, France	INRA-Bx
fungicola	VMX1	2002	Xalapa, Mexico	INRA-Bx
fungicola	V9503	1995	The Netherlands	PPO MRU
fungicola	V010404	2001	Limburg, The Netherlands	PPO MRU
aleophilum	V02	1999	Chester County, PA, USA	Collopy *et al.* (2001)
aleophilum	V07	1999	Chester County, PA, USA	Collopy *et al.* (2001)
aleophilum	V17	1999	Chester County, PA, USA	Collopy *et al.* (2001)

2.3. *Pathogenicity test in culture*

INRA tests: *Agaricus bisporus* was grown in 0.9 m^2 trays filled with commercial compost spawned at 0.8%. No fungicide was added, and cultures were covered with plastic film. Spawn-run took place for 13 days in a climatic room set at 24°C, 92% relative humidity before casing (85% limestone and 15% peat, v:v, not treated with fungicide) was applied. Nine days after casing the room temperature was decreased to 16°C. Eleven days after casing the cultures were ruffled and a conidial suspension of *V. fungicola* var. *fungicola* was sprayed onto the surface of the casing layer at a rate of 10^6 conidia / m^2. Each *V. fungicola* isolate was supplied to six trays. Bait cultures made of six uninfected trays were used to assess contamination caused by spores produced by affected mushrooms. The experiment was performed twice with two different batches of compost. Healthy mushrooms, spotted mushrooms (S), stipe blow-out (BO) and bubbles (DB) were harvested for 4 weeks and weighted separately. Data reported were means from the two cell tests. A first test compared the level of susceptibility of *A. bisporus* Amycel 2100 to six isolates of *V. fungicola*, VCF, VCTC, VF, VK, VMX1 and V95. In a second test, the isolate VCTC was used to assess the susceptibility of two wild strains (WA and WB) and three hybrids between wild strains

(HW1, HW3 and HW4). Bait cultures consisted in six crates of uninoculated WB.

MRU-test: *Agaricus bisporus* A15, U1, W1-5, W7 and W8 were cultivated in crates containing 15 kg of compost. After spawning, malathion was sprayed onto the compost. Spawn-run took place at 24°C. After one day, the cultures were covered with paper. After 17 days, casing soil (CNC, standard composition, not treated with fungicide) was applied. Subsequently, the cultures were infected with *V. fungicola* V9503, V010404, V02, V07 and V17 by pouring 100 mL of a suspension of freshly harvested conidia (1.5×10^6 spores / m^2) on top of the casing soil. Eleven days after casing the cultures were ruffled. Fourteen days after casing, the temperature was decreased down to 17°C. Cell tests, each with one crate per *A. bisporus*-V. *fungicola* combination and one control crate (uninfected) per *A. bisporus* strain, were repeated three times. Harvested mushrooms were divided into three categories: healthy, spotted and affected (stipe blow-out and bubbles) mushrooms and counted.

2.4. *Data analyses*

Data reported were percentages of the total crop but statistical analyses were performed on arc sinus transformed data using the general linear model provided by the SAS system (SAS Institute Inc., Cary, NC, USA).

3. Results and Discussion

3.1. *Factors of variation in the pathogenicity test of* Agaricus bisporus *strains*

Significant cell-test effects were detected (Table 2) which corroborated the observations of Sonnenberg *et al.* (2005) who reported that the infection levels of *A. bisporus* vary considerably from crop to crop for unknown reasons. Previous experiments carried out both at INRA facilities and PPO MRU facilities with a single strain of *A. bisporus* contaminated with a single isolate of *V. fungicola* have shown the homogeneity of the climatic cell (data not shown). We postulated that uncontrolled variations between batches of compost were responsible for the variations in susceptibility observed from crop

to crop. The absence of significant interaction between the cell tests and the treatments (Table 2) means that the classification of the isolates of *V. fungicola* for aggressiveness was the same in the two cell tests and consequently allowed us to consider mean values from the two tests.

Table 2
Analysis of variance for the various symptoms (INRA test 1).

Variable[1]	*Source*	*df*	*Mean square*	*F value*
S	Cell test	1	35.1736	3.59 ns
	Treatment	6	231.3690	23.58 **
	Cell test*Treatment[2]	6	15.7446	1.60 ns
	Crate	5	22.9408	2.34 ns
BO + DB	Cell test	1	1123.2729	71.08 **
	Treatment	6	1237.2523	78.29 **
	Cell test*Treatment	6	14.3596	0.91 ns
	Crate	5	8.3585	0.53 ns
S + BO + DB	Cell test	1	430.2089	31.39 **
	Treatment	6	1336.4944	97.50 **
	Cell test*Treatment	6	24.8501	1.81 ns
	Crate	5	21.5202	1.57 ns

1. S = spot, BO = stipe blow-out and DB = dry bubble. 2. *V. fungicola* or control (bait culture).
** = significant at $P < 0.01$, * = significant at $P < 0.05$ and ns = not significant at $P = 0.05$.

The disease levels of *A. bisporus* 2100 varied dramatically with the isolate of *V. fungicola* supplied. Sporophores with stipe blow-out were more abundant on crates inoculated with VCF, the less aggressive isolate, than on control crates used as bait culture, but the disease level did not differ significantly between both treatments. The percentage cumulating mushrooms with stipe blow-out and bubbles was nine times higher after inoculation of VCTC, V95 and VMX1 than after the supply of VCF. Differences observed for spotted mushrooms were also significant but of far less magnitude (Table 3). A significant correlation ($P< 0.01$) was found between the percentage of spotted mushrooms and that of stipe blow-out whereas none was detected between the percentage of these symptoms (S + BO) and that of bubbles.

The various symptoms depend on the stage of development of the mushroom at the time of infection (van de Geijn, 1982; North and Wuest, 1993; Rinker and Wuest, 1994). But as this cell-test was performed with a single strain of *A. bisporus* the propension in developing rather bubbles or rather other symptoms should be related to the pathogen. VF induced as numerous stipe blow-out and spotted mushrooms but far less bubbles than the aggressive isolates VMX1 and VCTC. Contamination by spores produced by bubbles cannot explain these differences because of the low symptom levels observed on bait cultures and crates contaminated with VCF.

Total mushroom production (including both diseased and healthy mushrooms), expressed as g/kg substrate, did not vary significantly with the treatment and was not significantly different from that observed for the control (Table 4).

Table 3
Response of *Agaricus bisporus* 2100 to various isolates of *Verticillium fungicola*

Treatment	*Percentages of*				
	S	*BO*	*DB*	*BO + DB*	*S + BO + DB*
VMX1	11.4 ab[1]	4.3 ab	20.9 a	25.2 a	36.60 a
VCTC	13.0 ab	4.7 ab	20.0 a	24.7 a	37.80 a
V9503	14.3 a	6.9 a	15.6 a	22.5 a	37.20 a
VF	12.6 ab	5.6 a	6.0 b	11.6 b	24.30 b
VK	10.1 b	3.1 bc	3.6 c	6.7 c	16.89 c
VCF	7.4 c	2.1 c	0.7 d	2.8 d	10.20 d
Control	2.9 d	0.7 d	0.6 d	1.3 d	4.20 d

1. Values within a column following by the same letter do not differ significantly by the Student-Newman-Keuls test ($P = 0.05$)

Table 4
Effect of contamination with *Verticillium fungicola* on the total crop

Treatment	*Total crop*	*(g/kg substrate)*
VMX1	261.0	ab[1]
VCTC	285.1	a
V9503	262.1	ab
VF	282.5	a
VK	285.4	a
VCF	289.8	a
Control	281.1	a

1. Values within a column following by the same letter do not differ significantly by the Student-Newman-Keuls test ($P = 0.05$)

The aggressiveness of the isolates of *V. fungicola* was not correlated with the date in collection ($r^2 = 0.21$ for BO + DB and 0.03 for S, df = 4). In the same culture conditions, the isolate CBS440.34, in collection since 1934, was not affected by long time storage and gave 38.2% of affected mushrooms including 22.9% of bubbles.

3.2. *Variability in susceptibility of* Agaricus bisporus *strains to* Verticillium fungicola

For the purposes of breeding a Verticillium resistant strain, an Agaricus strain identified as resistant or tolerant to *V. fungicola* in cell-tests must also be resistant or tolerant to any Verticillium isolate. To identify material for breeding programmes and to characterise hybrids we have chosen the aggressive isolate VCTC. In other cell-tests this isolate was as aggressive towards *A. bisporus* Euromycel 31 as towards 2100 (Largeteau *et al.*, 2004). Despite its high aggressiveness VCTC revealed significant differences in susceptibility within a group of wild strains and hybrids (Table 5).

As observed for *A. bisporus* 2100, the inoculation of *V. fungicola* had no significant influence ($P = 0.05$) on the total crop of WB; 1871 g and 1941 g $crate^{-1}$ were harvested on inoculated crates and bait cultures, respectively.

Other cell-tests are in progress to characterize the INRA collection of *A. bisporus* and have identified some wild strains of substantial yield and high tolerance to VCTC (not shown). The large scale screening of wild strains for susceptibility to *V. fungicola* performed by Sonnenberg *et al.* (2005) identified several strains showing less than 5% affected mushrooms after three flushes.

Table 5
Comparison of wild strains and hybrids for their susceptibility to *Verticillium fungicola* VCTC

A. bisporus	*Percentages of*				
	S	*BO*	*DB*	*BO + DB*	*S + BO + DB*
WA	1.1 b	7.5 a	6.2 a	13.6 a	14.8 ab
WB	8.5 a	4.1 ab	5.3 a	9.4 ab	17.9 a
HW1	1.9 b	10.4 a	4.2 ab	14.7 a	16.6 a
HW3	2.3 b	6.3 ab	2.1 abc	8.4 ab	10.7 b
HW4	4.3 ab	3.9 abc	2.9 ab	6.8 ab	11.2 b

1. Values within a column following by the same letter do not differ significantly by the Student-Newman-Keuls test ($P = 0.05$)

Two varieties of the pathogen were responsible for recent outbreaks, *V. fungicola* var. *fungicola* in Europe and var. *aleophilum* in USA and Canada. Previous experiments (Juarez del Carmen *et al.*, 2002) carried out in small closed cells to avoid dissemination of spores, have shown that the var. *fungicola* isolate VCTC produced less numerous bubbles than the var. *aleophilum* isolate V-35. Breeding for resistance to *V. fungicola* implies to identify *A. bisporus* strains resistant or highly tolerant to both varieties. It was with this aim that cultivars and wild strains were compared at PPO MRU facilities for their susceptibility to three var. *aleophilum* isolates collected during the 1999 outbreak in Pennsylvania and two var. *fungicola* isolates responsible for the disease in The Netherlands.

Significant differences in *A. bisporus* susceptibility and *V. fungicola* aggressiveness were detected by analysis of variance, and no significant interaction occurred between *A. bisporus* strains and *V. fungicola* isolates (Table 6). Consequently, percentages of affected mushrooms shown on Table 7 are means of data from the three cell-tests.

Table 6
Analysis of variance for the various symptoms

Variable	*Source*	*df*	*Mean square*	*F value*
S	*A. bisporus*	8	223.08	12.96 **
	V. fungicola	5	319.63	18.57 **
	*A. bisporus * V. fungicola*	40	19.11	1.11 ns
	Cell test	2	10.03	0.58 ns
BO + DB	*A. bisporus*	8	678.89	26.17 **
	V. fungicola	5	339.48	13.09 **
	*A. bisporus * V. fungicola*	40	19.53	0.75 ns
	Cell test	2	108.45	4.18 *
S + BO + DB	*A. bisporus*	8	769.13	36.01 **
	V. fungicola	5	699.82	32.76 **
	*A. bisporus * V. fungicola*	40	24.37	1.14 ns
	Cell test	2	60.62	2.84 ns

** = significant at $P < 0.01$, ns = not significant at $P = 0.05$.

Table 7
Effect of the various isolates of *V. fungicola* on the susceptibility of *A. bisporus*

Treatment		*Percentages of*[1]		
		S	*BO + DB*	*S + BO + DB*
var. *aleophilum*	V02	3.97 a[2]	7.2 a	11.2 a
	V07	4.39 a	7.4 a	11.8 a
	V17	4.25 a	6.0 a	10.3 a
var. *fungicola*	V010404	1.31 b	5.5 a	6.8 b
	V9503	0.69 bc	3.2 b	3.9 c
Control		0.37 c	1.1 b	1.5 d

1. Mean percentages for all the *A. bisporus* strains.
2. Values within a column following by the same letter do not differ significantly by the Student-Newman-Keuls test ($P = 0.05$)

Table 8
Comparison of the susceptibility of the *A. bisporus* strains to both varieties of *V. fungicola*

A. bisporus	*% S*		*% (BO + DB)*		*% (S+BO + DB)*	
	/ var. fung.	*/ var.* aleo	*/ var.* fung.	*/ var.* aleo	*/ var.* fung.	*/ var.* aleo
A15	0.5 b[1]	6.4 a	13.5 a	17.1 a	14.0 b	23.5 a
U1	2.1 b	9.8 a	12.9 a	17.4 a	14.9 b	27.2 a
W1	0.1 a	1.0 a	2.3 a	3.4 a	2.4 a	4.4 a
W2	1.0 a	2.6 a	0.5 a	1.1 a	1.5 a	3.6 a
W3	1.9 a	2.4 a	1.1 a	3.2 a	3.0 a	5.5 a
W4	1.6 b	8.7 a	2.7 a	3.0 a	4.3 b	11.7 a
W5	0.7 b	2.5 a	2.6 a	1.0 a	3.3 a	3.5 a
W7	0.7 b	3.4 a	2.5 a	7.0 a	3.1 b	10.4 a
W8	0.4 a	1.3 a	0.8 a	5.1 a	1.1 a	6.4 a

1. For a same strain and a same symptom values following by the same letter do not differ significantly at $P = 0.05$.

When all the strains of *A. bisporus* were taken as a whole, they were more susceptible to the three var. *aleophilum* isolates than to the two var. *fungicola* isolates (Table 7).

Looking at the strains individually showed that all, except W5, were more susceptible to var. *aleophilum* than to var. *fungicola* isolates. The low percentages of affected mushrooms produced by W1, W2, W3 and W8 may explain why the differences in susceptibility related to the variety of pathogen were not significant. The percentage of bubbles was slightly but not significantly higher after contamination with var. *aleophilum* isolates. The difference in *A. bisporus* susceptibility to both varieties was mainly related to the propension of the var. *aleophilum* isolates to induce a greater production of spotted mushrooms. Considering the high aggressiveness of the *aleophilum* variety, wild strains highly tolerant to *V. fungicola* were detected in the PPO MRU collection (Table 8).

4. Conclusion

Strains of *A. bisporus* very tolerant to *V. fungicola* exist and can be introduced in breeding programmes but this work shows the importance of the use of

several *V. fungicola* isolates to assess the level of tolerance of future hybrids. Even the differences in susceptibility of the more tolerant strains to both varieties of the pathogen were not significant, the resistance of the selected hybrids to var. *aleophilum* isolates would be determined.

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Sequence Analysis and Expresion of a RecQ Gene Homologue from *Lentinula edodes*

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We cloned and sequenced a *recQ* gene homologue from *Lentinula edodes*. This gene, named *Le.recQ*, was found to have a coding capacity of 945 amino acids (aa). The deduced Le.RECQ protein was clearly smaller than other fungal RecQ proteins such as *Neurospora crassa* QDE3 (1955 aa), *Schizosaccharomyces pombe* Rqh1 (1328 aa), and *Saccharomyces cerevisiae* SGS1 (1447 aa). It exhibited the highest homology to the *Arabidopsis thaliana* RecQl4A protein (1182 aa) in its size and aa sequence. Northern-blot analysis showed that the *Le.recQ* gene is transcribed at similar levels during mycelial development in *L. edodes* fruiting-body formation. The *L. edodes* dikaryotic mycelial cells were found to contain a clearly larger amount of *Le.recQ* transcript than the *L. edode*s two compatible monokaryotic mycelial cells. Results in situ RNA-RNA hybridization showed that subhymenium and outer region of trama contain larger amounts of *Le.recQ* transcript. Expression of *Le.recQ* cDNA in *S. cerevisiae* might partially complement defects associated with the loss of its homologue *S. cerevisiae SGS1* gene.

1. Introduction

RecQ helicases, a group of DNA helicases with a remarkable sequence conservation within all seven helicase motifs first reported in *Escherichia coli* RECQ (Nakayama et al. 1884; Irino et al. 1986), are widely found in organisms from bacteria to human. Whereas in *E. coli* and yeast (Gangloff et al. 1994; Stewart et al. 1997) only one RecQ protein is present, five different RecQ homologues have been found so far in human (Ellis et al. 1995; Yu et al. 1996, Puranam et al. 1994; Kitao et al. 1998) and six different RecQ homologues in plant *Arabidopsis thaliana* (Hartung et al. 2000).

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RecQ helicases have been reported to be involved not only in recombination, as in *sgs1* mutants in *S. cerevisiae* and *rqh1* mutants in *S. pombe* and humans affected by Bloom (BLM) and Werner (WRN) syndromes, but also in re-initiation of replication following DNA damages, as found in *E. coli*, *S. cerevisiae*, human etc. (reviewed by Cobb et al. 2002 and Wu and Hickson 2002). *N. crassa* QDE3 has been shown to be involved in post-transcriptional gene silencing, as the first evidence of a new function for a DNA helicase (Cogoni et al. 1999). All these evidences are related to the fundamental genetic processes: replication, recombination, repair and transcription. Expression in different tissues of *A. thaliana* six *RecQ* genes has been analyzed by RT-PCR method, showing that the expression of *RecQl1*, *RecQl2*, *RecQl4A* and *RecQl4B* genes is higher in shoots and flowers than in leaves and seedlings, but the expression of *RecQl3* gene does not differ much between all examined tissues (Hartung et al. 2000).

Although the genomic DNA fragment containing recQ sequence (not entire recQ gene) has been isolated from Ustiligo maydis, belonging to protobasidiomycetes (Sanchez-Alonso et al. 1998), there is no report on isolation of recQ homologue from the eubasidiomycetes. This led us to attempt to isolate recQ gene homologue(s) from Lentinula edodes, one of the typical eubasidiomycetes from which we have previously isolated various genes and analyzed their functions (Hori et al. 1991; Kajiwara et al. 1992; Endo et al. 1994; Kondoh et al. 1995; Kaneko et al. 1998; Zhou et al. 1998; Kaneko and Shishido 2001; Akiyama et al. 2001; Nishizawa et al. 2002), and to attempt to study the expression in L. edodes of recQ gene homologue in the course of fruiting-body formation, in both vegetatively growing binucleate-celled dikaryon and uninucleate-celled monokaryon and also in hymenophore (gill tissue). We also attempted to express the recQ homologue in S. cerevisiae and study whether the recQ homologue complements defects associated with the loss of S. cerevisiae SGS1 (recQ homologue) gene.

2. Results

2.1. *Cloning and ucleotide sequence(nt) analysis of* Le.recQ *gene*

L. edodes genomic DNA was digested with *Bam*HI, *Eco*RI or *Hin*dIII and the resulting digests were put through Southern-blot analysis at higher

(65°C) and lower (58°C) temperatures using the probe of the PCR-amplified 0.7-kb *recQ* conserved sequence (Probe 1 of Fig. 1). A single signal was detected in all three digests and at both higher and lower temperatures: 9.0 kb for *Bam*HI, 3.2 kb for *Eco*RI, and 8.0 kb for *Hin*dIII. We cloned the 3.2-kb *Eco*RI-*Eco*RI fragment (Clone 1 of Fig. 1). The nt sequence analysis suggested that the cloned 3.2-kb fragment contains the sequences encoding all seven RecQ helicase motifs (I, Ia, II, III, IV, V, and VI)(see Figs. 1 and 2), but it lacks 5'-terminal coding and promoter regions of *Le.recQ* gene (see Fig. 1). To clone these missing sequences of *Le.recQ,* the following inverse PCR was carried out. The *L. edodes* genomic DNA was digested with *Sal*I, *Sph*I, *Xba*I, or *Xho*I, all of which cut the aforementioned 3.2-kb *Eco*RI-*Eco*RI fragment at a single site. The resulting digests were subjected to Southern-blot analysis using the ^{32}P-labelled 1.2-kb *Eco*RI-*Sal*I fragment (Probe 2 of Fig. 1) within the 3.2-kb *Eco*RI-*Eco*RI fragment. A single signal was detected in all four digests: 1.4 kb for *Sal*I, 6.4 kb for *Sph*I, 15 kb for *Xba*I, and 10 kb for *Xho*I. Based on these data, a restriction map was constructed as shown in Fig. 1.

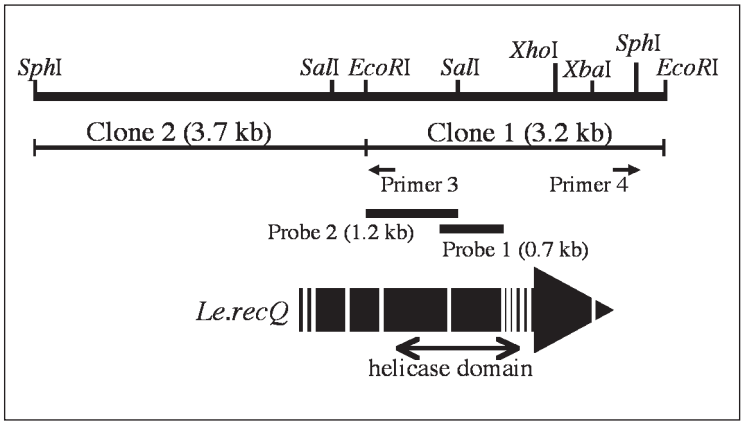

Figure 1. Restriction and gene maps of the region containing *Le.recQ* gene on the chromosome of *L.edodes. Le.recQ* gene is represented by arrow

The *Sph*I-digested *L. edodes* genomic DNA fragments were circularized by self-ligation and the resulting circular DNAs were subjected to inverse PCR using the primers 3 and 4, isolating the 3.7-kb *Sph*I-*Eco*RI fragment

(Clone 2 of Fig. 1). This 3.7-kb fragment was restriction mapped and sequenced. Based on the nt sequences of *Le.recQ* gene, we attempted to synthesize its cDNA by RT-PCR method using the total RNA prepared from *L. edodes* mature fruiting bodies. We succeeded in isolation of 3.2-kb cDNA sequences. The 6.9-kb genomic sequences (of 3.2-kb *Eco*RI-*Eco*RI fragment and 3.7-kb *Sph*I-*Eco*RI fragment) were compared with the cDNA sequences. Perhaps of the *Le.recQ* gene contains 3,396-bp coding region interrupted by 11 small (nt 49-59) introns and encodes 945 amino acids (aa). Putative transcription termination signal of AATAAA and the signal-like sequence of AATACAA were found between translation stop codon (TAG) and poly(A)-addition site.

2.2. *The transcription start point (tsp) of* Le.recQ *gene.*

To confirm the translation start codon of *Le.recQ* gene and analyze the structural feature of *Le.recQ* promoter, we determined the tsp of *Le.recQ* gene by primer extension method. The primer extension product of the *Le.recQ* transcript isolated from the *L. edodes* mature fruiting bodies gave a clear band at the position of 117-nt upstream of the suggested translation start codon (data not shown). The promoter region of *Le.recQ* gene contained a TATA-like sequence (TATACTAT) 40-nt upstream from the tsp, but not other eukaryotic (fungal) promoter consensus sequences such as GC-box, CAAT-box and CT-stretch.

2.3. *Comparison of the amino acid (aa) sequences of Le.RECQ and other RecQ proteins*

To determine the relationship between *Le.recQ* and other *recQ* genes, their derived aa sequences were compared (Figs. 2 and 3). *N. crassa* QDE3, *S. pombe* Rqh1, *S. cerevisiae* SGS1, *A. thaliana* RecQl4A, *E. coli* RECQ, Homo sapiens BLM, and Homo sapiens WRN consist of 1955, 1328, 1447, 1182, 610, 1417, and 1432 aa, respectively. Among these RecQ-type proteins, the *A. thaliana* RecQl4A was most homologous to the *Le.recQ* gene product, Le.RECQ (945 aa), in size. The RecQ-type helicases are known to have a

remarkably conserved helicase domain. So the aa sequences of the helicase domain of Le.RECQ were compared with those of other RecQ proteins. The *N. crassa* QDE3, *S. pombe* Rqh1, *S. cerevisiae* SGS1, *A. thaliana* RecQl4A, *E. coli* RECQ, H. sapiens BLM, and H. sapiens WRN showed 50%, 49%, 48%, 50%, 44%, 49%, and 36% identity to the Le.RecQ protein, respectively (Fig. 3). These data indicate that the *A. thaliana* RecQl4A protein is the most homologous to the Le.RECQ protein in its size and aa sequence (of the helicase domain). The RecQ-type proteins have been reported frequently to contain acidic aa-rich sequence(s) usually in their N-terminal region and C-terminal (to the helicase domain) conserved region. It was found that the Le.RECQ possesses both acidic aa-rich sequence and C-terminal conserved region.

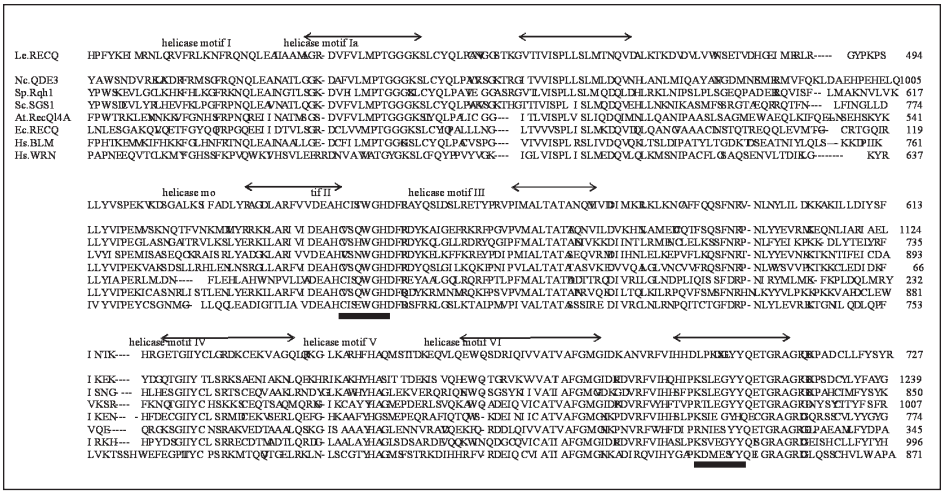

Figure 2. Comparison of the amino acid sequences of *L. edodes* RECQ (Le.RECQ) and other RecQ helicase proteins in their helicase domains

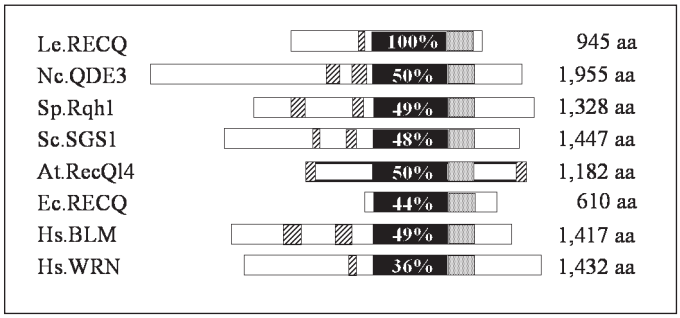

Figure 3. Schematic representation of members of the RecQ Helicase family of Le.RECQ, Nc.QDE3, Sp.Rqh1, Sc.SGS1, At.RecQl4A, Ec.RECQ, Hs.BLM and Hs.WRN. Acidic aa-rich sequences and C-terminal conserved regions are shown by striped bars and light-shaded bars respectively

2.4. *Transcriptional expression in* L. edodes *of* Le.recQ *gene*

Fruiting body was formed on saw-dust-corn bran medium. Total cellular RNA was isolated from preprimordial aggregated mycelia, primordia, immature fruiting bodies and mature fruiting bodies and subjected to Northern-blot analysis using ^{32}P-labelled probes of the PCR-amplified 0.7-kb *Le.recQ* conserved sequence (Probe 1 of Fig. 1) and the cDNA (1.2 kb) of *Le.ras*, which has been shown to be transcribed at similar levels during mycelial development in fruiting-body formation of *L. edodes* (Hori et al. 1991). The specific radioactivities of the two probes were almost the same. A single signal of 3 kb, corresponding to the size of *Le.recQ* cDNA, was detected in all RNA blots and the signal intensities were similar, though they were significantly weaker than those of the 1.2-kb *Le.ras* signals (Fig. 4A). The intensities of the *Le.ras* signals were similar in all RNA blots, ensuring an equal loading and transfer of RNA preparations. These results indicate that *Le.recQ* is constitutively transcribed during the fruiting-body formation of *L. edodes*, but the transcript levels are relatively low.

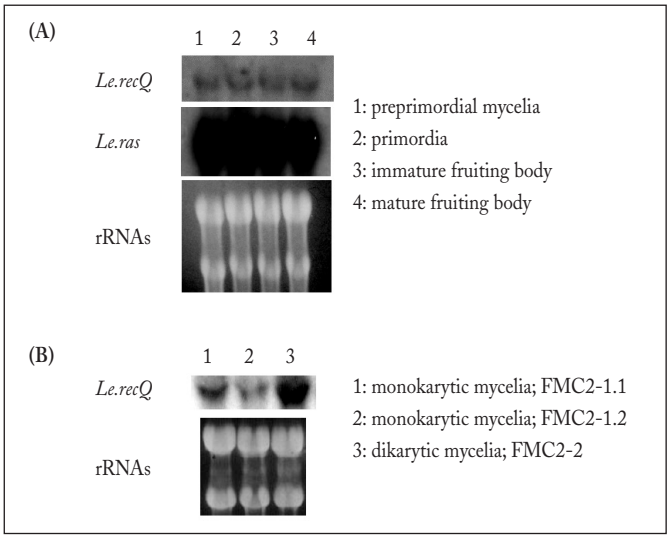

Figure 4. Transcriptional expression of *Le.recQ* gene in the course of fruiting-body formation (A) and in vegetatively growing dikaryotic and monokaryotic strains of *L. edodes* (B)

We investigated the transcript levels of *Le.recQ* gene in vegetatively growing mycelial cells of two compatible monokaryotic strains of FMC2-1.1 and FMC2-1.2 (Yasuda and Shishido, 1999) and dikaryotic strain FMC2-2 obtained by crossing FMC2-1.1 and FMC2-1.2. These strains were cultured in liquid SMY medium. As shown in Fig. 4B, FMC2-2 (lane 3) contained several times larger amount of *Le.recQ* transcript as compared with FMC2-1.1 (lane 1) and FMC2-1.2 (lane 2). It was also shown that FMC2-2 grown in the liquid medium with shaking (lane 3 of Fig. 4B) contains clearly larger amount of *Le.recQ* transcript than FMC2 (parental strain of FMC2-2) grown on the solid medium (lane 1 of Fig. 4A). These results suggest that *Le.recQ* gene might function much more actively in the binucleate-celled dikaryon in which growth is faster than the uninuleate-celled monokaryons (FMC2-2 grows approximately 1.3 times faster than FMC2-1.1 and 2 times faster than FMC2-1.2).

Quantitative RT-PCR analysis demonstrated that *Le.recQ* transcript is present under high density in hymenophore (gill tissue), which contains a large amoun of total RNA. Results in situ RNA-RNA hybridization showed

that subhymenium (on the top of which hymenium is formed) and outer region of trama (the region branching out into the suybhymenium) contain larger amounts of *Le.recQ* transcript (Fig. 5).

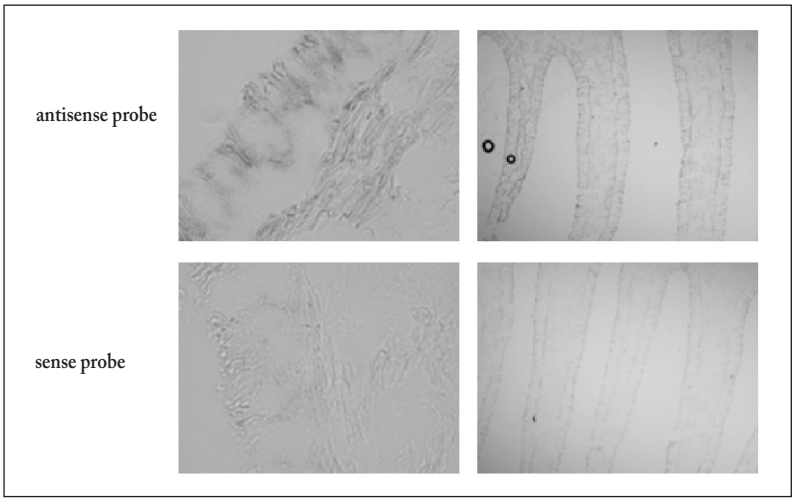

Figure 5. Expression of the *Le.recQ* gene in hymenophores of *L. edodes*

2.5. *Functional complementation of* Le.recQ *cDNA in* S. cerevisiae sgs1 *mutant*

S. cerevisiae possesses one *RecQ*-type gene, *SGS1*. The *sgs1* defective mutation causes *S. cerevisiae* a slight delay in growth and a sensitivity to DNA-damaging compound, methylmethanesulfonate (MMS) (Miyajima et al. 2000). We examined whether *Le.recQ* gene is able to complement these phenotypes of the *sgs1* mutant. *S. cerevisiae* 966NS-1 carrying the *Le.recQ* cDNA expression plasmid pYES2-Le.recQ, i.e., the 966NS-1[pYES2-Le.recQ] and the 966NS-1 carrying the *S. cerevisiae SGS1* expression plasmid pYES2-SGS1, i.e., the 966NS-1[pYES2-SGS1] were used for the experiments. The 966NS-1 carrying the vector pYES2, i.e., the 966NS-1[pYES2] was used as a control. The growth rates of these three strains were analyzed in the CM (without uracil) medium containing 2% raffinose and 0.2% galactose under the absence and presence of 0.001% MMS. The growth rate in the absence of MMS of the 966NS-1[pYES2-Le.recQ] was similar to that of the

966NS-1[pYES2-SGS1], and was higher than that of the 966NS-1[pYES2] (data not shown). In the presence of MMS, on the other hand, the 966NS-1[pYES2-Le.recQ] grew faster than the 966NS-1[pYES2], but slower than the 966NS-1[pYES2-SGS1] (data not shown). These results suggest that the *Le.recQ* cDNA can complement *sgs1* mutation of *S. cerevisiae*, but the complementation is not as efficient as that given by *S. cerevisiae SGS1* gene.

3. Discusion

As for the problem whether eubasidiomycete *L. edodes*, a multicellular filamentous fungus, possesses plural number of *recQ* gene, the following data appear likely to imply the presence of a single *recQ* gene homologue on *L. edodes* genome. Southern hybridization at higher (65˚C) and lower (58˚C) temperatures of *Bam*HI-, *Eco*RI-, or *Hin*dIII-digested *L. edodes* genomic DNA using the probe of 0.7-kb *recQ* conserved sequence gave a single signal. The 0.7-kb band in agarose gel of the PCR-amplified product was cut out from the gel and inserted into the pBluescript II vetor, followed by transformation of *E. coli*. Total 12 clones were selected and sequenced. The 10 fragments have an identical nt sequence of *Le.recQ* and other 2 fragments the nt sequences unrelated to *recQ* gene (data not shown). To verify the presence of a single *recQ* gene in *L. edodes*, other approaches, including a whole genome sequence analysis, are necessary. There exists a correlation between *Le.recQ* transcription level and growth rate of the mycelial cells. Efficient expression of *Le.recQ* gene is considered to be required for good growth of mycelial cells, implying a role in DNA replication.

A functional complementation test indicated that *Le.recQ* cDNA does complement slow growth phenotype of *S. cerevisiae sgs1* mutant, implying that *Le.recQ* play a role in DNA synthesize and cell divisions of the yeast. On the other hand, *Le.recQ* only partially complements the MMS-sensitivity of the *sgs1* mutant. The biological significance of *Le.recQ* gene in *L. edodes* totally remains to be determined.

4. Acknowledgements

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Genetic Variability of Flamulina velutipes Collections from Armenia

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Armenian collections of *Flammulina velutipes* were examined for DNA sequence variability within the ribosomal ITS1-5.8S-ITS2 region. Of 22 *F. velutipes* collections examined, several were heterozygous for two indels and were cloned to recover complete ribosomal ITS sequences. Genetic diversity was remarkably high in collections from Armenia when compared to collections from other parts of Eurasia. Haplotypes were assayed by phylogenetic analysis using maximum parsimony. At least 16 haplotypes were recovered (collections were defined as the same haplotype if they differed by no more than two base pairs). Most natural collections were heterozygous with respect to haplotype, suggesting an interbreeding, genetically divergent population of fungi. This high level sequence diversity may be a consequence of survival of ancient genetic variation in the Caucasus and in Armenia during periods of glaciation while in Europe, genetic variation was extirpated. A subset of Armenian genetic diversity is found in Europe.

1. Introduction

The genus *Flammulina* comprises several species commonly cultivated for food and for medicinal properties. Until 1970, *Flammulina* was thought to consist of a single species, *Flammulina velutipes,* which had a temperate Northern Hemisphere distribution (Buchanan 1993). *Flammulina* is collected throughout Europe as an edible, often in the winter, thus earning the epithet "the winter mushroom." and is widely cultivated in Asia as enoki-taki.

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The first species to be segregated from *F. velutipes* was *Flammulina ononidis* based on material from Germany growing on *Ononis spinosa* (Arnolds 1977). Bas (1983) proposed *F. fennae* and later (1995) summarized the known taxa of *Flammulina* in Europe as *F. ononidis*, *F. fennae* and *F. velutipes.* Based on morphology, Redhead and Petersen (1999) described *F. populicola*, *F. rossica* and a new combination, *F. elastica*. A New Zealand species, *Flammulina stratosa* was described (Redhead et al. 1998). Finally, *Flammulina mexicana* was assigned to a unique endemic growing at 9000' in the volcanic highlands of Mexico (Redhead et al. 2000). Petersen et al. (1999) examined crosses between and within these North Temperate taxa and identified mating groups as *F. velutipes*, *F. fennae*, *F. ononidis*, *F. populicola*, *F. mexicana*, and *F. rossica / F. elastica*. The latter two taxa are partially inter-compatible and inter-fertile but are separate morphological species. Morphospecies designations were confirmed by comparing DNA sequences for the ribosomal ITS1-5.8S-ITS2 (ITS) region using geographically diverse collections of each morphospecies where possible (Hughes et al. 1999). Following this, authentic *F. velutipes*, based on mating studies and DNA sequence information, has a pan-northern hemisphere distribution but is also found in Argentina, New Zealand and Australia where it is most likely an invasive species (Methven 2000).

The object of our study, *F. velutipes,* is a well-known medicinal mushroom. It contains different groups of active compounds (polysaccharides, protein-glucan complexes, sterols, lectins, phenolic compounds, etc) and substrate-specific enzymes. A large spectrum of pharmacological activity (immune-modulating, antitumor, antioxidant, etc.) and a number of medicinal properties have been attributed *F. velutipes*. Ethanol extracts of *F. velutipes* were reported to suppress hypersensitive immune responses such as inflammation in delayed allergy responses (Sano et al. 2002). Recently, a fruiting body protein from *F. velutipes*, Fve, was shown to possess immune-regulatory activity (Paavolainen et al. 2001; Wang et al. 2004). Polysaccharides (β-glucans) derived from putative *F. velutipes* have been shown in a number of studies to have significant anti-tumour activity (Yoshioka et al. 1973; Ikekawa 1995). Proflamin, a new antitumor glycoprotein was isolated from the culture mycelium of *F. velutipes* and was reported to be effective against the B-16 melanoma and adenocarcinoma (Ikekawa et al. 1982; Ikekawa 1995). Wine produced by fermentation using *F. velutipes* showed thrombosis-preventing activity, giving a prolonged thrombin clotting time

2.2-fold that of the control (Okamura 2001). Finally, antimicrobial compounds such as cuparene-type sesquiterpenes have been isolated from *F. velutipes* (Ishikawa et al. 2000; Ishikawa et al. 2001).

In our study of antibacterial activity of the cultural liquid of *F. velutipes* SBIII-2 and SB99 strains against *Staphyllococcus aureus* (209p) and *Salmonella typhimurium* (ATCC 1474), antiviral activity of fruiting body extract and obtained fractions against encephalomyocardial virus of mice have been observed (unpublished data). Antifungal/antagonistic activity of several mycelial strains against test-micromycetes pathogenic for men/animals (*Acremonium alternatum, Alternaria alternata, Aspergillus candidus, A. wentii, Chrysosporium keratinophilum, Fusarium tricinctum, Geotrichum candidum, Paecilomyces lilacinus, Penicillium aurantiogriseum, P. griseofulvum, Stachybotris chartarum, Verticillium lecani*), plants (*Bipolaris sorokiniana, Fusarium culmorum, Gaeumannomyces graminis* var. *tritici, Rhizoctonia cerealis*), as well as their antagonists (*Gliocladium roseum, Trichoderma viride, T. harzianum* and *T. pseudokoningii)* has been reported as well (Badalyan 2004). Antioxidant activity (AOA) of both mycelial and fruiting body samples of *F. velutipes* was detected (Badalyan 2003). Weak antiprotozoal activity (APA) and miotogenic effect (MGE) of cultural filtrate was also revealed (Badalyan and Sisakyan 2005). No APA was mentioned in mycelial and fruiting body extracts, as well as polysaccharide-protein fraction separated from fruiting body extract of *F. velutipes.* However, they were able to stimulate mitosis of *Paramecia* up to 2.2, 1.8 and 1.4 times, respectively.

The presence of APA, MGE and AOA completes the list of medicinal properties of in *F. velutipes* and makes it suitable for further development of new mushroom-based nutritional supplements with antiprotozoal, antioxidant and wound-healing properties. However, many reports concerning medicinal properties of fungi have not confirmed the species morphological identifications of their experimental materials, particularly mycelial cultures and have not retained voucher specimens. In many cases, incorrect species epithets have been used (unpublished data).

Screening of morphological and growth characteristics, fruiting body formation specificity in 21 different strains of *F. velutipes,* including Armenian collections, have been analyzed (Badalyan and Sakeyan, 2005). Two (A, B) species-specific morphological types of colony correlated with substrate nature and geographical origination of strains were described in *F. velutipes'*

collections. However, significant correlation between their genetic variability and colony morphology have not been revealed, yet (unpublished data).

We previously reported high genetic variability within the ribosomal ITS1-5.8S-ITS2 gene region in collections of *F. velutipes* from Armenia (Badalyan and Hughes 2004) when compared to collections from Europe and Asia. Here, we further explore this genetic variability using DNA sequences of the ribosomal ITS1-5.8S-ITS2 region.

2. Methods and Materials

Collections: Collections of *F. velutipes* used in this study are given in Table 1. Armenian samples were isolated from fruiting bodies collected in different part of country using a tissue culture method. To obtain polyspore cultures, a piece of the pileus from a fruiting body was suspended from the lid of a Petri dish and spores were deposited on malt extract agar (MEA: 15g/L Difco Malt Extract, 20g/L Difco Agar). Alternately, spores were deposited on paper and diluted in sterile water before plating on MEA. Cultures were stored on MEA slants at 10°C.

To obtain material for DNA extraction, dikaryotic cultures were grown in 30 mL PD broth (24 g/L Difco Potato Dextrose Broth) until the mycelial culture was ca. 2 cm in diameter. The culture was filtered through a fine mesh cloth and blotted to remove excess medium.

Approximately 0.3g tissue was ground in 750 uL Carlson lysis buffer (Carlson et al. 1991) and incubated at 75oC for 30 mins. Tissue debris was removed by centrifugation and the supernatant extracted with 750 uL chloroform: iaoamyl alcohol (24:1). The top layer was removed and measured, and an equal volume of isopropanol alcohol added to precipitate DNA. DNA was washed with 200 uL 70% cold ethanol, air dried and suspended in TE buffer. PCR amplification of the ribosomal ITS1-5.8S-ITS2 region was carried out with primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). Cycle parameters were as described by White et al. (1990). PCR products were visualized by gel electrophoresis in 1.5% TBE agarose gels. PCR products were sequenced with an automated ABI 3100 DNA sequencer (ABI Prism Dye Terminator cycle sequencing; Applied Biosystems, Foster City, California, USA) with primers ITS5, ITS4, ITS3 and ITS2 de-

pending on the position of insertions and deletions (White et al. 1990). Sequences of each gene were manually corrected and aligned using the SEQLAB program in the Genetics Computer Group package (GCG 2000). Some collections were heterozygous for several indels and were cloned to obtain an accurate sequence. Cloning was accomplished using the pGEM-T vector system) in JM109 high efficiency competent *E. coli* cells (Promega, Madison, Wisconsin, USA following manufacturer's directions. Colonies containing an insert were subcloned, plasmid DNA was extracted and PCR amplification was performed using primers ITS1F and ITS4 as described above. Products were analysed by gel electrophoresis as described above. Plasmids which contained the ITS insert were sequenced as described above.

Data Analysis: Maximum parsimony analysis was performed using Paup* version 10 (Swofford 2001). Gaps were few and were informative. For this reason, gaps were treated as a 5th base. One-hundred bootstrap replicates were performed. Trees were visualized in Tree View (Page, 1998). Because cloning may occasionally introduce an aberrant base, collections differing by 1-2 base pairs were considered to be the same haplotype.

3. Results

In previous studies, we attempted to determine the number of identical haplotypes which existed in Eurasia based on specific variable sites in the ribosomal ITS1 and ITS2 regions. As sequence data accumulated, it became clear that Armenian populations represented too much variability to be conveniently described by a discrete number of haplotypes. For this reason, parsimony analysis was used to group the haplotype sequences derived from individual collections. An unrooted phylogram representing relationships among haplotypes is given in Figure 1. Cloned ITS1-5.8S-ITS2 sequences of *F. velutipes* collections may appear in different clades. Cloned ITS sequences of collection SBI-2, collected near Abovian City are found in Figure 1, clades 4, 5, 6, 7 and 8. Base pair differences between sequences of SBI-2 are given in Figure 2. The SBIII-2 also collected near Abovian City appears in clade 6.

Cloned sequences of SBI'-4, collected near Kharberd appear in clades 9, 10 and 11 (Fig. 1). These clades are monophyletic, appearing on a common

stem. Cloned sequences of SBII-3 appear in divergent clades 3 (clone 12) and 7 (clone 11). Not all of the cloned haplotypes were recovered. Cloned sequences of SB99 appear in the same clade and the second haplotype, present in the dikaryon culture, was not recovered.

Collections from the Netherlands, the Ural Mountains (Russia) and England (the type location for *F. velutipes*) are together in a large clade together with Armenian haplotypes represented by SBII'-1, SBII-2 clone 10 and others but there is considerable genetic variability in the Armenian collections that is not present in the European collections. Clades 5 through 15 differ from European collections. A collection from France appears to be unique. Collections from China are most like SB99 from Armenia however, the Chinese collections represent a long divergent branch (data not shown).

4. Discussion

Ribosomal ITS sequences are maintained in a tandem repeat and are subject to recombination (Schlotterer and Tautz 1994; Hughes and Petersen 2001). Further, the ribosomal repeat is subject to an unknown homogenization process in which all copies of the ribosomal repeat become identical (Hillis, Moritz et al. 1991; Linares, Bowen et al. 1994; Schlotterer and Tautz 1994; Sanget al. 1995; Wendel et al. 1995; Cronn et al. 1996; Franzke and Mummenhoff 1999). The time required for homogenization is unknown but may be relatively quick, within a few generations (Hughes and Petersen 2001). In hybrids representing two different haplotypes, without recombination or homogenization, only two haplotypes should be recovered. Clones of collections from Armenia appear in more than two clades suggesting that; 1) the ribosomal repeat is undergoing recombination, and 2) following recombination, the ribosomal repeat has not completely homogenized. The cloning process will occasionally result in an aberrant base substitution. These can tentatively be identified by comparison to the parent sequence or absence of the "mutation" from all other sequences in a data set.

Cloning artifacts were identified in the data set but the observed natural genetic variability in cloned ITS sequences is in specific ITS1 or ITS2 variable regions and appears in several clones from different collections. In some instances, we were able to get corrected sequences without cloning. We con-

clude that cloning artifacts account for only a very small portion of the recovered sequence variability.

Only a subset of the observed genetic variability in Armenia is present in collections from Western Europe (represented by collections from Netherlands, Eastern Russia and England). One explanation for this genetic diversity is that Armenia was a refugium during periods of glaciation in the Pleistocene and that current genetic diversity in Armenia represents an old and divergent gene pool. Michaux et al. (2004) examined populations of the yellow-necked fieldmouse (*Apodemus flavicollis*) and demonstrated unique haplotypes in Turkey, the Near East and Middle East area. The authors suggested that *Apodemus flavicollis* survived glaciation in this region and was blocked from moving north after glaciation by the Caucasus and the Black Sea. Haynes et al. (2003) studied the common vole (*Microtus arvalis*) and demonstrated a unique genetic lineage in Armenia not present in Western European populations. They also suggested this region as a possible glacial refugium. The possibility that some of the observed genetic divergence in Armenia also may result from post-glacial migration into this region from other regions cannot be excluded. Another potential contributing factor could be rare hybridization between *F. velutipes* and other species of *Flammulina* leading to some level of introgressive hybridization. Studies by Petersen and Hughes have demonstrated that, while rare, interspecific hybridization is possible (Hughes and Petersen 2001).

The genetic variability of ITS sequences for Armenian collections of *F. velutipes* suggests that this is a region in which there is considerable variability, enhanced by interbreeding and recombination. Much of the genetic variability is not seen in western European populations. Regardless of the source of genetic variability, it would appear that Armenian collections offer a pool of unusual genetic variability that may be mined for medicinal purposes or used for strain improvement.

5. Acknowledgements

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Table 1
Collections of *Flammulina velutipes*

Culture Designation	*Country*	*Location*
SB F-1	France	Paris
SB R-9	Russia	Near Moscow
CBS 771.81	Netherlands	–
7200	England	London
9887	Russia	Ural Mountains
SB I-2 – Clone 1 – Clone 2 – Clone 3 – Clone 4 – Clone 8 – Clone 9	Armenia	Near Abovian City
SB I'-4 – Clone 31 – Clone 32 – Clone 33 – Clone 37 – Clone 46	Armenia	Near Kharberd
SB II'-1		
SB II-2 – Clone 9 – Clone 10		
SB II-3 – Clone 11 – Clone 12	Armenia	Yerevan
SB II-4 – Clone 29 – Clone 30		
SB III-2	Armenia	Near Abovian City
SB III'-3 – Clone 39 – Clone 40	Armenia	Near Kharberd

Culture Designation	*Country*	*Location*
SB IV'-1 – Clone 1 – Clone 8		
SB V-3 – Clone 41 – Clone 42	Armenia	Yerevan
SB VI-1 – Clone 13 – Clone 17	Armenia	Dilijan National Park
SB IX-1 – Clone 22 – Clone 27		
SB 10	Armenia	Yerevan
SB 20	Armenia	Dilijan National Park
SB 99 – Clone 37 – Clone 42	Armenia	Yerevan

Monstruosities under the Inkap Mushrooms

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Four different Inkcaps were isolated from horse dung and tested for growth on different medium. In addition to normal-shaped mushrooms, three of the isolates formed fruiting body-like structures resembling the anamorphs of *Rhacophyllus lilaceus*, a species originally believed to be asexual. Teleomorphs of this species were later found and are known as *Coprinus clastophyllus*, respectively *Coprinopsis clastophylla*. The fourth of our isolates also forms mushrooms but most of them are of crippled shape. Well-shaped umbrella-like mushrooms assigns this Inkcap to the clade *Coprinellus*. ITS sequencing confirmed that the first three strains and the *Rhacophyllus* type strain belong to the genus *Coprinopsis* and that the fourth isolate belongs to the genus *Coprinellus*.

1. Introduction

Inkcaps are a group of about 200 basidiomycetes whose mushrooms usually deliquesce shortly after maturation for spore liberation (see Fig. 1). Until recently, they were compiled under the one single genus *Coprinus*. However, molecular data divided this group into four new genera: *Coprinus*, *Coprinopsis*, *Coprinellus* and *Parasola* (Redhead et al. 2001).

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Figure 1. Mushrooms of *Coprinopsis cinerea* strain AmutBmut (about 12 cm in size) formed on horse dung, the natural substrate of the fungus. Left. Adolescent fruiting body at the stage of cap expansion. Right. Aging mushroom at autolysis

The edible, very tasty *Coprinus comatus* (Fig. 2) is the type species of the new genus *Coprinus* (corresponding to the former *Coprinus* subsect. *Coprinus* http://www. grzyby.pl/coprinus-site-Kees-Uljee/species/Coprinus.htm). Only two other species, *Coprinus sterquilinus* and *Coprinus spadiceisporus*, are known in this genus that clusters within the Agaricaceae. A typical characteristic of these species is a central cord suspended inside the stipe (Redhead 2000).

Figure 2. Young, not fully developed fruiting body of *Coprinus comatus* (Shaggy Mane, Laywer's Wig) with the typical ring at the stipe left from rupturing the veil at the start of expansion of the pileus (about 10 cm in size)

The three other newly defined genera all belong into the family of Psathyrellaceae. *Coprinopsis* forms the largest genus with more than hundred defined species (Hopple and Vilgalys 1999, Redhead et al. 2001, Keirle et al. 2004) and includes well known species such as *Coprinus cinereus* (Dungheap Inkcap, Dunghill Mushroom; Fig. 1), *Coprinus lagopus* (Woolly Inkcap, Hare's Foot), *Coprinus atramentarius* (Common Inkcap, Alcohol Inkcap) and *Coprinus stercoreus*, now termed *Coprinopsis atramentaria*, *Coprinopsis cinerea*, *Coprinopsis lagopus* and *Coprinopsis stercorea*, respectively (Redhead et al. 2001). These species scatter over the formerly defined *Coprinus* section *Coprinus* subsect. *Atramentarii* and *Lanatuli* and section *Veliformis* subsect. *Narcotici*, respectively. A typical character of the subsect. *Lanatuli* is an easily removed hairy veil composed of sausage-shaped elements. In contrast, species of subsect. *Atramentarii* have a scarce veil. Typical veil elements of the subsection *Narcotici* are persistent, warty cells and mushrooms have a strong gas smell (Orton and Watling 1979, Breitenbach and Kränzlin 1995). Other *Coprinopsis* species not listed here were grouped into *Coprinus* section *Coprinus* subsect. *Alachuani* or into *Coprinus* section *Veliformis* subsect. *Nivei* (http://www.grzyby.pl/coprinus-site-Kees-Uljee/ species/Coprinus.htm).

Coprinellus is the second largest group with more than 50 defined species (Redhead et al. 2001), most of which belong to the *Coprinus* section *Pseudocoprinus* subsect. *Setulosi*. Other members of the genus *Coprinellus* are found in the *Coprinus* section *Veliformis* subsect. *Domestici* and *Micacei*. Well known members of this genus are *Coprinus disseminatus* (Fairy Bonnet, Little Helmet), now *Coprinellus disseminatus*, from section *Pseudocoprinus* subsect. *Setulosi* and *Coprinus micaceus* (Glistening Inkcap), now *Coprinellus micaceus*, from section *Veliformis* subsect. *Micacei*, respectively. Species of the section *Pseudocoprinus* subsect. *Setulosi* have either no veil or very fine veils and hair-like structures (setulae or setae) on stipe and pileus. Veils of species of the section *Veliformis* subsect. *Micacei* are made up of small granular flocks and stipes may be smooth or pruinose due to surface covering by crystals (http://www.grzyby.pl/coprinus-site-Kees-Uljee/species/Coprinus.htm).
Fruiting bodies of *Coprinellus* species are often very delicate by a less fleshly cap and stipes are often brittle (Breitenbach and Kränzlin 1995).

Figure 3. *Coprinellus xanthothrix* fruiting body (about 5 cm in size) formed on beech saw dust

Parasola is the smallest of the three new genera within the Psathyrellaceae with currently 18 defined species. The type species is *Parasola plicatilis* (Pleated Inkcap), previously *Coprinus plicatilis* (Breitenbach and Kränzlin 1995, Redhead et al. 2001) grouped in the *Pseudocoprinus* subsect. *Glabri* like most other Parasola species. *Parasola auricoma* (formerly *Coprinus auricomus*) is the only species forming *Pseudocoprinus* subsect. *Auricomi* (http://www.grzyby.pl/coprinus-site-Kees-Uljee/ species/Coprinus.htm).

The new classification of the Coprinii agrees well with Ulje's *Coprinus* key based on macro- and micro-morphologies of fruiting bodies and spores (http://www.grzyby.pl/ coprinus-site-Kees-Uljee/species/Coprinus.htm). Nevertheless, species are often difficult to recognize beyond doubt and, most likely, many species are still not characterized. Next to mushroom and spore morphology, the habitat of a fungus can add to correct identification (Breitenbach and Kränzlin 1995). The Coprinii can be found growing on a wide variety of substrates, such as soil, dung of herbivores, living or dead wood, straw, leaf litter and other plant debris and organic litter. Mostly, the Inkcaps are fimicolous, meaning that they grow on dung. Here we present some isolates from horse dung with unusual fruiting body morphologies.

2. Materials and Methods

2.1. *Strains and culture conditions, microscopy and DNA techniques*

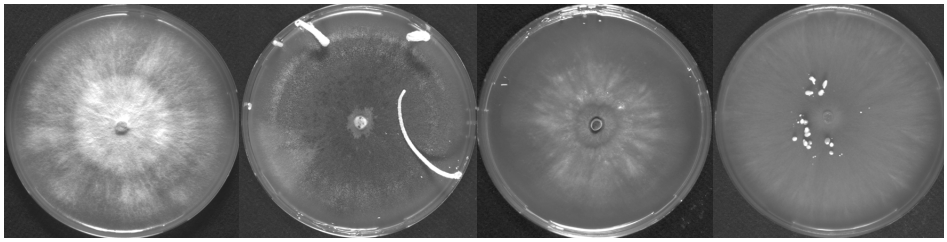
Four different *Coprinus* sp. isolates (sp. 1 to sp. 4) were obtained from horse dung from different localities close to Mainz, Germany (sp. 1 from Ober-Olm in year 2000; sp. 2 from Ginsheim-Gustavsburg in year 2003 and sp. 3 and 4 from Ingelheim am Rhein in year 2003). Isolates 3 and 4 came from the same collection of horse apples. *C. cinerea* strain AmutBmut (Swamy et al. 1984, Kertesz-Chaloupková et al. 1998) and *Coprinus xanthothrix* strain c144 (kindly supplied by Timothy James, Duke University) served in comparison of fruiting structures. The *C. clastophyllus* type strain was obtained from the CBS (473.70). Strains were cultivated on MEA (20 g malt extract, 1 g peptone, 20 g glucose, 10 g agar), solid YMG/T (Granado et al. 1997), solid corn meal-horse dung extract (HDE) according to Esser (2000), solid BSM (Hüttermann and Volger 1973) and sterilized horse dung either within a fungal growth chamber at constant 28°C in dark, ventilated boxes or in the laboratory at room temperature under normal day-night light conditions. Light microscopy was performed with a Zeiss Axiophot photomicroscope equipped with a Soft Imaging ColorView II digital camera. Lysomeres from caps were isolated with a scalpel and dispersed in distilled water. Samples were observed in light microscope at 20 x magnification. For observation of mycelia in the microscope, freshly grown agar pieces were squeezed on a glass slide. Aerial spores were harvested by pouring water over the surface of grown cultures. Genomic DNA was isolated by the protocol of Zolan and Pukkila (1986), ITS sequences amplified with primers ITS1 and ITS4 (Gardes & Bruns 1993) for sequencing at the Institute of Forest Genetics, Georg-August-University Göttingen.

3. Results and Discussion

3.1. *Culture and mycelial characteristics of the four newly isolated strains*

Cultivating on different agar media (YMG/T, MEA, BSM and HDE)

showed for all four new strains that HDE is best for mycelial growth in terms of speed of growth (Fig. 4). Strain sp. 1 was more dense and fluffy than the other three strains. Typical for strains sp. 2, sp. 3 and sp. 4 was thin growth within the agar in form of hyphal strands and very little or no aerial mycelium. Mycelia of the strains were observed. Strain sp. 1 had no clamp



cells at hyphal septa, unlike the three other strains (Fig. 5).

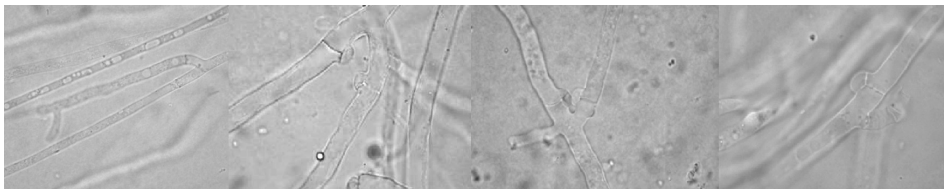


Figure 4. Colony morphology of isolates sp. 1, sp. 2, sp. 3 and sp. 4 (from left to right) on HDE medium grown at 28°C in the dark. Note the "etiolated stipe" structures on the plates of isolate sp. 2 (fully developed), sp. 3 (starting to develop), and sp. 4 (still growing)

Figure 5. Isolate sp. 1 had no clamp cells at hyphal septa in contrast to isolates sp. 2, sp. 3 and sp. 4 (shown from left to right)

Regularly in HDE cultures of strains sp. 2, sp. 3 and sp. 4, multi-cellular structures with long stipe and poorly developed caps appeared (Fig. 4) that

resembled etiolated stipes from *C. cinerea* strain AmutBmut (Fig. 6). Furthermore, cultures of the three isolates gave rise to white and black sclerotia (not shown). In the black forms, an outer melanized rind and an inner medulla were identified.

Figure 6. Etiolated stipes (also called dark-stipes) from *C. cinerea* strain AmutBmut that have an underdeveloped cap and an extra long stipe and form when the fungus has not enough light

3.2. *Fruiting abilities of the four new isolates*

In *C. cinerea*, etiolated stipes are formed when the fungus obtained a short light signal and then stayed in the dark. To correctly follow the sequence of developmental events leading to mature fruiting bodies with basidiospores, *C. cinerea* needs alternating light and dark phases synchronized to the normal day/night rhythm (Lu 1974, 2000, Kües 2000). Since the multi-cellular structures of sp. 2, sp. 3 and sp. 4 formed in the dark (Fig. 4) suggested that these came from aberrant fruiting body development possibly due to lack of light, new HDE cultures were incubated at room temperature on a bench in the laboratory under a natural day/night light regime.

Under the day/night light regime, production of multi-cellular, etiolated stipe-like structures with underdeveloped caps did not occur. Instead, the

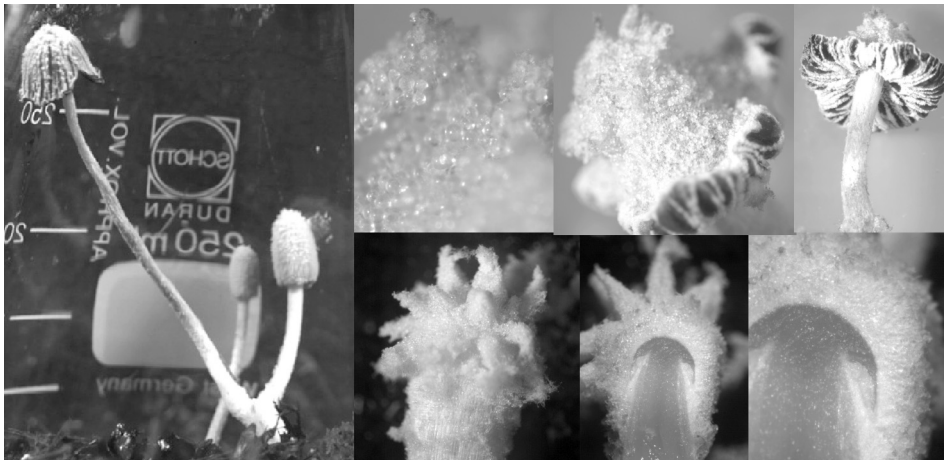


Figure 7. Fertile mushrooms of isolate sp. 4 on horsedung (photo at the left). At the right, upper panel of pictures: Masses of large round bodies are covering sterile fruiting-body-like structures of isolate sp. 4 (left). Transition structures form gills that partially carry basidiospores and are partially covered with large round bodies (middle). The more basidiospore, the less the amounts of large round bodies (right). At the right lower panel: Sterile fruiting-body like structures from the *Coprinopsis clastophylla* type strain are also covered by masses of large round bodies. The same structure is shown once intact (left) and longitudinal cut at two different enlargements (middle and right)

three strains regularly gave rise to many sterile fruiting body-like structures as well as some normal umbrella-shaped, fertile fruiting bodies carrying basidiospores (Fig. 7). Cultures obtained from germination of such basidiospores always gave rise to mycelium with clamps cells again able to form sterile and fertile fruiting structures (not shown). Cap tissues including the lamellae and partially the stipe of the sterile structures were covered by masses of large, round, glittering bodies as if cap and stipe have been dusted with icing sugar (Fig. 7). To different degrees, fertile structures had also such bodies (Fig. 7). Clemençon (1997) referred to unusual large round bodies on mushroom caps as lysomeres, Patouillard (1901) as bulbils.

Lysomeres observed by Clemençon (1997) and Reynders and Malençon (1969) contain many small cells and are probably different structures from what we see here. Lysomeres have been observed in the past in an anamorphic species forming sterile mushroom-like structures. This anamorph was originally called *Rhacophyllus lilacinus* (Berkeley and Broome 1871; Redhead et al. 2000). Later, an isolate of the species was observed to form also fertile

fruiting bodies. The sexual form was recognized as a *Coprinus* and the teleomorph obtained the name *Coprinus clastophyllus* (Maniotis 1964), respectively now described by Maniotis (1964) as *Coprinopsis clastophylla* (Redhead et al. 2001). The type strain of *C. clastophylla* was obtained from the CBS. This strain rarely formed fruiting bodies with basidiospores but very commonly sterile fruiting-body-like structures that resembled those of isolates sp. 2, sp. 3 and sp. 4 (Fig. 7). ITS sequencing confirmed the four strains to belong to one species.

We also observed the behavior of HDE cultures of isolate sp. 1 on the bench in the laboratory. This strain regularly produced many strangely shaped mushrooms of about 3 to 4 cm in size (Fig. 8). Usually, they carried basidiospores (Fig. 8) that germinated into monokaryons that constitutively produced oidia (Fig. 8). Typically, the stipe and often also the cap of the mushrooms split during development and often, inner stipe tissues shot up and appeared to pierce the cap during upward growth. As a result, mushroom caps look like "picked helmets". Amongst those and other misshaped mushrooms, there were always a few on a plate that look like a typical Inkcap (Fig. 8). These classically shaped mushrooms were delicate with thin, hollow stipes and fine umbrellas with little inner pileus trama. Brittle, hollow stipes and delicate caps are typical for *Coprinellus* species such as *Coprinellus xanthothrix* (Fig. 3). ITS sequences groups isolate sp. 1 into the genus *Coprinellus* (not shown).

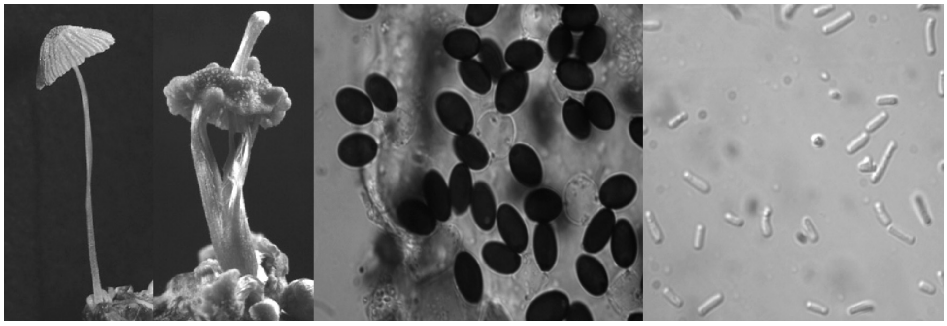


Figure 8. Classical umbrella-shaped mushroom of isolate sp. 1, a monstrous mushroom of the "picket

helmet"-type, basidiospores of isolate sp. 1 and oidia formed on monokaryons obtained from germination of basidiospores (from left to right)

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Overexpression of Laccases in *Coprinopsis cinerea*

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Laccases are versatile redox-enzymes that oxidize various phenolic compounds and aromatic amines. Because of the broad substrate specificity, these enzymes are of interest for many different biotechnological applications. White-rot fungi are the major source of laccases. Although basidiomycete species give rise to relatively high enzyme yields, these might not be optimal for applications. Basidiomycetous laccases have been reported to show hyper- or hypo-glycosylation when expressed in ascomycetes. In consequence, enzyme characteristics were found to be altered. Therefore, we use the basidiomycete *Coprinopsis cinerea* as an organism for laccase overproduction. We present a vector system for easy and rapid cloning of promoters and/or genes of interest and show that such constructs can be functional in laccase production.

1. Introduction

1.1. *Laccases: occurrence, structure and biological functions*

The family of multi-copper oxidases (Mco) includes laccases (1.10.3.2), ascorbate oxidases (1.10.3.3), ceruloplasmins (1.16.3.1), bilurubin oxidases, sulochrin oxidases, phenoxazinone synthases, ferroxidases, enzymes contributing to copper-resistance and a few yet not classified proteins (Messerschmidt 1997; Solomon et al. 1996; Hoegger et al. in preparation). Enzymes of this family catalyze the four electron reduction of oxygen to water. Laccas-

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es contain four copper atoms per monomer, which are bound to three distinct copper binding sites (mononuclear Type 1 and Type 2 sites and binuclear Type 3 site; Type 2 and Type 3 together form a trinuclear center). Type 1 Cu is the primary electron acceptor from the substrate. From there, electrons will be transferred to the Type 2/Type 3 cluster, where in two-electron steps the reduction of oxygen to water occurs (Claus 2003). Laccases, the enzymes we are studying, are phenoloxidases that catalyze oxidation of various mono- and di-phenols, ascorbic acid and aromatic amines (Messerschmidt 1997). Other enzymes oxidizing phenols are phenoloxidases are tyrosinases (1.14.18.1), heme peroxidases (1.11.1.7), manganese dependent and independent peroxidases (1.11.1.13) and lignin peroxidases (1.11.1.14). Laccases distinguish in phenoloxidase activity from the two-copper containing tyrosinases by lack of a hydroxylation reaction (cresolase activity). Different from peroxidases, laccase do not need hydrogen peroxide as cosubstrate (Messerschmidt 1997).

Laccases are widely distributed in plants and fungi and few are found in bacteria and insects. Plant laccases are believed to be involved in lignin synthesis (Gavnholt and Larsen 2002), whilst fungal laccases can participate in lignin degradation (Leonowicz et al. 2001). In confrontations with other organisms, laccases are produced as part of defense reactions (Baldrian 2004). Some of the fungal enzymes act in synthesis of melanin and other pigments (Langfelder et al. 2003; Pukkila-Worley et al. 2005). Others seem to have roles in fruiting body formation (Kües and Liu 2000; Wösten and Wessels 2005). Bacterial laccases function in formation of melanin-like pigments, in spore coat formation and in mediating copper tolerance (Endo et al. 2002; Roberts et al. 2002). In insects, laccases likely contribute to cuticle sclerotization (Dittmer et al. 2004).

1.2. *Laccases in industrial applications*

Apart from the broad range of biological functions, laccases are interesting enzymes for many biotechnological applications. By their wide substrate specificity, laccases have a great potential in water and soil bioremediation, for example in degradation of polycyclic aromatic hydrocarbons (PAH) and many other toxic phenolic and non-phenolic compounds (Johannes and Ma-

jcherczyk 2000; Mai et al. 2004). Laccases are very effective in degradation of recalcitrant dyes (Rodríguez et al. 1999, Svobodova et al. 2003). This property is of importance in clearance of effluxes from the textile industry (Kandelbauer et al. 2004) as well as in textile refining, for example in production of "stone-washed jeans" (Pazarlioglu et al. 2005). In addition in the textile industry, laccases might be used as bleaching agent to improve the whiteness of cellulosic fibers (Tzanov et al. 2003). In the pulp and paper industry, laccases are used in bleaching of pulp in order to increase the brightness and the tensile strength of the paper (Addleman and Archibald 1993; Chandra et al. 2004). Laccases have been shown to activate the natural binding forces of lignin, allowing production of medium-density fiber boards (MDF) and particle boards without addition of chemical adhesives (Hüttermann et al. 2001, Mai et al. 2004; Fig. 1). Potential applications in the food industry are in clearance of wine and juices and in enhancing the dough quality in bread baking and the softness of the bread product (Minussi et al. 2002). Laccases are employed in removing phenolic compounds from corks prior to bottling that otherwise would be released and spoil the tastes of wines during storage (Brenna et al. 1994). Laccases can serve as the measuring device in biosensors for detection of phenolic compounds in food, in pharmaceutical formulations and medical research, in waste waters and others (Gomes et al. 2004). Another interesting application of laccases is in the biofuel cell design (Heller et al. 2004).

Figure 1. Medium-density fiber boards (MDF) of 1 cm thickness produced with a raw laccase extract from *Pleurotus ostreatus* (left) and the MDF-pilot plant at the Institute of Forest Botany (right). Details of the laccase-MDF-production process are found in Kharazipour et al. (1997).

1.3. *Production of laccases*

White-rot fungi such as *Pleurotus ostreatus, Trametes versicolor*, and *Coprinopsis cinerea* are natural sources for laccase production (Table 1). Species differ in amount of laccase production (Table 1) and also different strains of a given species (Pelaez et al. 1995). Some species produce laccases in typical fungal growth media, e.g. MYPG containing yeast extract, malt extract, peptone and glucose (Nagai et al. 2002) or soy flour medium (Yaver et al. 1999). More commonly, high laccase production is induced by addition of toxic compounds such as copper or phenols (Palmieri et al. 2000, Terron et al. 2004) or sometimes by non-toxic but expensive vanillin (de la Rubia et al. 2002). Species may produce more than one laccase and individual laccases can be very different in their properties (Table 1). Often, fungal culture supernatants contain mixtures of laccase isoforms and enzymatic activities of different culture batches can vary according to the relative amounts of the different isoforms (Palmieri et al. 2000).

White-rot fungi are most efficient natural laccase producers, but for industrial applications enzyme yields are often not optimal. For example, 2-3 l of concentrated enzyme (8-10 IU/ml) per 10 kg fibre will obtain 1 m^2 of 1 cm-thick MDF boards with a density of 0.8 g cm^{-3} (Schöpper 2002, Schöpper and Rühl, unpublished). In developing the process, commercial available laccase (Novo SP504) or self-produced laccase from *T. versicolor* were used (Kharazipour et al. 1997). Our recent studies with *P. ostreatus* extracts show that enzymes from this species are also effective (Fig. 1A). However, whether enzymes of different sources are more suitable than others remains to be shown. On the market are enzymes from *Agaricus bisporus*, *Rhus vernificera*, *T. versicolor* and *P. ostreatus* (Minussi et al. 2002), indicating that there is an interest in obtaining enzymes from different sources and of different properties for various applications.

To overcome the various problems of laccase production with natural strains (too low enzyme yield, variable compositions of isoenzymes, production of enzymes with suboptimal properties, need for toxic and/or expensive inducers, poorly established fermentation processes, expensive growth media), laccase genes from white-rot fungi were overexpressed in heterologous ascomycetous hosts. Only in some of the cases, recombinant produced enzymes have been further characterized (see Table 2 for examples). Usually,

there are differences in glycosylation compared to the enzymes obtained from the native producer (Schneider et al. 1999, Yaver et al. 1996). Along with this, enzymes have altered properties e.g. in optimal pH and Km values (Sigoillot et al. 2004), making them less suitable for biotechnological applications. In other instances, properties of recombinantly enzymes have been described but data for the original enzymes are not available, e.g. for *Fome lignosus* Lcc, *Pleurotus sajor-caju* Lac4 (Table 2). More than one hundred laccase genes are identified from saprophytic and wood-rotting basidiomycetes (Hoegger et al. in preparation), but only very few of the corresponding enzymes have been identified and characterized. In turn, where specific properties of enzymes are known (e.g. *Trametes trogii*, *T. versicolor*; Garzillo et al. 1998, Claus et al. 2002, see Table 2), the corresponding gene has often still to be identified. From the high number of uncharacterized genes and from the available data on variations between the characterized enzymes, it is clear that the potential of different laccases for industrial applications is yet not exploited in the best possible way.

Table 1
Examples of laccase production in basidiomycetes and enzyme properties[1]

Enzyme	*Laccase activity (IU/ml) in cultutre supernatant[2]*	*Enzyme properties*				
		Mw (kDa)	*pI*	*Optimal pH*	*Optimal temperature (°C)*	*Km (mM)*
Agaricus blazei laccase	5.0	66	4.0	2.3	?	0.063
Ceriporiopsis subvermispora laccase L1	1.1	71	3.4	3.0	?	0.03
Coprinopsis cinerea Lcc1	?	63	3.7-4.0	4.0	?	?
Lentinula edodes Lcc1	1.4	72.2	3.0	4.0	40	0.1
Phellinus ribis laccase	?	76	?	5.0	?	0.207
Pleurotus eryngii laccase I	0.120	65	4.2	4.0	65	?
Pleurotus ostreatus						
POXA1	?	61	6.7	3.0	45-65	9.0
POXA2	?	67	4.0	3.0	25-35	1.2
POXC	?	59	3.3	3.0	50-60	2.8
POXA1b	?	61	6.9	?	?	4.7
POXA3a	?	67	4.1	3.6	35	7.0
POXA3b	?	67	4.3	3.6	35	7.4
Polyporus pinisitus laccase	?	65	3.0	4-5	?	?
Pycnoporus cinnabarinus laccase	18.0	76.5	3.7	?	?	?

1. Data have been taken from the following references: Claus et al. 2002; Eggert et al. 1996; Fukushima and Kirk 1995; Galhaup et al. 2002; Garzillo et al. 1998, 2001; Lomascolo et al. 2002; Min et al. 2001; Munoz et al. 1997; Nagai et al. 2002; Palmieri et al. 1997, 2003; Schneider et al. 1999; Ullrich et al. 2005; Xiao et al. 2003
2. For better comparison, values were converted into IU in cases, where in the original literature laccase activities (determined with ABTS) were given as nkat/ml or as arbitrary units.

Pycnoporus sanguineus GO5 laccase	71.0	70	3.5	?	?	?
Trametes AH28-2 laccase A	9.0	62	4.2	4.5	50	0.025
Trametes pubescens LAP2	15.5	65	2.6	3.0-4.5	50-60	0.014
Trametes trogii laccase	0.8	70	3.3-3.6	3.0-3.5	?	0.03
Trametes versicolor laccase	?	40	3.0	5.0	?	?

White-rot fungi usually have laccase multi-gene families. For example, four laccase genes have been identified in *P. ostreatus* and five non-allelic laccase genes in *Trametes villosa* and in *P. sajor-caju* (Giardiana et al. 1995; Yaver et al. 1996; Soden and Dobson 2001). Recently, we described eight non-allelic laccase genes from *C. cinerea* (Hoegger et al. 2004). Their deduced protein products contain all ten histidine and one cysteine residues spread over four highly conserved amino acid regions known as fungal laccase signature sequences L1-L4 (Fig. 2) that are needed for copper binding. Subsequently, from analysis of the *C. cinerea* genome established by the Broad Institute (http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/index.html), nine further non-allelic laccase genes were identified in this species (Kilaru et al., unpublished). On the protein level, only laccase Lcc1 has so far been described (Schneider et al. 1999; Table 1). Lcc1 is the main enzyme produced in liquid culture (Yaver et al. 1999). Designing laccase mutants from this enzyme with improved stability properties has been patented (Schneider et al. 2001).

Gene *lcc1* of *C. cinerea* has been overexpressed in *Aspergillus oryzae* and the recombinant enzyme was found to be hyperglycosylated (Schneider et al. 1999; Table 1, 2). Overexpression of laccases in basidiomycete hosts might not result in such problems. To date, the *Pycnoporus cinnabarinus lac1* and *Coriolus versicolor laccase III* genes were overexpressed in *P. cinnabarinus* and *C. versiocolor*, respectively (Alves et al. 2004, Kajita et al. 2004) but properties of the produced enzymes are not yet known. We now established *C.*

cinerea as a basidiomycete host for overexpression of homologous and heterologous laccases and developed a vector system for quick exchange of promoters and/or genes via *in vivo*-recombination in the yeast *Saccharomyces cerevisiae*. Unlike most other basidiomycetes, *C. cinerea* is very efficient in DNA transformation with up to 1000 transformants per µg DNA (Binninger et al. 1987; Granado et al. 1997). This allows easy screening of bulks of transformants (Granado et al. 1997) and will be of advantage at later stages, for example when screening randomly mutagenised libraries of specific laccase genes. The fungus grows fast in cheap liquid and solid media over a range of temperatures (20-42°C) with the optimum at 37°C, being of benefit for the use as a production organism.

Table 2
Examples of overexpressed basidiomycete laccases[1]

Host-Gene	*Laccase activity*		*Enzyme properties*			
	(IU/ml) in cultutre supernatant[2]	*Mw (kDa)*	*pI*	*Optimal pH*	*Optimal temperature (°C)*	*Km (mM)*
Saccharomyces cerevisiae						
- *°Trametes versicolor* lcc2	0.086	64	?	?	?	?
Pichia pastoris						
– *T. versicolor lcc1*	11.5	67	?	?	?	?
– *T. versicolor lcc4*	1.5	85	?	5.5	?	?
– *Fome lignosus lcc*	9.0	66.5	?	2.4	55	0.177
– *Pleurotus sajor-caju lac4*	?	59	?	3.3	?	2.5
Yarrowia lipolytica						
– *T. versicolor lac111b*	0.23	52	?	3.0	?	0.026
– *Pycnoporus cinnabarinus lac1*	0.44	90	?	?	?	?
Aspergillus niger						
– *P. cinnabarinus lac1*	11.0	70	3.7	4.0	65.0	0.055
Aspergillus oryzae						
– *P. cinnabarinus lac1*	8.0	70	3.5	4.5	65.0	0.055
– *Trametes villlosa lcc1*	3.0	60-70	3.5	2.7	?	0.058
– *Coprinopsis cinerea lcc1*	?	66	3.5	4.0	60-70	0.023

1. Data have been taken from the following references: Brown et al. 2002; Cassland and Jönsson 1999; Hong et al. 2002; Jolivalt et al. 2005; Liu et al. 2003; Madzak et al. 2005; Schneider et al. 1999; Sigoillot et al. 2004; Soden et al. 2002; Yaver et al. 1996.
2. For better comparison values were converted to IU in cases, where in the original literature laccase activities were given as nkat/ml or as arbitrary units.

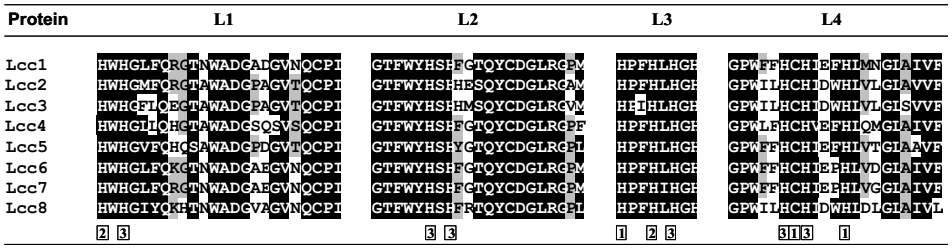

Figure 2. Fungal laccase signature sequences of *C. cinerea* laccases. The fungal laccase signature sequences L1-L4 were defined by Kumar et al. (2003) by comparison of 60 different enzymes. The sequences of the *C. cinerea* laccases are from Hoegger et al. (2004) where alignments of the whole proteins can be found. Numbers 1, 2 and 3 beneath the signature sequences refer to residues acting in copper binding at the Type 1, Type 2 and Type 3 sites of laccases, respectively (Piontek et al. 2002)

2. Materials and Methods

C. cinerea monokaryon FA2222 (*A5*, *B6*, *acu-1*, *trp1.1,1.6*; Kertesz-Chaloupková et al. 1998) was transformed by the protocol of Granado et al. (1997). *S. cerevisiae* RH 1385 (Mösch et al. 1990) was used for *in vivo*-recombination (Raymond et al. 1999), *Escherichia coli* XL1-Blue (Stratagene) for plasmid amplification. The yeast shuttle-vector pYSK2 (Kilaru et al., unpublished, Fig. 3) contains the yeast 2µm *ori* and *URA3* selection marker, the ColE1 *ori* (*ori Ec*) and amp^R from *E. coli*, the phage f1(+) *ori*, the *C. cinerea pab1* gene (for *para*-aminobenzoic acid synthesis; James et al. 2003) and the *C. cinerea lcc4* gene (Hoegger et al. 2004) under control of the *C. cinerea tub1* promoter (Cummings et al. 1999). pYSK2 can be used alone for transformation of *pab1* strains, but for selection of transformants in monokaryon FA2222, co-transformation with the $trp1^+$ vector pCc1001 (Binninger et al. 1987) is necessary. DNA work was performed by standard protocols (Sambrook et al. 2001). Promoter sequences and/or gene sequences were amplified with suitable chimeric primers from genomic DNA or plasmid subclones (Kilaru et al. unpublished). Recipes of *C. cinerea* media are given in Granado et al. (1997).

3. Results

3.1. *Cloning of promoters and/or genes into pYSK2 by in vivo-recombination in yeast*

The principle of directed cloning promoters and/or genes into vector pYSK2 is documented in Fig. 3. To exchange either a promoter or a gene, a one-step homologous recombination is sufficient. To replace both, promoter and gene, a two-step homologous recombination is required (see Fig. 3). For the latter, at least three chimeric primers have to be designed: i. a chimeric primer having at its 5'end a 30 bp homologous overlap to regions in the vector, e.g. to the *C. cinerea pab1* gene, linked to 20 bp of sequence for a promoter X fragment to be amplified by PCR; ii. a chimeric primer having at its 5'end a 30 bp homologous overlap to regions in the vector, e.g. to the f1(+) *ori*, linked to 20 bp of sequence for a gene Y to be amplified by PCR; iii. a chimeric primer covering the fusion point between a promoter X and a gene Y with a 30 bp extension at the 5' en into either the promoter sequences or the gene sequences, depending on whether the primer is designed for amplifying the gene or the promoter, respectively. A fourth primer required for amplification of the second DNA fragment of interest (either promoter or gene, respectively) does not need to be a chimeric primer but needs to allow amplification of the 30 bp homologous overlap to chimeric primer 3. Upon amplification of promoter X and gene Y by PCR, the DNA fragments are gel-purified and mixed with pYSK2 DNA for transformation into yeast. For suppression of background in yeast transformation by clones without new insert and to enhance recombination frequencies, pYSK2 is previously linearized by suitable restriction enzymes, for example through digestion with *BamH1* and *Kpn1* that cut in pYSK2 only in gene *lcc4* (see Fig. 3). The digested plasmid is purified by gel-electrophoresis. Transformants are plated on yeast selection medium. After two days, grown colonies are analyzed by colony PCR for presence of the expected construct. DNA from positive clones is isolated and transformed into *E. coli* for further plasmid amplification.

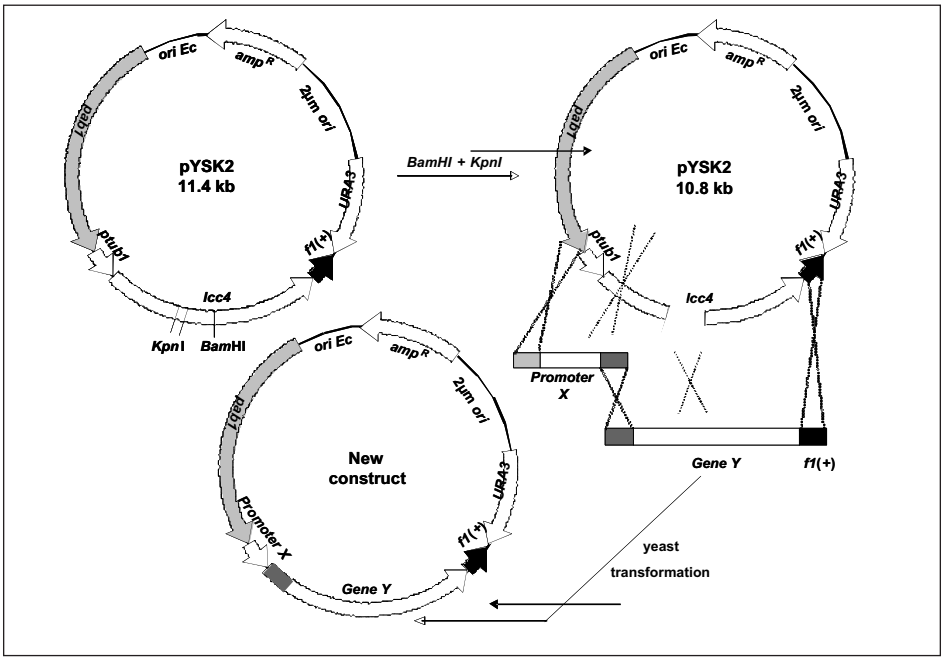

Figure 3. Yeast-shuttle vector pYSK2 and promoter and/or gene replacement strategy via homologous recombination in *Saccharomyces cerevisiae*

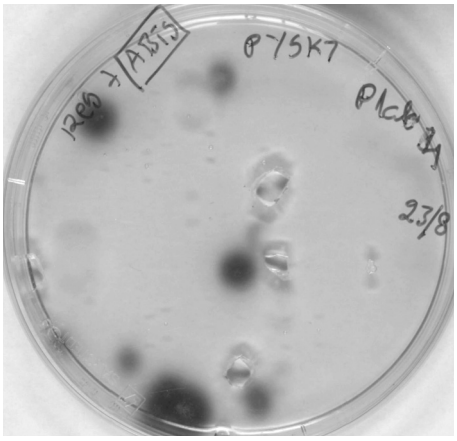

Figure 4. Laccase activity of transformants on regeneration medium plus 0.5 mM ABTS. The pYSK2 derived plasmid with a laccase gene was co-transformed into *C. cinerea* FA2222 protoplasts together with pCc1001 and plated on regeneration medium supplemented with ABTS. The green color around the colonies indicates oxidation of ABTS by secreted laccase.

3.2. Coprinopsis cinerea *transformation*

To test their efficiency in laccase production, pYSK2-derivatives are transformed together with pCc1001 into monokaryon FA2222. Upon transformation, protoplasts are plated onto regeneration medium with the laccase substrate 0.5 mM ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)]. Transformants producing functional laccase through activity of a constitutive promoter can be identified after 3 to 5 days of incubation at 37°C by green staining of the regeneration agar due to oxidation of ABTS (Fig. 4). When transferred onto YMG/T medium with 0.5 mM ABTS, constitutive expression of laccase by such transformants is seen throughout growth by staining the medium red-brown (Fig. 5). pCc1001 control transformants of FA2222 neither show staining activity on regeneration agar with ABTS (not shown), nor on YMG/T medium with ABTS (Fig. 5).

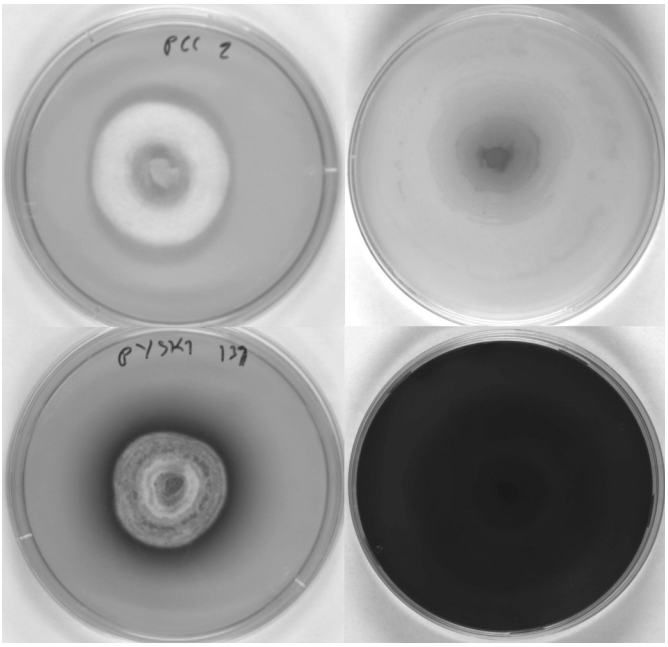

Figure 5. Overexpression of laccase during growth of a *C. cinerea* transformant on YMG/T complete medium plus 0.5 mM ABTS. Upper lane: pCc1001 control transformant at day 4 (left) and day 10 (right) of incubation at room temperature, respectively. Bottom lane: laccase transformant at day 4 (left) and day 10 (right) of incubation at room temperature, respectively.

4. Conclusion

Our study shows that we can express individual laccase genes in *C. cinerea* without other laccase activities in the background. We now can easily isolate individual enzymes for purification and biochemical characterization and, on a larger scale, for biotechnological applications. Enzymes can now be produced within this basidiomycete without the need to add any toxic or expensive laccase inducers. Our strategy for cloning promoters and/or genes via homologous recombination in yeast is simple and rapid. Several different promoters and/or genes from homologous or heterologous origin can be easily combined at the same time in parallel transformations.

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Heterologus Expression of Mating type Genes in Basidiomycetes

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Mating type genes in basidiomycetes encode two types of transcription factors (HD1 and HD2) and pheromones and pheromone receptors. Usually, mating type genes are so dissimilar in DNA sequence (allelic genes and genes from different species) that they do not cross-hybridize. In homobasidiomycetes, directly next to the *A* mating type locus encoding the transcription factors is a highly conserved gene *mip* that allows positional cloning. A candidate gene for positional cloning of the *B* mating type genes encoding the pheromone-pheromone receptor system is *cla4*/*ste20*. With more and more mating type loci cloned from different species, evolution of these loci and their genes can be addressed by sequence analysis and by function by transformation into other species, here *Coprinopsis cinerea*. Transformation of cloned mating type genes into heterologous hosts can lead to activation of mating type controlled development. Heterologous expression of mating type genes is especially interesting for species in which no transformation system exists. Since in *C. cinerea* an *A* null-mutant is available without functional transcription factor genes, self-compatibility of cloned *A* genes from homothallic species can also be tested.

1. Introduction

1.1. *Breeding systems in the basidiomycetes.*

An estimated 85-90% of all basidiomycetous species are heterothallic and need to undergo mating between two compatible monokaryotic strains for sexual development (karyogamy and meiosis) to occur. The remaining 10-

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15% of species are homothallic, i.e. self-compatible. These fertile homokaryotic strains undergo karyogamy and meiosis without mating to another strain. From such truly homothallic species, secondarily homothallic species have to be distinguished. Secondarily homothallic species carry two nuclei of opposite mating type in the basidiospores which germinate into fertile dikaryons (Whitehouse 1949, Quintanilha and Pinto-Lopes 1950).

Sexual development in heterothallic species is controlled by either one or two mating type loci. Species with one mating type locus are called bipolar, because two different mating type specificities segregate in the haploid progeny of a cross. These mating types correspond to the mating types of the parental strains of the cross. Species with two mating type loci are tetrapolar, and four different mating types are found among the progeny of a cross. Two of the four mating types are parental by passing on parental alleles at both mating type loci. The two alternate mating types are newly formed by recombination between the two mating type loci. Approximately 30-40% of heterothallic species are estimated to be bipolar and 60-70% to be tetrapolar (Whitehouse 1949, Quintanilha and Pinto-Lopes 1950).

In most cases, the single mating type locus of bipolar species are called *A*, the two mating type loci of tetrapolar species *A* and *B*. Usually, mating type loci in the basidiomycetes are multi-allelic. Different mating type specificities are indicated by numbers (*A1*, *A2*, *A3*, ...; *B1*, *B2*, *B3*...). In species with two mating type loci, every distinct *A* and *B* combination defines a specific mating type, i.e. *A1B1*, *A1B2*, *A2B1* and *A2B2* strains are all different in mating behaviour. Of these, *A1B1* and *A2B2* strains are compatible as are *A1B2* and *A2B1* strains. For a successful mating, fusing monokaryons need to be different at both mating type loci. In consequence, tetrapolar breeding systems promote outbreeding (Whitehouse 1949, Quintanilha and Pinto-Lopes 1950).

The *A* and the *B* mating type genes in tetrapolar species regulate different steps in sexual development explaining why both loci have to be different between two mates (for details see reviews by Casselton and Olesnicky 1998, Kües 2000, Kües et al. 2002a, 2004, Casselton and Riquelme 2005).

1.2. *The mating type loci in tetrapolar species*

Mating type loci have been cloned and functionally analyzed from the tetrapolar species *Coprinopsis cinerea*, *Schizophyllum commune* and *Ustilago maydis*.

The *A* mating type loci of *C. cinerea* and *S. commune* and the *b* mating type locus of *U. maydis* contain genes encoding two types of homeodomain transcription factors known as HD1 and HD2 proteins. Generally, to induce sexual development, an HD1 protein from one mate has to interact with an HD2 protein of the other mate (Fig. 1). In the simple case in *U. maydis*, there is one pair of divergently transcribed *HD1* and *HD2* genes (*bE* and *bW*) with about 25 different alleles. In a mating, there are therefore two compatible protein interactions: bE1-bW2 and bE2-bW1 (compare Fig. 1). However, one such protein combination is sufficient. In *C. cinerea*, there are at least three independently interacting *HD1-HD2* gene pairs. Each gene pair has a few alleles that are freely recombining with the alleles from the other gene pairs. This recombination generates the estimated number of 160 different *A* mating type specificities. For sexual development, it is, however, enough if mates carry different alleles at any one of the three possible *HD1-HD2* gene pairs. In *S. commune* with a high number of different *A* mating type specificities (>280), the situation is similar. So, far only one complete gene pair (*Aα* locus) has been characterized in this species and one non-allelic *HD2* gene (from the *Aß* locus) been cloned (for details see reviews by Hiscock and Kües 1999, Casselton and Olesnicki 1998, Casselton and Challen 2005).

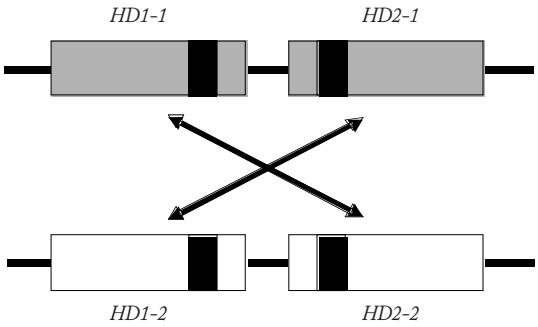

Figure 1. Schematic presentation of a mating type locus encoding HD1 and HD2 genes for homeodomain transcription factors. The simplest case with one pair of divergently transcribed genes are shown. Genes for proteins of the different classes of homeodomain transcription factors (HD1 and HD2) are specified by the first number. The second number defines the allele of a respective gene. DNA dissimilarities between alleles are indicated by boxes of different shading. The more conserved regions encoding the homeodomain DNA-binding motif are given in black boxes. The two possible functional interactions between products from allelic gene pairs are indicated by the arrowheads. These HD1-1/HD2-2 and HD1-2/HD2-1 interactions are redundant in function

The *B* mating type loci of *C. cinerea* and *S. commune* and the *a* locus of *U. maydis* contain genes for pheromones and pheromone receptors (Fig. 2). Pheromones and pheromone receptors of different specificity have to interact for induction of sexual development. In *U. maydis*, there are only two alleles of the *a* locus and each has a pheromone gene and a receptor gene. Three independent groups each comprising one pheromone receptor gene and up to three pheromone genes have been described in *C. cinerea*. Different combinations of the various alleles of these groups are expected to give rise to the 80 *B* specificities estimated to exist in nature. Two groups with each one pheromone receptor gene and up to 8 different pheromone genes are described for the *B* locus in *S. commune*. Similarly for this species, 80 different *B* specificities are expected worldwide to exist (for details see reviews by Casselton and Olesnicky 1998, Kothe 2001, Casselton and Challen 2005, Riquelme et al. 2005).

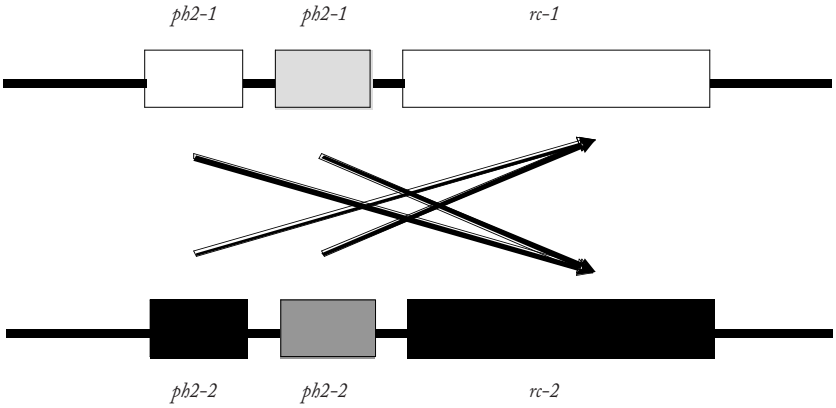

Figure 2. A hypothetical mating type locus encoding two different pheromones (Ph1, Ph2; second number = allele) and one pheromone receptor (Rc; second number = allele) is shown – in reality, there might be less or more pheromone genes and also genes unlinked to mating function that have been integrated into the locus by coincidence. Interactions of pheromones with the respective receptor from an allelic mating type locus are indicated by arrows

1.3. *Cloning mating type loci*

Alleles of mating type loci are very dissimilar in sequence so that they do not cross-hybridize. This feature has been used in cloning the *B* mating type

genes from *C. cinerea* by a genomic subtraction technique selecting unique DNA sequences for incorporation into a cloning vector (O'Shea et al. 1998). In other cases, as in cloning the *B* genes from *S. commune*, mating type genes were identified upon transformation through activation of developmental programs being under control of the mating type genes (Specht 1995). Chromosome walking from closely linked and easily to identify metabolic genes was applied in cloning *A* genes from both *C. cinerea* and *S. commune* (Giasson et al. 1989, Mutasa et al. 1989). Despite the reported homothallic behaviour of strains in *Phanerochaete chrysosporium* (Alic et al. 1987), mating type genes were identified in the genome sequence of this fungus (Martínez et al. 2004). All these approaches to obtain mating type genes are very laborious and often also difficult.

To understand the evolution of mating type loci and the different breeding systems in the basidiomycetes, genes from more species need to be cloned and analyzed. In higher basidiomycetes, a highly conserved gene for a mitochondrial intermediate peptidase (*mip*) is found directly next (< 1 kb) to the *A* mating type genes (James et al. 2004a). This gene has successfully been employed in positional cloning of *A* mating type genes from *Coprinopsis scobicola* (=*Coprinus bilanatus*; Kües et al. 2001), *Pleurotus djamor* (James et al. 2004b), and *Coprinellus disseminatus* (=*Coprinus disseminatus*; James 2003).

Fragments with *B* mating type gene sequences from *P. djamor* were initially identified through PCR using two pairs of degenerate primers, showing that such a PCR approach can be successful (James et al. 2004b). Another approach for isolating *B* mating type genes makes use of positional cloning. Previous studies indicated that in the ascomycete *Pneumocystis carini* (Smulian et al. 2001) and in the basidiomycetes *Cryptococcus neoformans* (Lengeler et al. 2002) and *P. chrysosporium* (James 2003) the p21-activated kinase gene *cla4*/*ste20* is closely linked to pheromone and pheromone receptor genes. The *cla4*/*ste20* gene was cloned from *P. djamor* and shown to reside at a distance of about 28 kb from to a pheromone gene and a pheromone receptor gene (James et al. 2004b). Close linkage between a *cla4*/*ste20* gene and the *a* mating type locus of *U. maydis* has now also been reported (Leveleki et al. 2004), and this syntenic arrangement is also conserved in *C. cinerea* as observed in the genome sequence (http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/). In conclusion, positional cloning through *cla4*/*ste20* appears to be a most promising strategy to obtain *B* mating type genes from many species.

1.4. *Functional analysis of cloned mating type genes*

Transformation systems that allow functional analysis of cloned potential mating type genes exist for only very few basidiomycetes. In the higher basidiomycetes, functions of cloned *A* and *B* genes from *C. cinerea* and *S. commune* have been tested in strains of the same species (for details see the reviews cited above). The first heterologous expression of *C. cinerea A* mating type genes was achieved in the closely related heterothallic fungus C. *scobicola* (Challen et al. 1993). Subsequently, function of *A* mating type genes from *C. scobicola* in *C. cinerea* was also demonstrated. *C. scobicola A* mating type genes are active with resident genes from various *C. cinerea* monokaryons (Kües et al. 2001; Fig. 3). Transformation experiments with heterologous

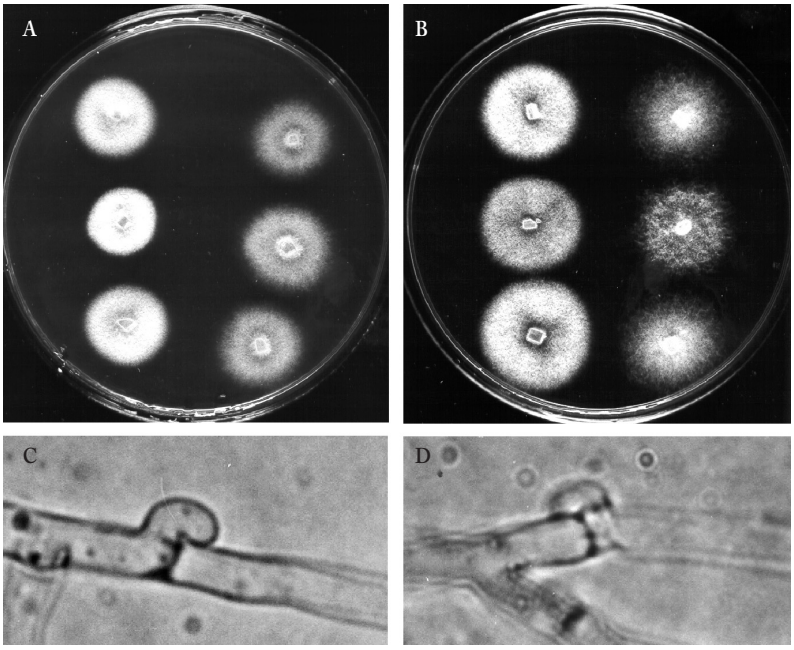

Figure 3. Expression of A mating type genes from a cosmid of *C. scobicola* in *C. cinerea* monokaryon LN118 (*A42, B42, ade-2, trp-1.1,1.6*) and FA2222 (*A5, B6, acu-1, trp1.1,1.6*). *A* and *B* show typical colony morphologies of densely grown monokaryotic clones (left) transformed with the *trp1*$^{+}$ plasmid pCc1001 (Binninger et al. 1987) and of less dense, more fluffy clones co-transformed with pCc1001 and a cosmid with A mating type genes from C. scobicola (right). *C* and *D* show unfused clamp cells found at hyphal septa in the A-activated transformants of LN118 and FA2222, respectively

genes from other species suggest that *C. cinerea* monokaryon 218 (*A3*, *B1*, *trp-1.1,1.6*) reacts best in tests with *A* mating type genes from foreign species (Srivilai, unpublished observation; James et al., in preparation). Different scenarios can be tested in strains that have endogenous mating type genes: the general function of an entire cloned *A* mating type locus within the heterologous species (Kües et al. 2001; Fig. 3) or the behavior of individual *A* genes and whether they interact with foreign *A* genes resident in the new host (Challen et al. 1993).

In *C. cinerea*, mutations in the *A* mating type locus are known that lead to self-compatibility. These mutations originate from large deletions of mating type DNA leading to in frame-fusions of an *HD2* and an *HD1* gene whose products normally do not interact. However, the product of the *HD2-HD1* fusion gene acts in the same manner as normal heterodimers of HD2 and HD1 proteins from different mating types. Activity but not self-compatibility of such fusion genes can be tested by clamp cell production in backgrounds of hosts with different mating type specificity. Self-compatibility can be shown by transformation into a wild-type self-background, i.e. by transformation into a monokaryon that carries the original unfused genes (Kües et al. 1994, Pardo et al. 1996). More elegant is the use of a *A*-null strain such as NA2 (Pardo 1995), where no resident *A* genes can interfere with action of the fusion gene (Fig. 4). The bipolar species *C. disseminatus* is found to have unlinked loci for homeodomain transcription factors and for pheromone genes and pheromone receptors, respectively, suggesting that one of these loci is either inactive or self-compatible (James et al., in preparation). Transformation into *C. cinerea* NA2 can help to clarify whether for example the locus for homeodomain transcription factors confers self-compatibility. Likewise, in strain NA2 one might test self-compatibility of genes for homeodomain transcription factors cloned from homothallic species. More-

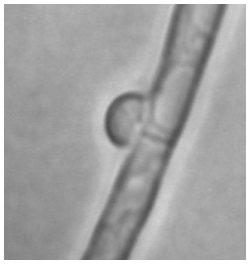

Figure 4. Clamp cell produced in *A* null mutant strain NA2 upon transformation with the self-compatible fusion gene *a2-1/d1-1* generated by deletion from an *A6* wildtype locus (Kües et al. 1994); photo by E. Polak.

over, co-transformation with *A* genes from two different mating types are possible in this strain enabling analysis of the interaction of two genes at a time (Kües et al. 1999).

Functionality of *B* mating type genes can be tested in *C. cinerea* by transformation into a monokaryon and subsequent mating to another monokaryon carrying the same resident *B* mating type locus but a different *A* mating type locus (O'Shea et al. 1998). In monokaryon 218, functional expression of compatible *B* mating type genes is also possible to recognize by a specific colony morphology on complete medium. Positive transformants show retarded colony growth, produce little aerial mycelium and irregular hyphal morphology, the so-called "flat" phenotype (Kües et al. 2002b). When simultaneously transformed with an *A* mating type gene of a specificity compatible to that already present in the monokaryotic strain, clamp cell production apical to a septum is induced by action of the *A* mating type genes and *B*-induced clamp cell fusion to the subapical hyphal cell can be observed. Usually in the mycelium of such transformants, more often than formation of fused clamp cells, *B*-regulated formation of subapical pegs is detectable on septa with non-fused clamp cells (Kües et al. 2002b, Badalyan et al. 2004; Fig. 5). Similar observations in monokaryon 218 can be made when transforming heterologous *B* genes into the strain (Srivilai, unpublished). A *B* null mutant from *C. cinerea* so far is unfortunately not available to test self-compatibility of cloned homologous and heterologous *B* mating type genes.

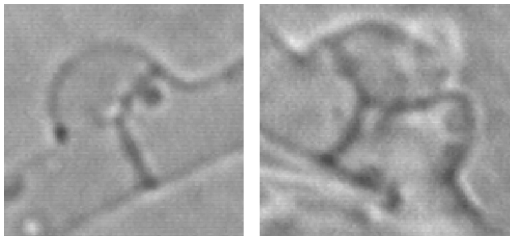

Figure 5. Fused clamp cell (left) and unfused clamp cell accompanied by a subapical peg (right) produced in monokaryon 218 upon co-transformation with compatible *A* and compatible *B* mating type genes.

2. Conclusions

Mating type genes have now been cloned from several different basidiomycete species. Cloning of mating type loci from further species is now

possible by positional cloning. The function of these mating type genes from other species can be addressed by transformation into *C. cinerea*. Our work showed that at least in some cases, foreign mating type genes are functionally expressed. Foreign gene products appear to interact with those from resident genes. Self-compatible *A* mating type genes and specific combinations of pairs of *A* mating type genes can be analyzed in an *A* null background of *C. cinerea*. For the first time, foreign *B* mating type genes are also seen to function in *C. cinerea*.

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Multiple Hydrophobin Genes in Mushroom

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Hydrophobins are small secreted fungal proteins that form amphipathic films on the hyphal surfaces. In the wood-rotting fungus *Schizophyllum commune*, four different hydrophobins are known with well established functions during vegetative growth and fruiting body development. Our study aims at elucidating the role of these proteins in wood penetration and lignocellulose degradation. Blast searches of the genome of the dung fungus *Coprinopsis cinerea* revealed a surprising number of 34 different hydrophobin genes in this species. Functional analysis of these genes is in progress.

1. Introduction

Schizophyllum commune, the Split-Gill Mushroom, is one of the most widely distributed wood inhabiting basidiomycete found throughout the tropical and temperate regions (Raper et al. 1958, James et al. 1999, James and Vilgalys 2001). The species is regarded as mild rot in temperate regions and severe wood destroyer in the tropical regions (Schmidt and Liese 1980). Most commonly, *S. commune* is found in nature on fallen trunks and branches of deciduous trees (Fig. 1, left), less often on wood of conifers (Cooke 1961, Breitenbach and Kränzlin 1991). Moreover, the fungus can act as a pathogen on standing trees (Latham 1970, Adaskaveg et al. 1993; Fig. 1, middle and right).

Genetics and Cellular Biology of Basidiomycetes-VI. A.G. Pisabarro and L. Ramírez (eds.)
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Figure 1. *Schizophyllum commune* fruiting bodies found on a fallen beech tree in the Billingshäuser Schlucht, Göttingen (left; May 2004) and on a living *Juglans ailantifolia* tree growing next to the GZMB (Göttinger Zentrum für Molekularbiologie) on the ground of the Georg-August-University Göttingen (middle; November 2004). Note that in spring 2005 the tree produced leaves on all healthy branches but not on branches infected with *S. commune* (right; May 2005)

S. commune is usually considered to be a white rot fungus. However, wood decay tests in the laboratory in most instances did not result in considerable weight losses (Hegarty et al. 1987, Nsolomo et al. 2000, Humar et al. 2001, 2002, Schirp et al. 2003). Schmidt and Liese (1980), Nilsson and Daniel (1983) and Leithoff and Peek (2001) measured between 1 to 6% weight loss for different strains and concluded the fungus is a rather weak wood-destroyer. In rare cases, mass losses of nearly 50% were observed mainly due to lignin degradation and to a low degree due to cellulose degradation (Hong 1982). Although there might be no weight loss in wood decay tests, toughness of wood can be negatively affected. 32% strength loss had been measured (Schirp et al. 2003). In flake board tests with *S. commune*, weight loss of 9.6% occurred together with strength loss (67.5% modulus of rupture loss; Hadi et al. 1995). A monokaryon of *S. commune* was shown to some extent to demethylate lignin, but this was not due to production of laccases, peroxidases or ligninases (Trojanowski et al. 1986). Poor lignin degradation in another study was thought to result from the inability of the fungus to solubilize lignin (Boyle et al. 1992). On wheat straw, some tropical isolates were shown to degrade lignin (up to 15%) and to cause simultaneous mass loss of up to 23.5%. Another strain showed even higher mass loss (26.7%) but no lignin decay (Capelari and Zadrazil 1997). Lignin in olive pomace was efficiently degraded by a *S. commune* isolate (up to 52.7% of lignin breakdown). During pomace degradation, high laccase activities were recorded (Haddadin et al.

2002). Many *S. commune* strains have xylanase and cellulose activities (Schmidt and Liese 1980, Clarke and Yaguchi 1986, Hegarty et al. 1987, Bray and Clarke 1995, Haltrich et al. 1995, Thygesen et al. 2003) and most of them have also laccase and peroxidase activities (Schmidt and Liese 1980). de Vries et al. (1986) described laccase activity specific to the dikaryotic state of *S. commune* whilst in the parental monokaryons, they could not detect this activity. Phenoloxidase activities were not found in other studies, also not upon treatment with phenolic inducers (Boyle et al. 1992, Nsolomo et al. 2000). By the well established xylanase and cellulose activities it has been suggested that the main role in nature is to recycle carbon by breaking down cellulose and xylans in fallen wood (Raper and Fowler 2004). In conclusion, the ability of *S. commune* to degrade wood remains to be a puzzle.

S. commune is used as a model fungus to study mating types and fungal development in basidiomycetes (Raper and Fowler 2004). Well known is the discovery of hydrophobins in this fungus, small-secreted fungal proteins of about 100 to 140 amino acids with 8 conserved cysteine residues. Upon secretion, hydrophobins self-assemble into amphipathic stable films that cover fungal cells and make their surfaces hydrophobic. Therefore, these films enable fungal structures to grow into the air and protect them from adverse environmental conditions. Due to the hydrophobin coating, aerial hyphae are repellent towards water (Fig. 2). Within mushrooms, hydrophobin films coat the air channels and prevent them from filling with water. In total, four different hydrophobins have been described in *S. commune*. SC3, the best characterized hydrophobin, is expressed in the vegetative mycelium of monokaryons and dikaryons. The other three, SC1, SC4 and SC6 are dikaryon specific. SC4 is low expressed in the mycelium and well in mushroom tissues. SC1 and SC6 are specific to fruiting stages (Wösten 2001, Walser et al. 2003).

It is not known whether hydrophobins participate also in wood colonization and pathogenicity of *S. commune* and in degradation of lignin as a hydrophobic component (Wösten et al. 1994). Is it necessary that the hyphae have a hydrophobic surface when growing in wood? Hyphae of the species have been shown to grow within the lumen of tracheids and vessels and attack the wood by loosening the S_3 layer from the rest of the wood cell walls before localized dissolution of cell wall substance results in narrow slits within the S_2 layer. Pronounced lamellation of the S_2 layer occurs in later stages of degradation and only then hyphae were found in the slits of the S_2 layer (Nilsson and Daniel 1983).

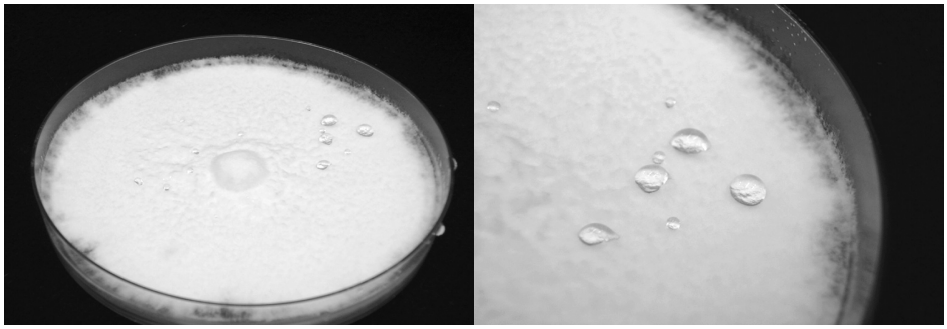

Figure 2. Aerial mycelium of *S. commune* monokaryon 4-39 is water-repellent due to the amphipathic SC3 film covering the surfaces of the hyphae

Another model fungus for studying development in basidiomycetes is the dung fungus *Coprinopsis cinerea* (Kües 2000, Kües et al. 2002, 2004). So far, only one hydrophobin (CoH1; Fig. 3) has been described in this species. CoH1 is expressed in the vegetative mycelium of monokaryons and, less efficient, of dikaryons. Although shorter in length than the *S. commune* hydrophobin SC3, the hydropathy plots of the two proteins are very similar (Fig. 4). A gene for another potential hydrophobin (CoH2) had been detected by sequencing on a 10 kb DNA fragment carrying gene *coH1* (Ásgeirsdóttir et al. 1997). Studies in our lab aim at further understanding roles of *S. commune* and *C. cinerea* hydrophobins in growth and development.

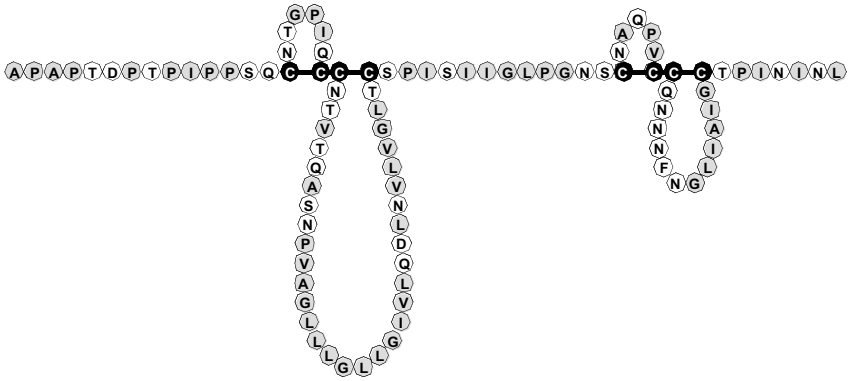

Figure 3. Model of *C. cinerea* hydrophobin CoH1 analogous to a *S. commune* SC3 model presented by Wessels (2000). The eight conserved cysteines (shown as white letters in filled black circles) are thought to interact to give the typical hydrophobin folding

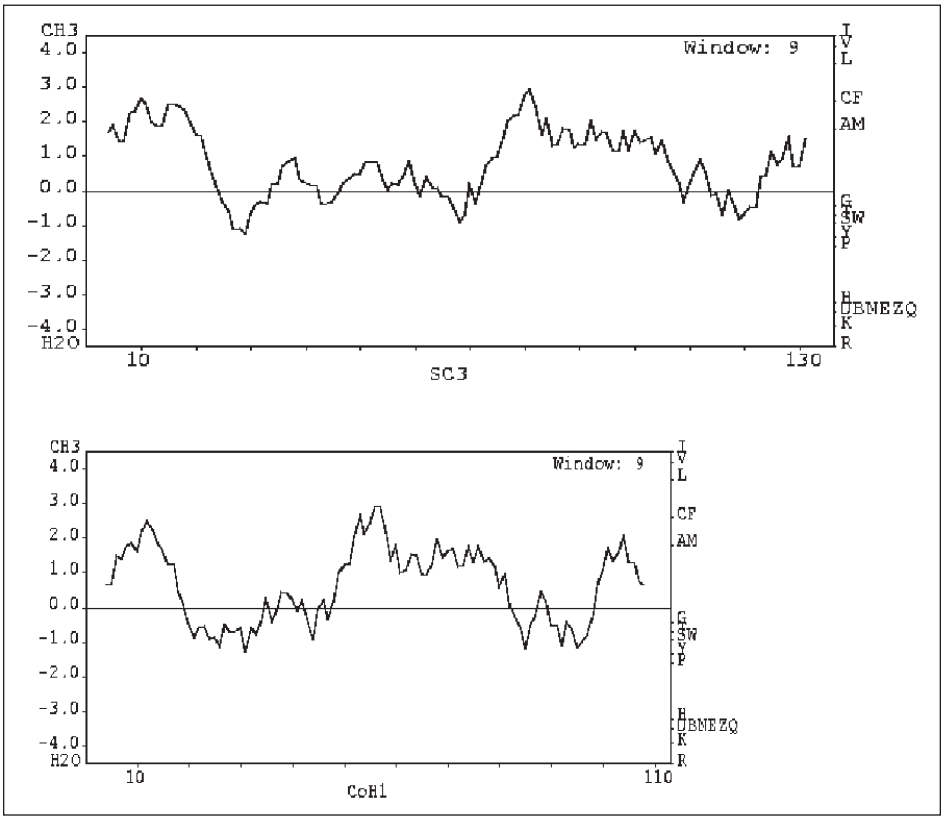

Figure 4. Hydropathy plots for *S. commune* hydrophobin SC3 (top) and *C. cinerea* hydrophobin CoH1 (bottom) calculated according to Kyte and Doolittle (1982)

2. Materials and Methods

2.1. *Strains, culture conditions and light and FTIR microscopy*

S. commune monokaryon 4-39 (*A41 B41*) was kindly supplied by Han A.B. Wösten. To infest wood, the strain was grown at 25°C in light on minimal medium (Dons et al. 1979). Sterilized beech wood blocks (3 x 1 x 0.5 cm) were placed onto 1 mm thick stainless steal grids layed on established mycelium to avoid direct contact between the wood and the agar. Wood blocks were incubated with the fungus for 20 weeks before sections of 25 µm

thickness were prepared with a microtome. Sections were transferred onto gelatin-coated glass slides, stained for 10 min with lactophenol blue (Esser 1976), dried at 60°C and cleaned with water. Coverslips were fixed on the samples with Depex (Serva Electrophoresis, GmbH, Heidelberg) and dried overnight with a weight placed on top of the cover slip. Samples were analyzed with a Zeiss Axiophot photomicroscope equipped with a Soft Imaging Color View II digital camera. For FTIR microscopy, mycelium grown on the surface of the wood blocks was air-dried. An FTIR spectrometer Equinox 55 in combination with an IR microscope Hyperion 3000 (Bruker Optics, Ettlingen, Germany) with a single channel MCT detector was used to record FTIR spectra of the mycelium on KBr windows (2 mm) in transmission mode with a 15 x Cassegrain-objective, knife edge aperture of 20 x 45 µm, resolution of 4 cm^{-1}, 16 scans (Naumann et al. 2005).

C. cinerea strain AmutBmut is a self-compatible homokaryon that forms fruiting bodies without prior mating to another strain (Boulianne et al. 2000) under standard fruiting conditions (Granado et al. 1997). Primordia and fruiting bodies were harvested for hydrophobin isolation following the protocol of Ásgeirsdóttir et al (1998). Isolated proteins were separated by SDS-PAGE (Garfin 1990).

3. Results

3.1. *Growth of* Schizophyllum commune *strain 4-39 in beech wood*

In sections of beech wood incubated for 20 weeks with *S. commune* strain 4-39, lactophenol-stained fungal hyphae were visible within vessels (Fig. 5). Signs for attack of the wood by the fungus were not obvious from optical inspection of the samples.

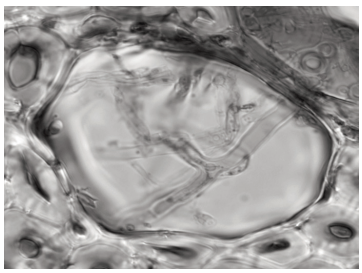

Figure 5. Lactophenol blue-stained hyphae of *Schizophyllum commune* strain 4-39 in a beech wood vessel

Wood and mycelium grown in and on the beech wood in unstained sections was analyzed by Fourier transform infrared microscopy (FTIR) microscopy (Naumann et al. 2005). FTIR microscopy allows local resolution of the chemical composition of a sample. The absorption of infrared light by dipolar molecular bonds of polysaccharides, proteins, lipids, aromatic and many other compounds results in a typical absorption spectrum for the sample (Fig. 6).

Spectra recorded by FTIR microscopy distinguish wood and mycelium from each other (Naumann et al. 2005). Spectra from mycelium grown within vessels are similar to each other (results not shown) as well as spectra from mycelium grown on the wood surface (Fig. 6). The two groups of spectra relate to each other. However, compared to mycelium within vessels, spectra of surface mycelium showed a more pronounced peak at 1640 cm^{-1} (Naumann et al. 2005). This wave length corresponds to the amide I band of peptide bonds (de Vocht et al. 1998). Purified SC3 hydrophobin, the most abundant protein on the aerial hyphae of *S. commune* 4-39 strain (Wessels et al. 1991, de Vries et al. 1993), has a characteristic peak at this wave length (de Vocht et al. 1998).

Next to wildtype strains, there exist *SC3* knockout strains of *S. commune* (van Wetter et al. 1996) that will be very useful in analyzing whether the fungus needs hydrophobins for growth in wood. By comparison, these mutants will be useful to establish in the FTIR spectra of *S. commune* wildtypes specific peaks corresponding to the hydrophobins. Knowing this, it might be possible to deduce whether detected differences in spectra of mycelium grown in and on wood result from differences in hydrophobin expression.

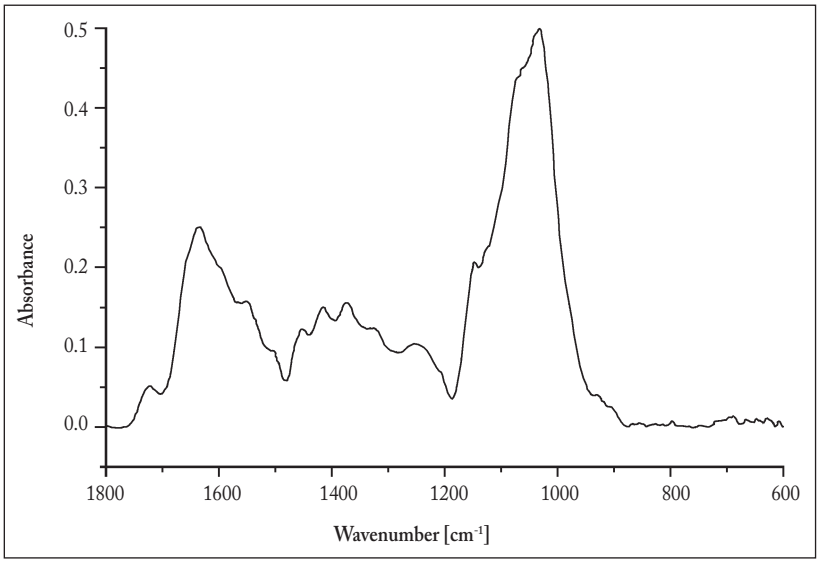

Figure 6. FTIR spectrum of mycelium of *Schizophyllum commune* grown on the surface of wood blocks, recorded with an FTIR microscope with MCT single channel detector. The spectrum has been baseline corrected. The arrow points to 1640 cm^{-1} where hydrophobin SC3 has a characteristic peak.

3.2. *Hydrophobins and hydrophobin genes in* Coprinopsis cinerea

Hydrophobins in *S. commune* and other basidiomycetes have important structural functions in mushroom development (Wösten 2001, Walser et al. 2003). Since in *C. cinerea* nothing was known on hydrophobins in mushroom development, by standard protocols (Ásgeirsdóttir et al. 1998) we tried to isolate such proteins from young fruiting bodies from homokaryon AmutB-mut (stages of rapid stipe elongation and basidiospore pigmentation). However, we were unable to bring hydrophobins properly into solution that were isolated from such late stages in fruiting body development. Such probes were contaminated with melanin that probably was produced for spore blackening in the mushroom maturation process. Since not properly been dissolved, on SDS gels such hydrophobin samples caused smear (Fig. 7). In contrast, we could solubilize hydrophobins isolated from earlier stages of primordia development prior to spore formation and separate them on SDS gels (Fig. 8). Multiple hydrophobin bands were visible ranging in sizes from

10 to 15 kDa, which is the typical size for this class of proteins (Wösten 2001, Walser et al. 2003) with the only exception of *S. commune* SC3 that is a 24 kDa protein (Wösten et al. 1993).

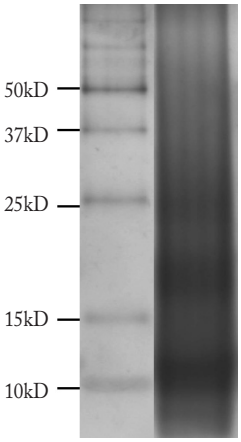

Figure 7. 15% SDS-PAGE showing poorly dissolved hydrophobins from young fruiting bodies of *Coprinus cinerea* homokaryon AmutBmut in a dark smear (right lane). At the left, molecular size marker

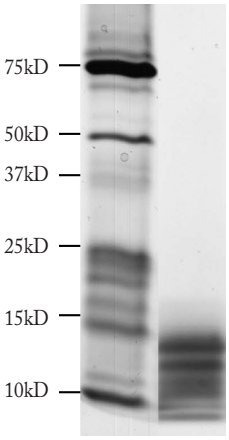

Figure 8. 15% SDS-PAGE showing well dissolved and several well separated hydrophobins from primordia of *Coprinus cinerea* homokaryon AmutBmut (right lane). At the left, molecular size marker.

With the release of the genomic sequence of *C. cinerea* strain Okayama 7 by the Broad Institute (http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/), we were able to Blast search the genome for hydrophobin genes and found the amazing total number of 34 different genes. All potential gene products contain the eight conserved cysteines involved in protein folding (Fig. 3) and their hydropathy plots resemble those of *S. commune* SC3 and *C. cinerea* CoH1 (Fig. 4). Expression studies of the different *C. cinerea* genes will have to verify whether they are all functional or whether there are pseudogenes amongst them. The protein gel shown in Fig. 8 supports that many of them will be functional.

The situation of multiple genes as in *C. cinerea* appears not be uncommon in higher basidiomycetes since in the genome of the wood-rotting fungus *Phanerochaete chrysosporium* (Martinez et al. 2004), we found 20 different hydrophobin genes by Blast searches. Whilst four different hydrophobin genes are currently known in *S. commune* (Wessels et al. 1995), also this fungus may have many more than is so far evident from experimental work.

4. Conclusion

Basidiomycetes can have many different hydrophobins. Work in *S. commune* showed before that hydrophobins are differently expressed and have been evolved for different development functions (Wösten 2001, Walser et al. 2003). So far, it is not known whether hydrophobins are needed for growth in and degradation of lignocellulosic substrates such like wood and straw. In *C. cinereus*, we found the amazing number of 34 different hydrophobin genes and it will be a demanding task to determine all their functions in growth and development.

5. Acknowledgements.

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Cell Wall-Associated Redox Enzymes in White Rot Fungi

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Many enzymes of white rot fungi involved in wood degradation belong to the class of redox enzymes. The most important are laccase (copper-containing polyphenol oxidase), lignin peroxidase, manganese-dependent peroxidase and manganese-independent peroxidase. However, the role of these enzymes in wood degradation remains unclear and complex redox processes or unknown redox enzymes also may contribute to this process. Several oxidative enzymes secreted by white rot fungi into the environment have been studied in the past, but little attention has been paid to the cell wall-associated redox enzymes. Cell wall-associated laccase activity in the purified cell walls of copper induced cultures of *Trametes versicolor* has been found. Laccases have been extracted by establishing new methods for cell wall purification and for protein release from the cell walls of basidiomycetes.

1. Introduction

The shape of a fungal cell is determined by the cell wall that functions to maintain the integrity of the cell, to protect the cell against the environment and to interact and communicate with the surrounding environment. The cell wall shields the cell against osmotic, chemical, physical and biological injuries. It is involved in growth and morphogenesis, in cell-adhesion and in cell-cell interaction. The cell wall is not inert but a robust, highly elastic and permeable structure (Cabib et al. 1988, Gooday 1995, Latgé and Calderone 2005, Sietsma and Wessels 2005).

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The main components of the fungal cell wall are α- and ß-glucans and chitin that form the cell wall skeleton. In addition, glycoproteins, chitosan, polyuronids, inorganic salts and pigments are found. The skeletal polysaccharides comprise approx. 80% of the cell wall dry matter whilst the protein content varies typically from 3% to 20% (Ruiz-Herrera 1992, Latgé and Calderone 2005, Sietsma and Wessels 2005). Numerous cell wall-associated proteins reveal enzymatic activities. Identified cell wall associated fungal exoenzymes fall into the following biochemical classes, respectively subclasses:

- oxidoreductases
- O-glycosyltransferases
- hexosyltransferases
- aminoacyltransferases
- phosphoric monoester hydrolases.

Of these, hexosyltransferases and O-glycosyltransferases were most often described in an extraprotoplastic location of the cell. The evidences presented for cell wall-association of particular enzymes generally were indirect, i.e., inferred from simple histochemical tests or immunocytochemical analyses or from genome analysis (Rast et al. 2003). Most studies concerning fungal cell wall enzymes were performed on ascomycetous yeasts and filamentous ascomycetes and a few on zygomycetes. Some specific cell wall-associated enzymes detected in these fungi are:

- ß(1-3)-glucanosyltransferase in *Candida albicans* (Hartland et al. 1991)
- acid phosphatase in *C. albicans*, *Aspergillus fumigatus* and *Botrytis cinerea* (Molloy et al. 1995, Weber et al. 1997, Bernard et al. 2002)
- phospho- and lysophospholipases in *C. albicans* (Chaffin et al. 1998)
- chitinase in *Saccharomyces cerevisiae, C. albicans* and *Kluyveromyces* sp. (Iranzo et al. 2002, Bahmed et al. 2002)
- ß-glucosidase in *C. albicans*, *Acremonium persicinum* and *Aspergillus kawachii* (Ram et al. 1984, Iwashita et al. 1999, Pitson et al. 1999)
- trehalase in *C. albicans* and *Neurospora crassa* and in the zygomycete *Mucor rouxii* (Hecker et al. 1973, Ram et al. 1984, Molloy et al. 1995, Lucio et al. 2000)
- ß(1-3)-glucanase in *C. albicans* and *A. persicinum* (Ram et al. 1984, Pitson et al. 1999)
- ß(1-6)-glucanase in *A. persicinum* (Pitson et al. 1999)
- N-acetylhexosaminidase in *C. albicans* and *M. rouxii* (Rast et al. 1991, Molloy et al. 1995).

Most of the cell wall glycoproteins are considered to be catalytically active (Mrsa et al. 1999). Therefore, many other enzymatic activities are expected to be present in the subcellular location of the cell wall that will have to be detected in future work.

In the basidiomycetes, there are some well characterized structural cell wall proteins such as hydrophobins (Wösten 2001, Walser et al. 2003, Peddireddi et al. 2005) and galectins (Walser et al. 2003, 2004, 2005). However, in comparison to ascomycetes much less is known about the enzymes residing in cell walls of basidiomycetes. Many cell wall associated enzymes will be responsible for basic reactions in cell wall synthesis (Latgé and Calderone 2005, Sietsma and Wessels 2005). Others will contribute to breakdown of growth substrates.

Particularly in the wood inhabiting wood, oxidative and hydrolytic enzymes for lignocellulose degradation might be expected to reside in the fungal cell wall. For example, presence of laccase has been demonstrated by coprecipitation of gold particles with a phenolic laccase substrate in the outer sphere of the hypha of *Pycnoporus cinnabarinus* (Jones et al. 1999). Various redox- and hydrolytic enzymes have also been localized by immuno-gold labeling to the cell wall in e.g. *Phanerochaete chrysosporium*, *Trametes versicolor*, *Rigidoporus lignosus*, *Phellinus pini*, *Lentinula edodes, Volvariella volvacea,* and *Phlebia radiata* (see review by Daniel 1994). In *P. chrysosporium*, presence of lignin peroxidase and manganese peroxidase (MnP) was detected both within degraded wood and on the surface of the fungal hypha (Ruel et al. 1991, Srebotnik et al. 1988). MnP secreted by *Ceriporiopsis subvermispora* whilst growing on wheat straw has been shown to be entrapped within the hyphal polysaccharide sheath due to direct interaction of the enzyme with polyglucan. Diffusion of the enzyme towards the lignocellulosic substrate was restricted to the extend of the hyphal polysaccharide network (Ruel et al. 2003). Laccases entrapped within the exopolysaccharide shields of *C. subvermispora* and *R. lignosus* hyphae and within the cell walls in *P. radiata* have also been reported (Nicole et al. 1992, Ruel et al. 2003). Furthermore, aryl-alcohol oxidase, pyranose oxidase, cellulases, glucanases and xylanase have been detected by gold-immunolabeling in association with the cell wall or the extracellular glucan sheath in wood degrading fungi (e.g. Barrasa et al. 1998, Cai et al. 1999).

Whilst immunolabelling proved to be very helpful for specific enzyme localization and monitoring of mobility within substrate, antisera prepared

against purified enzymes do not allow detection of unknown enzymes and may not respond to modified forms of the enzymes. Efficient and reliable methods for purification of cell walls and isolation of proteins from hyphal sheaths and from cell walls are therefore needed. In ascomycetes, commonly applied methods to obtain pure of cell wall fractions employ homogenization of the fresh material, followed by salt, detergent and/or enzyme treatment of the pure cell walls to isolated the associated enzymes or other proteins (Bruneau et al. 2001, Pitarch et al. 2002). In basidiomycetes, reports are so far few. From sporocarps of the edible basidiomycete *Agaricus bisporus*, a total of 19% of cell wall-associated phenoloxidase activity was liberated by NaCl extraction steps whilst up to 48% was set free by digitonin treatment. In this case, enzymes were obviously non-covalently bound to the cell wall (Sassoon and Mooibroek 2001). In the human pathogen *Cryptococcus neoformans*, cell wall-associated laccase activity retained in the cell wall after sequential extraction with salt, urea and SDS. However, about 40% of the activity was released from intact cells or cell wall fractions upon enzymatic treatment with glucanases suggesting a strong association of the enzymes to the cell wall carbohydrate via hydrolysable bonds (Zhu et al. 2001, 2003).

2. Materials and Methods

Trametes versicolor strain Nr. 6 from our institute collection, were cultivated on Basidiomycete-Medium (BSM, Hüttermann et al. 1973) with 1% agar. Liquid standing cultures were prepared by inoculation of 100 ml BSM medium in 500 ml flasks and cultivated for 14 days at 25°C in the dark. Laccase secretion was induced after 7 days of cultivation by 0.5 mM 2,5-xylidine (Fahreus et al. 1967) or 150 µM $CuSO_4$.

Mycelia were separated from liquid supernatant by filtration. Culture medium remaining after filtration of mycelium was centrifuged for 20 min at 3200 g and proteins precipitated with four volumes of 10% trichloroacetic acid (TCA) in acetone (w/v) and kept at -20 °C overnight. Protein pellets were separated by centrifugation at 16.000 g for 20 min, washed four times with acetone and air dried. Supernatant protein samples for native PAGE were concentrated using PES-Vivaspin concentrator 10.000 MWCO (Vivascience, Hannover, Germany). Mycelia were grinded in liquid nitrogen and

pure cell wall fractions were obtained after several washing steps and sorbitol gradient purification (Dwivedi et al., in preparation).

Laccase activities were determined at room temperature by monitoring the oxidation of 5.0 mM ABTS at 420 nm in 120 mM sodium acetate buffer pH 5.0 (Matsumura et al. 1986). One unit of enzyme activity (IU) represents the amount of laccase that oxidized 1 µmol substrate per minute. Amounts of total protein were determined by using Pierce Coomassie Plus Reagent (Perbio, Germany) using bovine serum albumin (BSA) as a standard.

For 2D-electrophoreisis protein samples were solubilised in loading buffer containing 8 M urea, 4% (w/v) CHAPS 50 mM DTT, 5% (v/v) Triton X100 and 0,67% (v/v) ampholyte buffer. In the 1st horizontal dimension, isoelectric focusing was performed using 18 cm IPG-strips pH range 3-10 (Amersham) according to the manufacture's protocol. In the 2nd dimension, proteins were separated on 12% PAGE using Ettan DALTsix (Amersham). Gels with native proteins were washed for 30 minutes with 100 mM sodium acetate buffer pH 5.0 and stained with 6.9 mM α-naphthol and 8.4 mM of N,N,N',N'-tetramethyl-p-phenylenediamine (TMDA) to visualize the laccase activity (Sterjiades et al. 1993). To visualize all proteins, gels were silver-stained (Blum et al. 1987). For peptide identification by LC-MS analysis (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=MIS), proteins spots were excised from gels and in gel-digestion with trypsin was performed at 58°C according to Havlis et al. (2003).

3. Results

In liquid culture with or without suitable inducers, *T. versicolor* secretes numerous extracellular proteins belonging to the class of redox oxidases (e.g. laccases) (Xiao et al. 2003), lignin-peroxidases, Mn-dependent and Mn-independent peroxidases (Collins et al. 1999), cellobiose dehydrogenases (Roy et al. 1996) and possible other enzymes important for lignocellulose degradation. Proteins in supernatants of standing BSM cultures of *T. versicolor* strain 6 were separated by 2D-electrophoresis. About 180 protein spots were detected. Most of them had a low pI-value and molecular weights in the range from 40 to 130 kDa (Fig. 1). Spots were eluted from gels, digested by

trypsin and analysed by LC-MS. Amongst other proteins, various peroxidases and a laccase were identified (Dwivedi et al., unpublished).

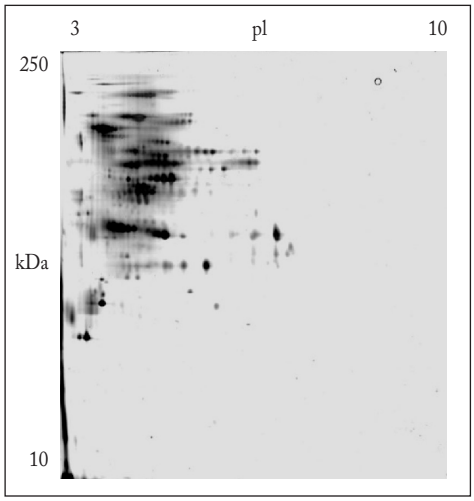

Figure 1. 2D-SDS-PAGE gel showing proteins from supernatants of standing *Trametes versicolor* BSM cultures at day 14 of cultivation

Laccase activities in the BSM culture supernatants were 42 ± 2.4 mU/ml. Laccase activity staining of 2D-PAGE gels showed a single spot at pI 3.5 ± 0.4 (not shown). Upon addition of 150 μM $CuSO_4$ and 0.5 mM 2,5-xylidine, laccase activities in culture supernatants raised to 260 mU/ml and 1.6 U/ml, respectively (Fig. 2). In 2D-gelelectrophoresis, both with copper and with 2,5-xylidine more protein spots were detected in silver-staining but laccase activity staining identified only the one spot known already from non-induced cultures (Fig. 3).

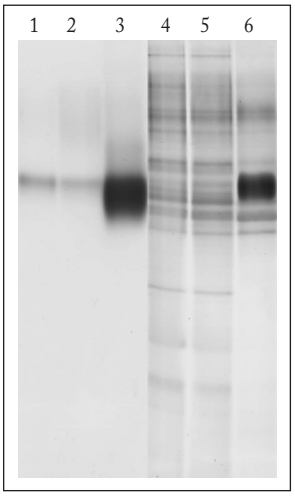

Figure 2. Native PAGE zymograms of laccases (lanes 1-3) and corresponding silver-stained proteins on 12 % PAGE (lanes 4-6). Equal sample volume of supernatant from 14 days-old *Trametes versicolor* cultures were loaded (1, 4: non-induced; 2, 5: copper-induced; 3, 6: 2,5-xylidine-induced)

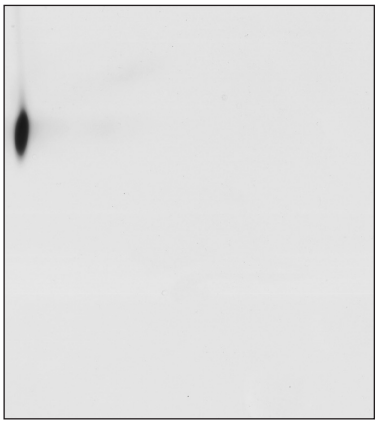

Figure 3. Native 2D-gel-electrophoresis of supernatant proteins from a 2,5-xylidine-induced, 14 days-old culture of *Trametes versicolor* stained for laccase activity

Laccase activity was also measured with mycelium of the cultures. Laccase activities per g mycelial fresh weight were 0.26 U, 0.60 U and 2.70 U in non-induced, copper-induced and 2,5-xylidine-induced cultures, respectively. In disrupted and washed mycelium, about a third of this activity remained in the cell wall fraction. Various buffers, salt concentrations, reducing agents, and cell wall-hydrolyzing enzymes were tested alone or in combination to extract the native enzymes. In the best case, 77% of enzymatic activity was

released from the cell walls. Two laccase activity bands were detected after separation of released enzymes by native 1D-gels, one of which seems to correspond to the enzyme found in large quantities secreted in the culture supernatant after 2,5-xylidine induction (Fig. 4).

Figure 4. Laccases from 2,5-xylidine-induced culture supernatant of *Trametes versicolor* (1) and isolated from the cell wall fraction of the mycelium from the same culture (2)

4. Conclusions

Our work shows that *T. versicolor* strain 6 secretes various oxidizing enzymes into culture media but also retains enzymatic activity in the cell walls. We have established protocols for cell wall purification and for release of enzymes from the cell walls of the species. Currently, we modify the protocols for optimal use in other basidiomycetes such as *Pleurotus ostreatus*, *Irpex lacteus* and *Coprinopsis cinerea*. Moreover, we proceed with biochemical characterization of the isolated cell wall-associated laccases from *T. versicolor*. Additionally, we characterize the enzymes in their natural cell wall bound form and compare the data with those obtained for laccases from the culture media.

5. Acknowledgements.

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Genetic analysis of *Coprinosis cinerea* mutants with defects in fruiting body development

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Few genes have so far been cloned and characterized in fruiting of the heterothallic mushroom *Coprinopsis cinerea*. Fruiting body development normally occurs on the dikaryon. However, the binucleate state of the mycelium hinders easy access of genes. Self-compatible mutants with defects in the mating type pathways can form fruiting bodies without prior fusion to another strain. Uninucleate haploid oidia of such mutants can easily be mutagenized and germinated mycelia tested for defects in fruiting. Mutants can be produced from oidia by classical techniques such as UV treatment or by modern REMI (restriction enzyme-mediated integration) mutagenesis via transformation. Such mutants of self-compatible strains have now been successfully appointed in cloning genes acting in sexual development. Co-isogenic strains of compatible mating types support in genetic characterisation of the mutants.

1. Introduction

1.1. *The wild-type life cycle*

Coprinopsis cinerea (formerly called *Coprinus cinereus*, Redhead et al. 2001) is an excellent model to study fruiting body development in the basidiomycetes. It easily grows in the laboratory and completes its life cycle (Fig. 1, Kües 2000) within two weeks on its natural substrate horse dung as well as

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on artificial substrates on yeast extract – malt extract – glucose basis (Walser et al. 2001).

The life cycle of the heterothallic *C. cinerea* starts with germination of basidiospores that contain one type of haploid nuclei. The resulting primary mycelia are called monokaryons. They have simple septa and one or sometimes two genetic identical haploid nuclei in their hyphal compartments. Monokaryons constitutively produce in high numbers single-celled, uninucleate haploid mitotic spores (oidia) on specialized aerial structures, the oidiophores (Polak et al. 1997, 2001, Kües et al. 2002a, Fischer and Kües 2003). As long as nutrients are available, monokaryons can grow indefinitely. In nature however, dikaryons are prevailing because as soon as they meet, monokaryons of different mating type will fuse to form this secondary mycelia. The dikaryon is characterized by a vigorous mycelium of usually faster growth compared to the parental monokaryons. It has two distinct haploid nuclei in the hyphal compartments (Iwasa et al. 1998) and clamp cells at the hyphal septa (Buller 1933, Badalyan et al. 2004). Under specific environmental conditions, fruiting bodies are formed on the dikaryon (Moore 1998, Kües 2000, Wösten and Wessels 2005). Oidia production on the dikaryon is repressed in the dark. In light, oidia are produced but in much lower numbers than on the monokaryons (Kertesz-Chaloupková et al. 1998, Kües et al. 2002b). The uninucleate haploid oidia are short-lived and serve in distribution of the species to new substrate and as fertilizing agent in

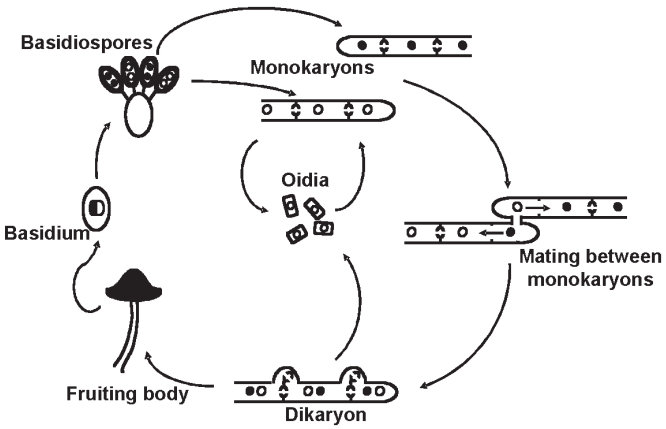

Figure 1. Life cylce of *Coprinopsis cinerea* (modified from Kües 2000). Filled and open circles indicate haploid nuclei of different mating type, a larger half filled-half open circle in the basidium the diploid nucleus obtained by fusion of two haploid nuclei of different mating type. For simplicity of the diagram, oidia production is only shown for one monokaryon.

fusion with monokaryons of different mating type (Brodie 1931, Kemp 1977, Kües 2002, Fischer and Kües 2005).

Light is also needed for induction of fruiting on the dikaryon (Tsusué 1969, Morimoto and Oda 1973, Lu 1974). However, fruiting occurs only under high humidity when nutrients are low and temperatures are in the range of 25-28°C (Madelin 1956, Walser 1997). Tissue formation within the primordium and fruiting body maturation including karyogamy, meisois and basidiospore production is adapted to the daily dark/light rhythm (Lu 1974, Moore et al. 1979). For the process to correctly proceed, further to initiation, light signals as well as distinct dark phases are required at specific points of development (Lu 1974, 2002, Kamada et al. 1978). Once all tissues in the primordium are established, light induced karyogamy parallels induction of fruiting body maturation. Meiosis directly follows karyogamy in the basidia. Stipes elongate and caps open with proceeding meiosis and basidiospore production and maturation. The mature fruiting body appears black by the colour of the melanized cell walls of the ripe basidiospores. The fruiting body is short-lived. Within a few hours, it quickly undergoes autolysis for spore liberation (Moore 1998, Kües 2000, Kamada 2002).

1.2. *Mutants in fruiting body development on the dikaryon*

Fruiting bodies normally develop on the dikaryon, which hinders genetic analysis of the process. Upon mutagenesis of the dikaryon, one would expect to only detect dominant mutations and a very low total number of mutants in screenings for defects in fruiting. For detection of recessive genes, principally two different nuclei would have to be mutagenised and this in the same cell and in the same gene. Such double mutants should be hard to find, particularly when using for mutagenesis mycelium with many dikaryotic cells in which many nuclei will be left non-mutagenised.

Takemaru and Kamada used macerated mycelium of a *C. cinerea* dikaryon in UV mutagenesis and chemical mutagenesis with NG (*N*-methly-*N*'-nitro-*N*-nitroso-guanidine). Surprisingly, they found abnormalities in fruiting body development in frequencies of over 10% of tested clones, in total 1,594 developmental variants amongst 10,641 tested isolates (Takemaru and Kamada 1969, 1970, 1972). Takemaru and Kamada (1972) suggested three causes for

the high amount of variants in their studies: i. influence of factors other than genes, ii. mutations in dominant genes and iii. easy access of fruiting genes in mutagenesis. Later on, Moore (1981) pointed out, that there are already many recessive genetic defects in fruiting present within the natural genetic pool of *C. cinerea*. For example, he observed in his wild-type strain collection defective alleles in four different genes acting in fruiting body initiation. In addition, we found in strain Okayama 7 (http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/) a natural defect in an essential fruiting initiation gene due to insertion of a transposon (unpublished). In support of Moore's view, other natural defects in sexual development have been described in tissue formation of the primordium (Muraguchi and Kamada 1998), in formation of basidiospores (Pukkila 1993, Kües et al. 2002b), in activation of fruiting in homokaryons (Uno and Ishikawa 1971, Murata et al. 1998a,b, Muraguchi et al. 1999) and in the process of nuclear exchange during mating (May and Taylor 1988). Furthermore, during crosses, new defects spontaneously arise (see below).

Few genes in fruiting have been cloned and analysed since Takemaru and Kamada did their mutagenesis study on the dikaryon. A respectable reason for the low number of cloned genes in fruiting is certainly the normally required dikaryotic state that requests an enormous work load and clever combinations of classical and molecular approaches when wanting to identify a gene (Muraguchi and Kamada 1998, 2000).

As a first gene, the pileus-specific *ich1* gene (for *ichijiku*, the Japanese word for fig) was cloned by first identifying the chromosome it locates on and then complementing the spontaneous recessive *ich1* mutation through transformation of a chromosome-specific library into an *ich1* defective monokaryon followed by crosses of transformants to another compatible *ich1* strain. *ich1* mutants fail to differentiate pileus tissue at the apex of the primordial stipe. Lack of pileus tissue causes a dent in the normally egg-shaped primordia giving the structure a fig-like shape. The abnormal *ich1* primordia rupture during stipe maturation and basidiospores are not formed, unless the defect is complemented by transformation with the wild-type gene. Ich1 is a large protein of 1353 amino acids that contains a potential nuclear targeting signal and has therefore been suggested to act within the nucleus (Muraguchi and Kamada 1998). Moreover, the protein has in its N-terminal half a potential S-adenosyl-methionine (SAM) binding domain similar to known O-methyltransferases (Kües 2000).

Dominant genes might be obtained in analogous strategies but by transformation into a wild-type monokaryon prior to mating of transformants to a compatible wildtype strain. *eln2* (*elongationless 2*) is a constitutively expressed gene that encodes a novel type of microsomal cytochrome P450 enzyme termed CYP502. A dominant *eln2* mutation (originally identified in a self-compatible background, see below) affects stipe tissue formation in the primordia and results in dumpy fruiting bodies with short stipes. The mutant gene was found by altered phenotype on the dikaryon after transforming a wild-type monokaryon and crossing to another strain (Muraguchi and Kamada 2000).

1.3. *Self-compatible mutants in studying fruiting body development*

Fruiting body development has been shown to be controlled by the mating type genes (Tymon et al. 1992, Kües et al. 1998, 2002b). The genes at the *A* mating locus, encoding heomeodomain transcription factors (Hiscock and Kües 1999, Casselton and Challen 2005) control light-induced initiation of fruiting. However, development arrests after tissue formation in the primordia and before karyogamy occurs in the basidia (Tymon et al. 1992, Kües et al. 1998). The genes at the *B* mating type locus, encoding pheromones and pheromone receptors, respectively (Kothe 2001, Casselton and Challen 2005), support the *A* mating type genes in their function in initiating fruiting body development. Primordia are formed in higher numbers and at an earlier time when both pathways are active. Moreover, after completion of tissue formation in the primordia, development continues leading to mature fruiting bodies. This suggests that the *B* mating type genes are required for induction of karyogamy (Kües et al. 2002b).

Consistent with the above results from monokaryons transformed with heterologous mating type genes, mutants with defects in the two mating type loci are self-compatible and have a simplified life-cycle (Fig. 2). Such homokaryotic *Amut Bmut* strains form fruiting bodies with basidiospores without mating to another strain (Swamy et al. 1984, Boulianne et al. 2000; Fig. 3 and Fig. 4). These basidiospores germinate into a self-compatible vigorous mycelia of dikaryon-like appearance (Fig. 3, left photo). The mycelium of *Amut Bmut* homokaryons has clamp cells at most septa (Fig. 4, photo at the left), but only one type of haploid nuclei in its hyphal cells. In submerged

medium, there are mostly two nuclei per hyphal cell and in there aerial mycelium, there is often only one nucleus in a hyphal cell (Swamy et al. 1984, Polak et al. 1997). *Amut Bmut* homokaryons produce uninucleate haploid oidia that again grow into self-compatible mycelia (Swamy et al. 1984; Fig. 3, 2nd photo from left). However, oidia are not constitutively produced in the aerial mycelium as in monokaryons, but asexual spore formation needs illumination as in dikaryons (Polak et al. 1997, Kertesz-Chaloupková et al. 1998). Also like in dikaryons, light induces fruiting on the established mycelium when nutrients are exhausted (Walser et al. 2003, Kües et al. 2004). Upon primordia formation, karyogamy of genetic identical nuclei occurs in the basidia. Meiosis follows and, during fruiting body maturation, the production of four identical basidiospores (Swamy et al. 1984, Kanda et al. 1989a; Fig. 3 photos to the right, Fig. 4). Nowadays, *Amut Bmut* homokaryons [either the original homokaryon AmutBmut from Swamy et al. (1984) or *A43mut*, *B43mut* homokaryon 326 created by Pukkila (1993, 1996) from homokaryon AmutBmut through repeated backcrosses to monokaryon 218] are in most instances used in mutant production (Kanda and Ishikawa 1986, Kanda et al. 1989a,b Chiu and Moore 1990, Pukkila 1994, Granado et al. 1997, Cummings et al. 1999, Inada et al. 2001, Arima, et al. 2004, Kües et al. unpublished;see below). Several genes have been cloned from UV and REMI mutants of such self-compatible homokaryons (Celerin et al. 2000, Inada et al. 2001, Arima et al. 2004, Liu et al. submitted). Amongst cloned functions acting in mushroom formation is a gene *cfs1* for a cyclopropane fatty acid synthase (Liu 2001, Liu et al. submitted) and a gene *eln3* for putative membrane protein with a general glycosyltransferase domain (Arima et al. 2004).

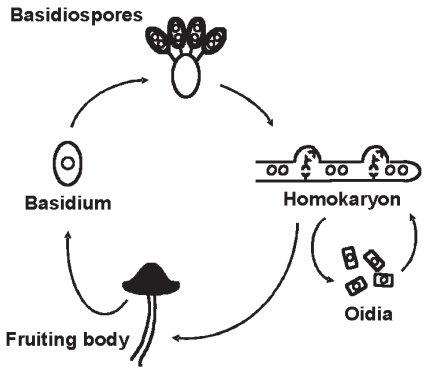

Figure 2. Life cycle of the self-compatible *Coprinopsis cinerea* homokaryon AmutBmut that is defective at both mating type loci (Swamy et al. 1984). Small open circles indicate haploid nuclei, a large open circle the homozygotous diploid nucleus in the basidium

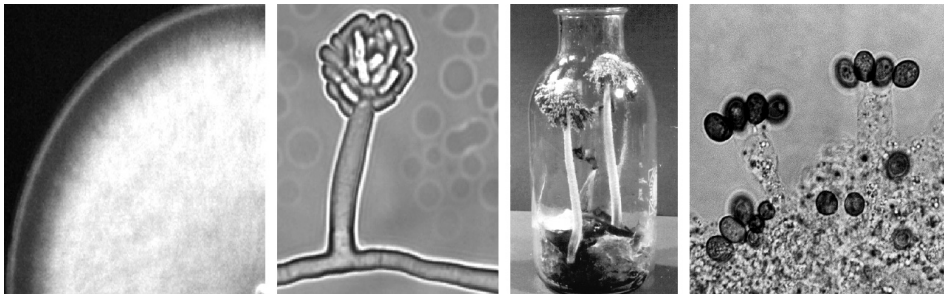

Figure 3. *Coprinopsis cinerea* strain AmutBmut (*A43mut, B43mut, pab1-1*), a homokaryon originally isolated by Swamy et al. (1984). From left to right: vegetative mycelium, an oidiophore produced upon light induction, mushrooms on horse dung and basidia with each four basidiospores. Photos are of courtesy of Yi Liu, Eline Polak, Markus Aebi and Jose Granado

Alternatively to *Amut Bmut* homokaryons, self-compatible strains with defects in the mating type pathways downstream of the mating type genes might be used for mutant production and gene recovery, e.g. the Cop5D mutant being defective in a gene *pcc1* for an HMG box transcription factor acting in the *A* mating type pathway (Murata et al. 1998a,b, Muraguchi et al. 1999, Muraguchi and Kamada 2000). The dominant *eln2* gene is an example of a gene detected through mutagenesis of homokaryon Cop5D (Muraguchi and Kamada 2000, see above).

2. Material And Methods

C. cinerea homokaryon AmutBmut (*A43mut*, *B43mut*, *pab1-1*; Swamy et al. 1984, May et al. 1991) was used in mutant production of UV mutant 6-031 (*A43mut*, *B43mut*, *pab1-1*, *skn1*, *mat*, *bad*) and REMI mutant B-1918 (*A43mut*, *B43mut*, *pab1-1*, *dst3*) (Liu et al. 1999 and submitted, Chaisaena et al. unpublished). Monokaryons JV6 (*A42*, *B42*), 218 (*A3*, *B1*, *trp-1.1,1.6*, *bad*) and PS001-1 (*A42*, *B42*; co-isogenic to homokaryon AmutBmut) were used in crosses (Kertesz-Chaloupková et al. 1998, Srivilai et al. in preparation). R1428 (*A8*, *B7*, *dst1-2*) was kindly supplied by T. Kamada. Growth conditions and all genetic methods are given in Walser et al. (2001).

3. Results And Discussion

3.1. *Mutant production with self-compatible* Coprinopsis cinerea *homokaryons*

The *pab1-1*-auxotrophic homokaryon AmutBmut (Fig. 3, Fig. 4) carrying the mating type alleles *A43mut* and *B43mut* (Swamy et al. 1984, May et al. 1991) has repeatedly been used in the past to create mutants in fruiting body development including meiosis and basidiospore production (Kanda and Ishikawa 1986, Kanda et al. 1989a,b Chiu and Moore 1990, Pukkila 1994, Granado et al. 1997, Kües et al., unpublished). Mutagenesis is easy since the haploid oidia can be used both for classical UV mutagenesis (Kanda et al. 1989 a,b) as well as for transformation in modern REMI (restriction enzyme-mediated integration) mutagenesis (Granado et al. 1997). Upon light illumination, oidia production on *A43mut*, *B43mut* homokaryons is abundant with numbers of up to 10^9 spores per plate (Kertesz-Chaloupková et al. 1998). Established UV and REMI mutagenesis protocols of oidia from homokaryon are given by Walser et al. (2001).

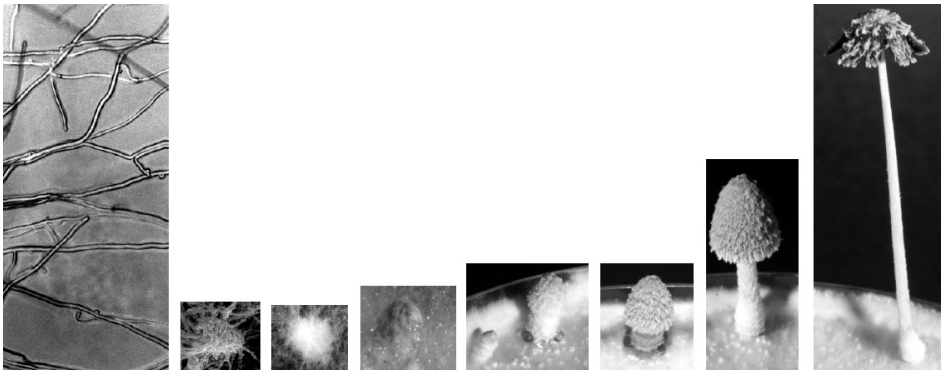

Figure 4. Stages in fruiting body development of *Coprinopsis cinerea* homokaryon AmutBmut (not to scale; adapted with alterations from Boulianne et al. 2000). Within the established mycelium (note the clamp cells at some of the septa in the left photo), loose aggregates (primary hyphal knots) form in the dark by localized intense formation of short hyphal branches with restricted tip growth. Upon reception of a light signal, hyphae aggregate into the compact secondary hyphal knots, in which tissue formation occurs. Correct tissue formation in the primordia needs changing day-night rhythms. Karyogamy in the basidia is induced by a light signal and directly followed by meiosis and basidiospore formation. Parallel to meiosis and basidiospore formation, the stipe elongates and the cap opens. Since it is a highly synchronized process, the developmental stages within the basidia can be predicted by the outer appearance of stages in mushroom development (further details can be found in Lu 1974, 2002, Moore et al. 1979, Kües 2000, Liu 2001, Walser et al. 2003, Kües et al. 2004).

Both UV and REMI mutagenesis has been performed by our group with homokaryon AmutBmut. About 10,000 mutants were screened for behaviour in fruiting. More than 1,200 mutants were detected that were affected in mycelial growth, asexual sporulation and/or fruiting body development (Granado et al. 1997, Kües et al. unpublished, Polak 1999). Using the scheme in Fig. 4, mutants were classified into three major groups with each several hundreds of mutants. The first group comprises defects in fruiting body initiation (block in primary hyphal knot formation and block in secondary hyphal knot formation), the second defects in primordia development up to the stage of karyogamy and the third defects in fruiting body maturation including defects in meiosis and basidiospore formation, respectively (Kües et al. unpublished). The frequencies of mutant production in homokaryon AmutBmut is thus as high as in the original mutant screens with dikaryons performed by Takemaru and Kamada (1972) and as high as in screens with other self-compatible homokaryons of *C. cinerea* (Cummings et al. 1999, Muraguchi et al. 1999).

3.2. *Gene cloning with mutants of self-compatible* Coprinopsis cinerea *homokaryons*

A gene in secondary hyphal knot formation (*skn1*) has recently been cloned by direct complementation of the AmutBmut UV mutant 6-031 (Liu et al., submitted). For transformations, an AmutBmut genomic library was used present in a cosmid carrying the wild-type *C. cinerea pab1* gene (Bottoli et al. 1999). *pab1* complements the *pab1-1* auxotrophy of homokaryon AmutBmut and encodes a para-aminobenzoic acid synthase (James et al. 2002).

Whilst the defect in the early step of fruiting was complemented in the original UV mutant, complete fruiting body development was not achieved (Liu et al. submitted). Crosses of mutant 6-031 with unrelated monokaryons suggested further mutations in later stages of fruiting to be present in the mutant. However, the results of crosses were difficult to interpret because of large progeny fractions were unable to initiate fruiting or development arrested at different stages in development. Large quantities of progeny from parallel crosses between homokaryon AmutBmut and the same monokaryons also failed to initiate fruiting whilst others initiated but did not complete

fruiting. Therefore, failure of initiation and completion of fruiting body development in the progenies of crosses of mutant 6-031 and monokaryons were in many instances likely not due to the *skn1* defect (Liu et al. 1999, Liu 2001, Srivilai et al. in preparation). Sequencing of the DNA fragment complementing the defect in fruiting body initiation in the *skn1* mutant identified the wild-type *cfs1* gene for a potential cyclopropane fatty acid synthase (Liu et al. submitted).

Handling REMI mutants might also not be as easy as originally thought. REMI mutants can carry more than one insertion (Granado et al. 1997, Liu et al. 1999), requesting separation by crosses prior to cloning the interesting insertion by plasmid rescue or PCR-mediated approaches (for techniques of inserted DNA recovery see Cummings et al. 1999 and Walser et al. 2001). However, genetic analysis of progeny of AmutBmut REMI mutants with unrelated monokaryons can be as difficult as with the UV mutants (Liu et al. 1999). As another hindrance found by other researchers (Inada et al. 2001), REMI insertions in some instances are unlinked to the mutant phenotype.

In conclusion, careful genetic analysis is advisable for both UV and REMI mutants before starting cloning genes. So far, this was difficult to perform for mutants of homokaryon AmutBmut.

3.3. *Creating monokaryons with different mating type specificities that are co-isogenic to* Coprinopsis cinerea *homokaryon AmutBmut*

In the past, few attempts have been made to create co-isogenic monokaryons in *C. cinerea* that distinguish just by mating types (Pukkila 1993). Therefore, we crossed *A43mut*, *B43mut* homokaryon AmutBmut to monokaryon JV6 with an *A42*, *B42* mating type and to monokaryon 218 with an *A3*, *B1* mating type. In the first generations, the fruiting abilities of *A43mut*, *B43mut* strains were very poor. In contrast, in higher filial generations of backcrosses to homokaryon AmutBmut, the mycelial appearance of clones in the progenies resembled that of homokaryon AmutBmut and the fruiting abilities raised above 90% of all *A43mut*, *B43mut* clones (Srivilai et al. in preparation). As a positive side effect from the first filial generation of the cross with monokaryon 218, we isolated a spontaneous *A43mut*, *B43mut* mutant with dumpy mushrooms (UFO1, see Fig. 5).

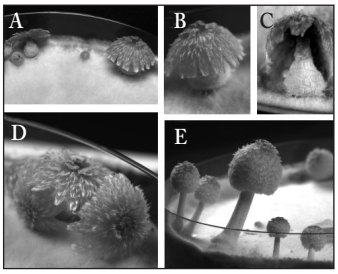

Figure 5. A.-D. Mutant UFO1 (*A43mut*, *B43mut*, *pab1-1*) forms dumpy mushrooms due to a semi-dominant defect in stipe elongation (*eln*). Moreover, it has no basidiospores due to a *bad* defect in basidiospore formation obtained from monokaryon 218 (Pukkila 1993, Kües et al. 2002). **E.** The UFO1 x 218 dikaryon forms medium-sized mushrooms suggesting that the *eln* defect in mutant UFO1 is semi-dominant. Mushrooms have white caps by lack of basidiospore production due to the homozygotous *bad* situation in the dikaryon.

3.4. *Co-isogenic, mating compatible monokaryons in crosses with the secondary hyphal knot UV mutant 6-031 of* Coprinospis cinerea *homokaryon AmutBmut*

When crossing the *skn1* mutant 6-031 with the compatible co-isogenic monokaryons, it was easy to separate the *skn1* mutation from a *mat* mutation (primordia maturation) and a *bad* mutation (basidiospore formation) that were also present in the mutant. Patterns of inheritance of such crosses were clear (Liu et al. submitted, Srivilai et al. in preparation, Fig. 6).

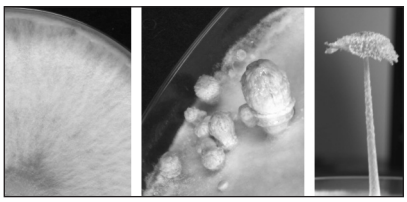

Figure 6. Phenotypes of *A43mut, B43mut* progeny of UV mutant 6-031 crossed with the compatible co-isogenic monokaryon PS001-1. From left to right: a *skn1* clone unable to initiate fruiting, a *mat* clone unable to produce mature fruiting bodies and a *bad* clone forming white mushrooms without spores.

3.5. *AmutBmut REMI mutant B-1918*

B-1918 is a REMI mutant of homokaryon AmutBmut that forms in light "etiolated stipes", also called "dark stipes" (Liu et al. 1999; Fig. 7). In the wild-type, etiolated stipes appear when a strain did not receive enough light (Lu 1974). T. Kamada kindly supplied monokaryon R1428 (*A8*, *B7*, *dst1-2*) that carries a recessive defect in a light receptor and causes in dikaryons etiolated stipe formation in light when present in both type of haploid nuclei (Yuki et al. 2003). A B-1918 x R1428 dikaryon forms mature fruiting bod-

ies (Fig. 7), indicating that the two mutants do not carry the same *dst* defect. In the homokaryotic *A43mut*, *B43mut* situation, the *dst1-2* gene gives in light rise to etiolated stipes (Fig. 7).

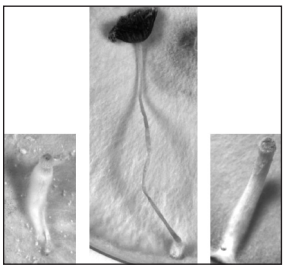

Figure 7. Mutant B-1918 forms etiolated stipes in the dark (left). In crosses with R1428, mature mushrooms arise on the dikaryon. *A43mut, B43mut, dst1-2* clones from the progeny AmutBmut x R1428 form etiolated stipes in the light and do not give rise to mature fruiting bodies.

4. Conclusions

The self-compatible *C. cinerea* homokaryon AmutBmut has been used for producing mutants in fruiting body development. In the past, genetic analysis of these mutants was difficult to perform by lack of co-isogenic compatible monokaryons. We now have co-isogenic strains that allow fast genetic access of mutants and clear-cut interpretations of inheritance of mutant genes in progenies of crosses with AmutBmut mutants.

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

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Galectins are ß-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-

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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 Wessels 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 immunomodulatoric 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 ß-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 ß-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 ß-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

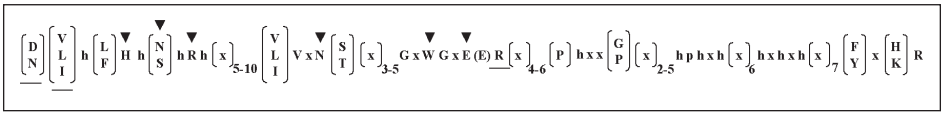

Figure 1. Conserved sequence elements within the carbohydrate recognition domain of the galectin family. Arrows mark residues with invariant bonding to carbohydrate ligands. Underlined positions indicate residues that coordinate ligands in extended binding site (e.g. acetamido group of N-acetyllactosamine or substituted ß-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).

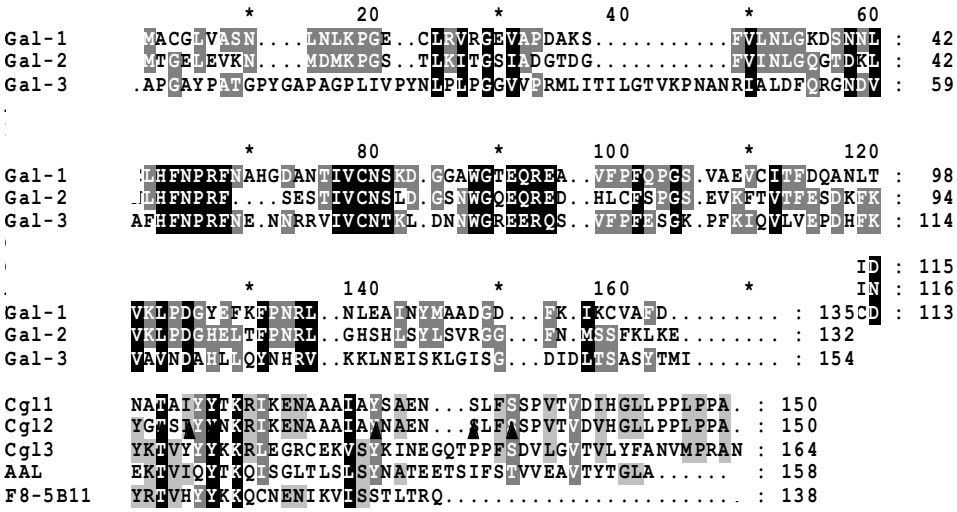

Figure 2. Sequence comparison of human and fungal galectins. Sequences of human and of fungal proteins were aligned separately from each other in order to better maintain the impression of sequence similarities between the fungal proteins. Arrows mark residues with invariant bonding to carbohydrate ligands (see Fig. 1). Protein and gene accession numbers in the NCBI GenBank: Cgl1 of *Coprinopsis cinerea* (Q06100), Cgl2 of *C. cinerea* (Q9P4R8), AAL of *Agrocybe aegerita* (Q6WY08), F8-5B11 of *Heterobasidion annosum* (BM346916), human Gal-1 (NM_002305.2), human Gal-2 (NM_006498.2), human Gal-3 (NM_002306.1). Cgl3 was deduced from contig 1.28 (position 715943 to 715452) from the *C. cinerea* genome 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 ß-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 ß-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).

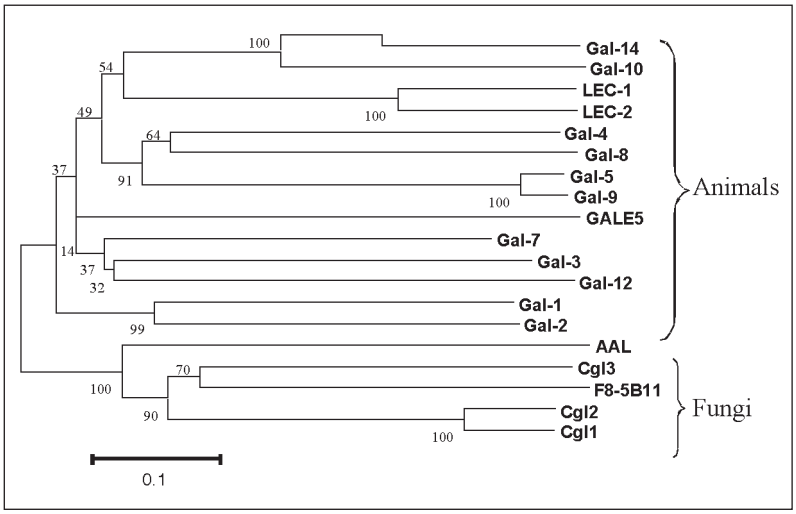

Figure 3. Phylogenetic tree of galectins from humans (GenBank accession numbers: Gal-1: NM_002305.2, Gal-2: NM_006498.2, Gal-3: NM_002306.1, Gal-4: NM_006149.2, Gal-5: AAH73889.1, Gal-7: NM_002307.1, Gal-8: NM_201545, Gal-9: NM_002308.2, Gal-10: Q05315, Gal-12: AAG40864.1, Gal-13: NM_013268.2, Gal-14: 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, Kües 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).

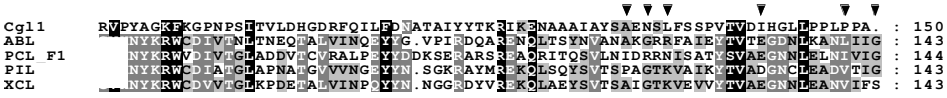

Figure 4. Comparison of fungal ß-galactoside binding lectins from various basidiomycetes. Cgl1 is a galectin from *Coprinopsis cinerea*. All other lectins belong to a recently defined family of fungal lectins that have structural similarities to actinoporins (Birck et al. 2004). Arrows above the sequences mark residues in galectin Cgl1 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: Cgl1 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 ß-galactoside binding lectins other than Cgl1 and Cgl2 are expressed in these tissues. At least one candidate exist in *C. cinerea* (see results). Remarkedly, the galectin ligands are of glycolipid nature (Walser et al. 2004, 2005). The ß-galactoside binding lectin SRL from sclerotial bodies (mycelial aggegrates serving in dormany) of *Sclerotium rolfsii* 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 ß-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 *cgl1* and *cgl2* will be analyzed.

2. Material And Methods

pYSK2 (Kilaru et al. 2005) is a yeast shuttle-vector that contains the yeast 2µm *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 receipies, growth and fruiting conditions are given by Granado 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 *cgl1* and *cgl2* 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 *cgl1* promoter region and with the *cgl2* terminator region, 495 bp of the *cgl1-cgl2* interim region present the *cgl1* terminator sequence and 871 bp the *cgl2* promoter region (Fig. 6). The corresponding promoter (*cgl1*) and terminator (*cgl2*) sequences are 841 bp and 522 bp long, respectively.

Bertossa et al. (2004) found minimal *cgl2* promoter activity to reside within 627 bp. The authors defined a number of potential promoter elements for *cgl2*, of which at least direct repeats of the sequence TGGAAG (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.

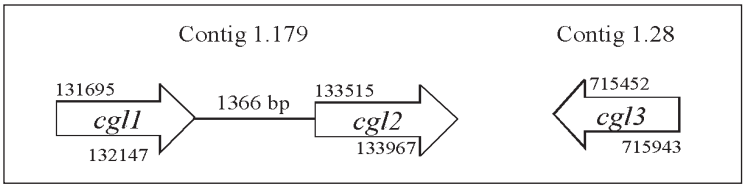

Figure 5. Galectin genes: localization in the genome of *Coprinopsis cinerea*. Sequences were identified by tBlast searches of the *C. cinerea* Okayama 7 genome (http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/) with Cgl1 from strain AmutBmut. Identical results hits were obtained in tblastn searches with Cgl2 and Cgl3.

When comparing the *cgl1* and *cgl2* 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 *cgl2* promoter. Most of the non-conserved elements locate in a 120 bp sequence that mediates induction of *cgl2* expression in the dark (Bertossa et al. 2004; Fig. 6). *cgl1* and *cgl2* are differentially regulated by light and dark signals. *cgl2* is dark induced, whilst *cgl1* is light induced (Boulianne et al. 2000) and may need other, yet to be defined regulatory elements.

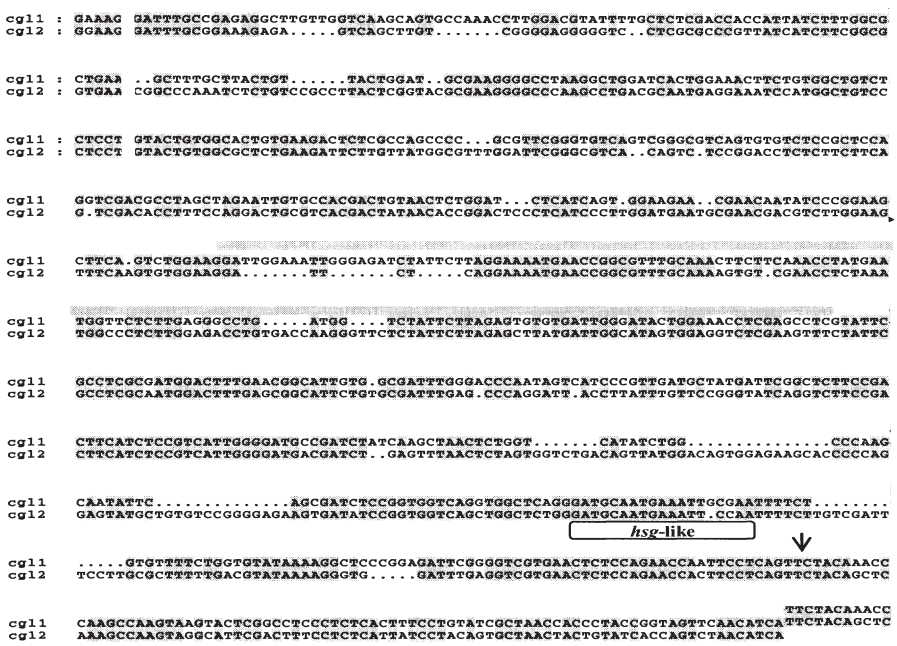

Figure 6. Comparison of the *cgl1* and *cgl2* promoter sequences. A black triangle indicates the maximal extension of the *cgl2* promoter − upstream sequences belong to the *cgl1* terminator region. The minimal *cgl2* promoter sufficient to confer regulated expression is marked by an open triangle. The 120 bp sequence needed for dark induction of the *cgl2* promoter is indicated by a grey bar. Within the *cgl2* promoter sequence, two identical, equal-spaced sequence stretches are outlined by open boxes. Boxes underneath the *cgl2* 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 *cgl1* and *cgl2* 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 c*gl1* and *cgl2* in front of laccase gene *lcc1* (see Fig. 7). The resulting *cgl1* and *cgl2* 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 *cgl2* promoter produce laccase at the edges of the plates when cultures are kept in dark. In contrast, transformants of the *cgl1* 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 *cgl1* and *cgl2* 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.

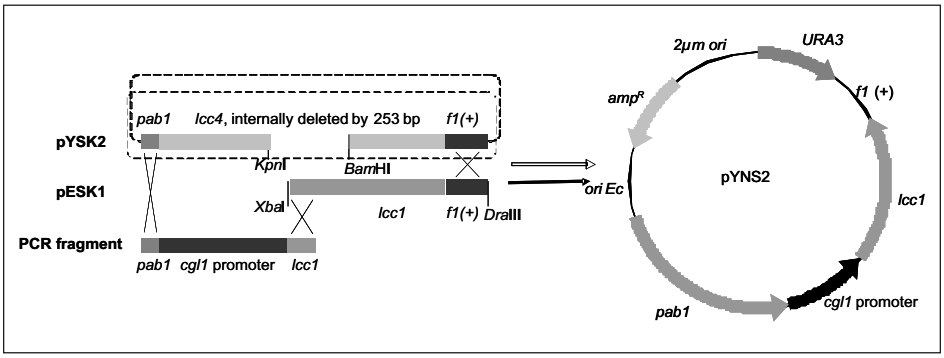

Figure 7. For *in vivo*-recombination in *Saccharomyces cerevisiae*, the yeast-*Escherichia coli* shuttle vector pYSK2 (Kilaru et al. 2005; only simplified map) was digested with *Bam*HI and *Kpn*I that cut within the *C. cinerea* gene *lcc4*. The linearized and purified vector was mixed with i. a 3.0 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 ß-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 ß-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 ß-galactoside binding lectins also gave no positive result. This does not exclude that there are other types of ß-galactoside binding lectins, both in the analyzed fungi and/or in other basidiomycetes.

4. Conclusions

Various types of ß-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 quantitave information.

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Verticillium fungicola Cell Wall Glucogactomannan-binding of the Lectin from the *Pleurotus ostreatus* Fruit bodies

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The *Verticillium fungicola* mycoparasitism on *Agaricus bisporus* fruit bodies appears to be a complex process made up of successive steps in which the recognition and binding between complementary molecules, the *A. bisporus* fruit body lectin and the *V. fungicola* cell wall glucogalactomannan, have recently been demonstrated. *P. ostreatus* fruit bodies have been described as containing a lectin and also presenting the "dry bubble" or the Verticillium disease. The aim of the present work is to purify and characterize the *P. ostreatus* lectin and compare the properties of both lectins in an attempt to confirm if the specific glucogalactomannan-lectin recognition and binding is the necessary step for the *V. fungicola* mycoparasitism process in *P. ostreatus*.

The characteristics and properties of the purified *P. ostreatus* lectin together with those also previously described by us on *A. bisporus* lectin show that, although both lectins present different chemical structures, they behave very similarly in relation to their glucogalactomannan-binding, thus confirming the existence of the specific recognition and binding step in the Verticillium disease on *P. ostreatus* fruit bodies.

1. Introduction

"Dry bubble" or Verticillium disease, the most serious fungal disease of the commercially grown strains of the white mushroom *Agaricus bisporus*, is

Genetics and Cellular Biology of Basidiomycetes-VI. A.G. Pisabarro and L. Ramírez (eds.)
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caused by *Verticillium fungicola*. The losses in yield of *A. bisporus* fruit bodies in Europe produced by *V. fungicola* are estimated at millions of euros annually. This mycopathogen infects not only *A. bisporus* but also other cultivated mushrooms such as *Pleurotus ostreatus* (Marlowe and Romaine, 1982). The only fungicide that is now used to control the disease, prochloraz, will probably be banned in the near future from commercial mushroom growing because *V. fungicola* has developed a resistance towards it (Gea et al. 1996). So, to elucidate the interaction between the mycopathogen and its host it will be necessary to know the molecular mechanisms of the infection.

Bernardo et al. (2004) described that an *A. bisporus* fruit body purified lectin recognized and binded the isolated glucogalactomannan from cell walls of *V. fungicola,* suggesting the specific interaction between both organisms, prior to the secretion of *V. fungicola* extracellular hydrolytic enzymes conductive to the development of the disease and the further *A. bisporus* fruit bodies necrosis.

This paper describes the characteristics and properties of a *P. ostreatus* fruit body lectin comparing them with those of *A. bisporus* fruit body lectin, in an attempt to confirm that the same molecular mechanisms of the infection occur in both mushrooms.

2. Materials and Methods

2.1. *Organisms and culture conditions*

Pleurotus ostreatus fruit bodies (commercial strain Amycel 3000) were grown in the CIES (Centro de Investigación, Experimentación y Servicios del Champiñón, Quintanar del Rey, Cuenca, Spain).

2.2. *Purification and characterization of* P. ostreatus *lectin*

Purification of the lectin was carried out by ammonium sulfate precipitation and ion-exchange chromatography as described previously (Bernardo et al. 2004). All procedures for characterization of the lectin (SDS-PAGE, MALDI-TOF mass spectrometry, chemical analysis and hemagglutination assays) have also been described before (Bernardo et al. 2004).

3. Results

The purification of the *P. ostreatus* lectin carried out following ammonium sulfate precipitation and ion-exchange chromatography is shown in Table 1. Preliminary experiments with ammonium sulfate fractionation showed that the hemagglutinating activity was distributed mainly in the 30%-100% saturated fraction. In the first anion-exchange chromatography all the coloured materials were absorbed by the column and the protein was eluted with the NaCl continuous gradient. In the cation-exchange chromatography the protein with the hemagglutinating activity was bound to the column, and it was eluted by means of the corresponding NaCl continuous gradient. The hemagglutinating activity evaluated at each step of purification is also shown in Table 1.

SDS-PAGE analysis of the purified lectin showed that the single band obtained was a pure protein, of an apparent molecular mass of 40±4 kDa (Fig. 1). On the basis of gel filtration the native molecular mass obtained was around 80 kDa. The molecular weight of the lectin was confirmed by MALDI-TOF mass spectrometry, obtaining a peak of 44270 m/z (Fig 2). The sugar composition analysis showed that this protein contained a 8.15% of carbohydrate content, so it was concluded that this lectin is a dimeric glycoprotein.

The sugar binding specificity of the lectin examined by hemagglutination inhibition assay is shown in Table 2. Some neutral sugars had no antagonizing activity against hemagglutination, however lactose and galactose showed some effect (50 and 25 mmol L^{-1} respectively), and N-acetylgalactosamine and glucogalactomannan from *V. fungicola* cell walls treated and not treated with the fungicide prochloraz (Bernardo et al. 2002) behaved as the best inhibitors (3.12, 6.25 and 12.5 mmol L^{-1} respectively).

4. Discussion

The chemical characteristics of the *P. ostreatus* lectin purified through this work are in good agreement with those described by Kawagishi et al. (2000) in a different strain of *P. ostreatus*. The specificity of this protein towards sugars has been established, and the results show that it deals with a galactose-binding lectin.

In a previous work, Bernardo et al. (2004) showed that the *A. bisporus* fruit body lectin recognized and binded the glucogalactomannan from *V. fungicola* cell walls, suggesting that this specific interaction was essential for the further secretion of *V. fungicola* hydrolytic enzymes and the development of the Verticillium disease on *A. bisporus* fruit bodies. In this report, we present evidence that the *P. ostreatus* fruit body lectin, although showing different chemical structure, behaves very similarly to the *A. bisporus* lectin in relation to its carbohydrate-binding specificity, and particularly towards the *V. fungicola* glucogalactomannan, thus indicating the same molecular mechanism for the *V. fungicola* mycoparasitism process in both *A. bisporus* and *P. ostreatus.*

The strongest inhibition effect shown by the glucogalactomannan isolated from cell walls of prochloraz pretreated *V. fungicola* mycelium can be explained by the increase of the terminal galactose residues of the molecule caused by the fungicide (Bernardo et al. 2002).

Further investigations may be needed to establish if the function related to the Verticillium disease is a general role of these lectins.

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Table 1
Purification of the *Pleurotus ostreatus* fruit body lectin

Fraction	*Protein (mg)*	*Hemagglutinating activity*		*Recovery (%)*
		Total (units)	*Specific (units/mg protein)*	
$(NH_4)_2SO_4$	335	12000	35.8	100.0
Anion-exchange	95.6	8256	86.4	68.8
Cation-exchange	12.3	4480	364.2	37.3

Table 2
Inhibition of hemagglutinating activity of *Pleurotus ostreatus* lectin by several carbohydrates

Carbohydrates	*Carbohydrate concentration (mmol/L)*									
	0.78	**1.56**	**3.12**	**6.25**	**12.5**	**25**	**50**	**100**	**200**	**PBS**
Glucose	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	-	-	-	+
Galactose	+	+	+	+	+	-	-	-	-	+
Arabinose	+	+	+	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+	+	+
Rhamnose	+	+	+	+	+	+	+	+	+	+
N-acetyl-D-glucosamine	+	+	+	+	+	+	+	+	+	+
N-acetyl-D-galactosamine	+	+	-	-	-	-	-	-	-	+
Glucogalactomannan*	+	+	+	+	-	-	-	-	-	+
Glucogalactomannan+F**	+	+	+	-	-	-	-	-	-	+

* glucogalactomannan of *V. fungicola*; ** glucogalactomannan of *V. fungicola* treated with the fungicide Prochloraz-Mn; +, hemagglutination positive; -, hemagglutination negative

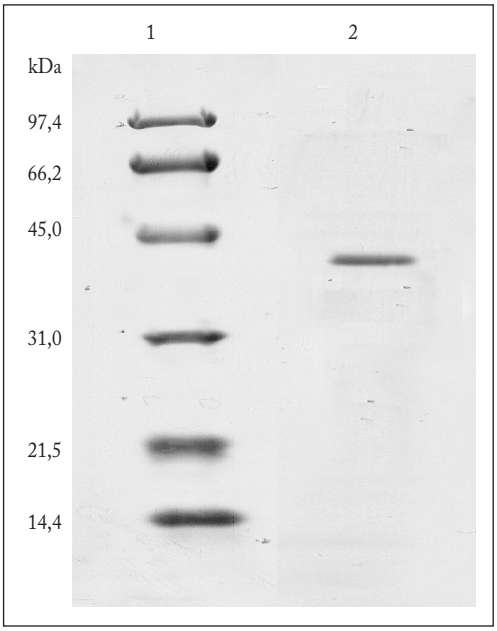

Figure 1. SDS-PAGE of the purified lectin from *Pleurotus ostreatus* fruit bodies: lane 1, molecular weight standards; lane 2, purified lectin.

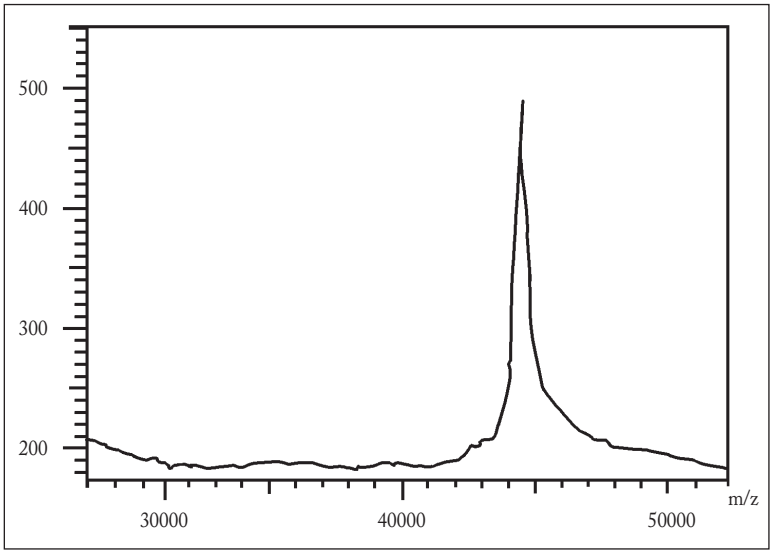

Figure 2. Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) analysis of the purified lectin.

Mushroom Breeding Program In Iran

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Mushroom cultivation is a newly established, but growing industry in Iran. There are about 120 producers with a total of about 20,000 tones per year in the country. *Agaricus bisporus* consists of more than 85 percents of the total production of all mushrooms produced in Iran. Its yield average is about 12-15 kg/m^2, while in the global production it is about 27-30 kg/m^2. This is mainly due to using strains of genetically weak performance.

Since ten years ago a breeding program was started with emphasis on breeding high yielding strains in Mashhad. The short-term effort consisted of selection among single spore isolates and multispores cultures with a better performance in yield. The long-term effort consisted of employing of heterosis in hybrid strains. To reach the aim, more than 350 homokaryone isolates were selected through RAPD markers followed by yield trials from commercial and domestic strains, and crosses were made in many combinations using diallel method.

Selection among spores of commercial strains could somehow recover their potential genetic capacity, so that an average of 22 Kg/m^2 was recorded in the selected strains. Using growth type as a marker, it was possible to decrease the number of isolates in final stages of selection for homokaryones in solid medium or spawn. Cluster analysis based on average of band numbers emerged by RAPD markers, could separate homokaryotic and heterokaryotic isolates in two distinct groups. Some hybrids showed a better mycelia growth and a considerable higher yield than their parents. Efforts are now being made to collect wild strains of *Agaricus bisporus* in Iran and evaluating them for desirable genes including resistance to pest and diseases.

1. Introduction

Among the edible fungi, the white button mushroom, *Agaricus bisporus* (Lange) Imbach (= *A. brunnescence* Peck) holds a unique position, with a

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world wide production in large amounts. The economic value of *A. bisporus* in the late 1980s amounted 3.5 billion US dollars (Horgen *et al.,* 1992) while at the beginning of the new millennium has exceeded 10 billion US dollars. Now, the world production of this mushroom is near 38 percent of the world total production of edible mushrooms.

In spite of the economic and agronomic importance of *A. bisporus*, its breeding programs have always been problematic. Little information existed on the basic biology of this mushroom before 1970s. In the years 1970 and 1971 it was known that *A. bisporus* behaves as a secondarily homothallic (Miller, 1971, Elliott, 1972, Raper *et al.,* 1972), or more recently as an intramictic (Kerrigan, 1990) fungus during its life cycle. It means that each dikaryotic basidium cell performs a transient diploid followed by a meiosis division and production of four haploid meiotic nuclei. Majority of basidiospores receive two non-sister post meiotic nuclei and are therefore heteroallelic in over 90% of loci similar to the parents (Kerrigan *et al.,* 1993, Horgen *et al.,* 2002). The basidiospores germinate and rise to self-fertile heterokaryotic mycelium. Small percentage of basidiospores receives one of four post-meiotic nuclei and the smaller percentage receives two similar daughter nuclei. These two group of basidiospores rise to self- infertile homokaryotic mycelium (Kerrigan *et al.,* 1987, Khush *et al.,* 1995, Miles *et al.,* 1997). Thus, the homokaryons which are much important to the breeding programs are infrequent and this is an impediment to the button mushroom breeding (Horgen *et al.,* 1992, Khush *et al.,* 1995). In this species, there is no evidence to nuclear migration or fusion until fruit bodies are formed (Raper *et al.,* 1972, Kush *et al.,* 1995). In a heterokaryotic mycelium, each cross wall is multinucleate in which there are several copies of both non-sister haploid nuclei (Paul A. Horgen, Personal Communication). Homokaryons in *A. bisporus* and *A. bitorquis* are also multinucleate and each cross wall contains several copies of one haploid nucleus (Miles *et al.,* 1997). In these two species, the lack of clamp connections makes it difficult to distinguish heterokaryons (or dikaryons) from homokaryons and this is another impediment to their breeding (Chang *et al.,* 1989, Khush *et al.,* 1995, Loftus, *et al.,* 1995, Miles *et al.,* 1997). The other problem is that the basidiospore germination is a variable and slow process. There are also some problems with contamination in the germination particularly with bacteria, even under controlled laboratory conditions, so that bacteria colonize the medium before basidiospores can

germinate. Some germination kinetics of the basidiospores has been already studied (Horgen *et al.,* 1989, Kokorwicz *et al.,* 1994).

Strain improvement in *A. bisporus* has more been on the basis of selection and hybridization (Mehta *et al.,* 1994, Pandey *et al.,* 1994, Pathak *et al.,* 1998). Selection makes use of the variation which isolates perform in growth rate, colony type, yield and other characteristics, while hybridization often includes simple mixing and cross breeding (or strain hybridization). Simple mixing has not had a significant role in strain improvement of *A. bisporus* during its history of breeding. Cross breeding is often based on collecting the desired genes to novel strains in which the heterosis phenomenon will be probably observed. In this approach, crosses among compatible homokaryons, so-called anatomists, are needed (Castle *et al.,* 1988, Horgen *et al.,* 1992, Mehta *et al.,* 1994) and therefore, recovering the homokaryons is very important. Several traditional and modern molecular approaches are applied to recover and to confirm homokaryons (Castle *et al.,* 1988, Summerbell *et al.,* 1989, Horgen *et al.,* 1992, Kerrigan *et al.,* 1992, Khush *et al.,* 1995, Horgen *et al.,* 2002).

Mushroom cultivation is a newly established, but a fast growing industry in Iran. There are about 120 producers with a total of about 20,000 tones per year in the country. *Agaricus bisporus* consists of more than 85 percents of the total production of all mushrooms produced in Iran (The Iranian Mushroom Growers Association, 2004). Its yield average is about 12-15 kg/m^2, while in the global production it is about 27-30 kg/m^2. This is mainly due to using strains of genetically weak performance.

So far, no breeding program of *A. bisporus* had been conducted in Iran. Since ten years ago a breeding program was started with emphasis on breeding high yielding strains in Mashhad. The short-term effort consisted of selection among single spore isolates and multispores cultures with a better performance in yield. The long-term effort consisted of employing of heterosis in hybrid strains, using molecular markers to assist selections (Kerrigan, 2000).

The main objective of this program was to find approaches to produce high yielding strains with more adaptability to the country conditions. Thus, it was necessary to carry out a complex study to get a better understanding of the mushroom breeding and related problems. In this complex study, we attempted to find out the problems and to establish a framework for research on the *Agaricus bisporus* breeding in Iran.

Our new main objective is to collect wild strains of *Agaricus bisporus* in Iran and evaluating them for desirable genes including resistance to pest and diseases. Molecular tools will be also used in differentiating homokaryons from heterokaryons, evaluating strains and bred hybrids, and patenting the bred strains.

2. Materials and Methos

Basidiospore germination as the first step in a mushroom breeding program was first optimized by our lab conditions. Twenty days after transferring the single colonies, the mycelia of each single spore filled the medium enough and a pure isolate generated through a single spore (or a single spore isolate=SSI) was prepared. These cultures were used to inoculate the grains. Growth type and growth rate of colonies were measured on petri dishes. Investigations were made during 15 days after spore culture.

Spawn running was considered as a criterion for measuring variation among spawned beds. The observed yields were classified as follows: high, moderate, low and zero. Growth types (in solid medium and spawn) were classified as follows: strandy-fast, strandy-slow, fluffy-fast, fluffy-slow, appressed-slow and appressed-very slow. Then the relationship between the yield and the classes of growth type was measured. For study of some breeding characteristics, the variation of basidiocarps in terms of the ratio of cap diameter to stripe height, general and specific combining ability and different variations for several breeding traits were investigated. RAPD markers (Williams *et al.* 1990) were used to assist the selection of homokaryons, based on Khush's work (khush *et al.,* 1992). More than 350 homokaryotic isolates were also selected through RAPD markers. Cluster analysis (Ward method) was used to separate homokaryons and heterokaryones based on RAPD bands and to select furthest isolates for hybrids parents.

3. Results

The rate of generating single colonies between days 5-12 was more than other days. The rate of spore germination in PDA medium was more than that

of the CYM medium ($p \leq \%1$). Generally, after twelve days, the colony of each single spore could be observed with naked eyes. At this time, it was possible to transfer the single colonies to a fresh medium. Fifteen days after spore culture, the colonies of basidiospores grew together well and filled the medium.

A considerable variation was observed among SSIs. They varied in colony type and growth rate. Four classes of colony types including strandy, fluffy, cottony and appressed were observed. The observed growth types included fast slow and very slow. Most SSIs with a fast or slow growth in solid medium had a corresponding spawn running in the bed. The light microscopic observations also showed the different features of mycelia related to different growth type (Li *et. al.,* 1994 and Heath *et al.,* 1995).

Heterokaryons and homokaryons were distributed in all classes of growth types and were overlapped. However, selection based on growth type, lowered the number of isolates involved in the final stages of breeding program, So that the frequency of desirable homokaryons increased in final stage before going to yield trials. Then isolates that meet the requirements (a high yield or a homokaryotic status) were used in yield trials for further investigations.

Fourteen days after inoculation, considerable variations were observed among spawns. In each SSI, the observed colony type and growth rate in solid medium and spawn was the same ($p \leq \%1$).

Not a significant correlation was found between growth types such as slow or very slow growing appressed isolates, and their yields. However, most of them had a low or zero yields. There was also not found any significant relationship between growth types such as fast growing strandy or fluffy isolates, and yield, but the majority of such isolates produced a high yield, while the minority of them had a low yield. Therefore, it is possible to decrease the number of isolates involved in selection in the stage of solid medium or spawn based on growth type. It looks like the most effective approach is both excluding of very slow growing appressed isolates and retaining of fast growing strandy ones in the stage of solid medium.

Selection of isolates that did not produce any mushrooms (i.e. homokaryons) is also challengeable. Based on this study, omitting of fast growing strandy and fluffy isolates (which most of them produce a considerable mushroom) and selection of slow or very slow growing isolates (which most of them produce no mushroom) increased the efficiency of screening of homokaryons (Kerrigan *et al.,* 1992).

In this study, in addition to single spore isolates, multi spore cultures were also used. In any multi spore culture, several growth types were observed. Samples obtained from these cultures showed also different growth types. However, all classes of growth types were not observed similar to SSIs and also there was not a significant relationship between growth types and yield. But selection based on multi spore culture can have an effective influence upon strain improvement in *Agaricus bisporus*, because several growth types could be observed simultaneously in one petri dish.

Some hybrids obtained from this study, showed a higher performance than their parents and showed a considerable high yield in further fruiting tests (22 kg/m^2).

Here it should be stated that the fruiting test is often necessary for final verifying of any breeding program (Paul A. Horgen, Personal Communication). However, some homokaryons produced a few mushrooms and also some heterokaroyns produced no or a few mushrooms, due to the effect of some environmental conditions or other unknown factors (Kerrigan *et al.,* 1992) and this makes the matter more complex.

Cluster analysis based on average of band numbers emerged by RAPD markers, could separate homokaryotic and heterokaryotic isolates in two distinct groups.

Use of RAPD markers in the breeding programs of *A.bisporus,* was first reported by Khush *et al*. (Khush *et al.,* 1991, 1992, 1995). By having two different types of nuclei, heterokaryons have more sites for annealing of primers and consequently more segments of DNA can be amplified. Conversely, homokaryons have one type of nucleus and thus have fewer sites for annealing of primers. In this study, RAPD markers showed that they are able to distinguish homokaryon from heterokaryon and also high yielding isolated from the others. Efforts are now being made to collect wild strains of *Agaricus bisporus* in Iran and evaluating them for desirable genes including resistance to pest and diseases.

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Molecular analysis of two acidic proteinases pumAe and pumAi and aminopeptidase pumAPE from *Ustilago maydis*: enzymes purification and differential expression

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Proteolytic system of *Ustilago maydis* was recently partially described (Mercado-Flores *et al*., 2003). Two acidic proteinases pumAe (extracellular) and pumAi (intracellular) and aminopeptidase pumAPE were detected and purified from the haploid phase of *U. maydis*. Purification consisted of ammonium sulphate fractionation and different chromatographic steps. Molecular masses were estimated: 58 kDa for pumAPE, 72 kDa for pumAe and 35.3 kDa for pumAi. Enzymatic activity was optimal at pH 7.0 and 35 ºC for pumAPE and 4.0 for the two proteinases. pumAPE was inhibited by EDTA-Na_2, 1,10-phenanthroline, bestantin, PMSF and several divalent cations, while proteinase pumAi was inhibited by pepstatine A, also finding that yeast-to-mycelium transition was inhibited by Pepstatine A in the culture medium. Primers were designed in order to amplify the gene *APEum* encoding pumAPE and *PRAum* gene encoding pumAi, and they were used as probes in a Southern blot. One copy of each gene was detected by genome in several strains. Differential expression of *APEum* was assessed under different physiological conditions, detecting high expression levels on media supplemented with corn infusion, proline, peptone and ammonium sulphate. *PRAum* is expressed when cells are exposed to corn infusion and ammonium sulphate.

1. Introduction

The phytopathogenic basidiomycete *Ustilago maydis*¸ the etiological agent of corn smut disease, has a complex life cycle with three major cell types: a non-

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pathogenic haploid unicellular form, a pathogenic dikaryotic filamentous form, and the teliospore, a diploid cell form (Banuett 1995). *U. maydis* produces an intracellular acid proteinase (pumAi) during its exponential growth, mainly when proline is added as nitrogen source. Pepstatin A, a potent aspartyl proteases inhibitor, affects the enzyme activity in the crude extract. Moreover, a strong inhibition of yeast-to-mycelium transition is observed when the inhibitor is added to a differentiation culture medium (Mercado-Flores et al. 2003). *U. maydis* produces an extracellular acid proteinase (pumAe) during exponential growth that is induced exclusively in acid conditions. This work focused on the purification and biochemical characterization of the recently reported pumAi and pumAe of *U. maydis* that might play an important role during infection of plant tissue. *U. maydis* produces at least two intracellular aminopeptidases (pumAPE) primarily associated with the exponential growth phase of the fungus. Maximal activity has been found in the soluble fraction and this activity is blocked by two metalloprotease inhibitors, EDTA-Na_2 and 1-10, phenanthroline (Mercado-Flores *et al.*, 2002).

This work focuses mainly on the purification and biochemical characterization of the recently reported intracellular aminopeptidase pumAPE and the acidic proteinase pumAi (intracellular) and pumAe (extracellular) from *U. maydis*, and the determination of the number of copies of the genes *APEum* (gene encoding pumAPE) and *PRAum* (encoding acidic intracellular proteinase pumAi) in the fungus genome and the differential expression of these genes when growing with several nitrogen sources. The properties of the purified enzymes and also their differential expression are discussed to elucidate the possible role of these enzymes during the plant-fungus relationship, maturation of proteins, and/or nitrogen uptake.

2. Materials and methods

2.1. *Organism and culture conditions*

The *U. maydis* haploid strains FB1 (*a1b1*), FB2 (*a2b2*), and diploid D12 (*a1b1/a2b2*) used in this study were kindly provided by Dr. Flora Banuett, University of California at San Francisco, CA, USA. For purification pur-

poses *U. maydis* FBI was grown in YEPD medium (1% yeast extract, 2% peptone and 2%dextrose) in a 1.5 Fernbach glass at 28˚C using an orbital shaker at 150 rpm. The medium was inoculated with an overnight culture and incubated for 24 h. For DNA isolation, the strains were grown in YEPD in Erlenmeyer flasks at 28ºC with orbital shaking for 24 to 48 h. For RNA isolation, logarithmic-phase cultures (4×10^8 - 6×10^8 cells/ml) were exposed to different sources of nitrogen in YNB (0.17% Yeast Nitrogen Base) supplemented with different nitrogen sources (2% proline, 0.5% $(NH_4)_2SO_4$, 2% peptone, corn infusion with 4 μg protein/mL) at different times (10, 20, 30, 60, 120, 360 minutes) at 28˚C.

2.2. *Standard enzyme assay and protein determination*

Proteinases pumAe and pumAi were determined as previously reported (Hirsch et al. 1989) using 2% acid-denatured hemoglobin (Sigma Chemical Co. St. Louis, MO) as substrate. One unit of enzyme activity was defined as the amount that released 1 μg of tyrosine per minute. Protein determinations were performed according to Bradford's method (Bradford, 1976). Aminopeptidase activity was measured using L-lysine-*p*-nitroanilide (Lys-pNA) (Bachem, King of Prussia, PA, U.S.A.) as substrate. The incubation mixture contained 100 mM Tris-HCl buffer (pH 7.5), 1 mM Lys-pNA and the enzyme, in a total volume of 500 μl. After incubation at 37˚C for 10 min, the reaction was stopped by adding 500 μl of 5% $ZnSO_4$ and 100 μl of 7.5% $Ba(OH)_2$. The mixture was centrifuged (10,000 X *g* for 10 min), and absorbance of the released p-nitroaniline was determined at 405 nm in the clear supernatant. One unit of aminopeptidase pumAPE corresponded to the amount of enzyme that releases 1μmol/min of p-nitroaniline under test conditions (Arbesú *et al*., 1993). Protein determinations were performed according to Bradford's method (Bradford, 1976).

2.3. *Enzyme purification*

The proteinase pumAi from *U. maydis* FB1 from was purified from biomass. Biomass was recovered by centrifugation and fragmented in Braun's mill us-

ing a mixture of 7.5 g of glass beads (0.5 mm diameter) and 14 mL of 0.1 M Tris-HCl, pH 7.5, for each 6 g of cells. The crude extract was removed from the glass beads and centrifuged at 10,000 X *g*. The supernatant was centrifuged at 100,000 X *g*. The supernatant obtained was saturated to 60-80% ammonium sulfate. The precipitate was colleted by centrifugation at 12 000 X *g* and dissolved in 50 mM KH_2PO_4/K_2HPO_4, pH 7.0. The sample was applied onto a Phenyl Superose HR 5/5 column (Amersham Biosciences, Ltd, UK). Proteins were eluted at 0.5 ml/min, by using a linear gradient from 1.7 to 0.0 M of ammonium sulfate. The active fractions were pooled and applied onto a HiPrep 26/10 prepacked desalting column (Amersham Biosciences, Ltd, UK). Eluted proteins were pooled, collected and applied onto a Mono Q HR 5/5 column. The proteins were eluted at 1 ml/min with a linear NaCl gradient (0.0-1.0 M). The active fractions were pooled and applied onto a prepacked Superose 12 Prep grade column (Amersham Biosciences, Ltd, UK), and eluted at 3 ml/h. The proteinase pumAe from *U. maydis* FB1 was purified from a culture supernatant by fractionation with ammonium sulphate and a step performed on Supherose 12 FPLC column (Amersham Biosciences Ltd, UK). Proteins were eluted at 3 ml/h and the fractions of highest activity were pooled.

For the protease pumAPE purification, biomass from the culture medium was recovered by centrifugation (5,000 X *g*, 4°C for 10 min). Cells were fragmented as described previously. The crude extract was carefully removed from the glass beads and centrifuged (10,000 X *g* for 4°C, 10 min). The supernatant was removed and ultracentrifuged (100,000 X *g*, 4°C for 90 min). The corresponding supernatant was used in the enzyme purification procedure. All enzyme manipulations and chromatographic separations were performed in a fast protein liquid chromatographic (FPLC) system (Amersham Biosciences Ltd., UK) at 4°C. The supernatant obtained was saturated to 60-80% ammonium sulphate. The precipitate was colleted by centrifugation at 12 000 X *g* and dissolved in 50 mM KH_2PO_4/K_2HPO_4, pH 7.0. The sample was applied onto a Mono Q HR 5/5 column. The proteins were eluted at 1 ml/min with a linear NaCl gradient (0.0-1.0 M). Eluted proteins were pooled, collected and applied onto a Phenyl Superose HR 5/5 column (Amersham Biosciences, Ltd, UK). Proteins were eluted at 0.5 ml/min, by using a linear gradient from 1.7 to 0.0 M of ammonium sulphate. The active fractions were pooled and applied onto a HiPrep 26/10 prepacked desalting

column (Amersham Biosciences, Ltd, UK).The active fractions were pooled and applied onto a prepacked Superose 12 Prep grade column (Amersham Biosciences, Ltd, UK), and eluted at 3 ml/h.

2.4. *Enzymes characterization*

Molecular weight of the enzymes was estimated by SDS-PAGE (Laemmli, 1970), and FPLC gel filtration performed on Superose 12 prep grade column. Proteins of known molecular weight were used as standards in (Andrew, 1965). The pI's of purified proteinases pumAe and pumAi and aminopeptidase pumAPE were estimated by isoelectric focusing with the Rotoford system (BioRad, USA), using a rotolite pH range from 3.0-11.0, running time was 5 h at 12 W.

Enzymatic activities for proteinases were performed upon acid-denatured hemoglobin at a final concentration of 2% were determined, while enzymatic activity for aminopeptidase was performed against Lys-*pNA*. The optimal pH of the proteases was determined at 37˚C with different buffers at 50 mM. For the pH ranges of 2.0-7.0, 7.0-10.0, and 9.0-11.0, McIlvaine, Tris-HCl, and glycine-NaOH buffers were used, respectively. The pH stability was determined by overnight preincubation of the purified enzyme in the appropriate buffer at different pH values ranging from 2-10 at 4ºC followed by standard enzyme assay. Optimal temperature was examined between 5 and 80˚C by standard enzyme assay. Thermal stability was evaluated by incubation of the enzyme solution at 5, 25, 30, 35, 40, 45, 50, 55, 60, 70, and 80˚C for 60 min, before performing the standard enzymatic assay. Activity was always expressed as a percentage of the activity obtained at either the optimal pH or temperature.

2.5. *Effects of protease inhibitors on proteases and metal cations on aminopeptidase pumAPE activity*

The effects of potential inhibitors, such as bestatin, pepstatin, leupeptin, Pefabloc, E-64, PMSF, 1-10 phenanthroline, and EDTA-Na_2 (Roche, Switzerland) were tested, as well as 0.5 or 1.0 mM metal cations only for

pumAPE. The purified enzyme was preincubated with the respective compound for 30 min at 37ºC, followed by the standard enzyme assay. Activity was expressed as a percentage of the activity obtained in the absence of the added inhibitor or metal salt.

2.6. *Substrate specificity*

The acid-denatured hemoglobin in the standard enzymatic assay was replaced by albumin (Milewski *et al*., 1994), resorufin-labeled casein (Twining 1984), gelatin (Kunits 1947), and Hide Powder Azure (HPA) collagen-type substrate (Hirsch *et al*., 1989), at pH 4.0, 5.0, and 7.0. The relative activities of the aminopeptidase pumAPE against several aminoacyl-p-nitroanilide (pNA) substrates were determined by standard activity assay. The reaction mixture consisted of 450 µl of 50 mM Tris-HCl (pH 7.5), 25 µl 25 mM of each substrate and 25 µl of enzyme. Absorbance at 405 nm was determined after 20 min of incubation.

2.7. *Determination of kinetic parameters*

Kinetic parameters of the purified enzyme were estimated for Lys-*pNA* by using concentrations ranging from 0 to 1.5 mM. Activity was measured continuously as described above. K_m and V_{max} values of the purified proteinase (pumAe) was estimated using 0.015 to 0.200 mM Suc-R-P-F-H-L-L-V-Y-MCA as substrate. Kinetic parameters were calculated from Lineweaver-Burk plots.

2.8. *Isolation and enzymatic restriction of DNA from U. maydis strains*

DNA from *U. maydis* was isolated using the method described by Hoffman and Winston (1987). DNA was digested with restriction endonucleases (Roche, Invitrogen), for Southern blot analysis. Putative genes encoding pumAi (*PRAum*) and pumAPE (*APEum*) were located in the *U. maydis* genome. Both genes *PRAum* and *APEum* were cloned into TOPO-TA 2.1 plasmid (Invitrogen). The cloned genes were used as probes in Southern blot analysis.

2.9. *RT-PCR analysis*

RNA isolation of each sample exposed to the specific nitrogen source at the specific time was performed by heating, freezing and then thawing them with phenol and SDS (pH 5.2), followed by twice extractions with phenol-chloroform-isoamyl alcohol (25:24:1) and phenol chloroform (24:1). RNA was precipitated overnight with 2.5 volumes of ethanol and 0.1 volumes of 3 M sodium acetate. RNA's were treated with RNase-free DNase I as recommended (Invitrogen). cDNA's were synthesized with reverse transcriptase (SuperScript™ II Reverse Transcriptase), using the reverse primer ACT 1 of *U. maydis* and the primer dT. *PRAum* and *APEum* were amplified from cDNA using specific primers (to be published), using an amplified actin gene fragment for normalization.

3. Results and discussion

3.1. *Purification of enzymes*

The proteinase pumAi from *U. maydis* FB1 was purified. The final yield was 6.8%, and the enzyme was purified 90.2-times. The proteinase pumAe from *U. maydis* FB1 was purified from a culture supernatant getting final yield of 7.7%, and it was purified 15.1 folds. Aminopeptidase pumAPE from *U. maydis* FB1 was purified getting final yield of 23.0%, and the enzyme was purified 69.3-fold. Purified anzymes show a single band in SDA-PAGE.

3.2. *Enzymes characterization*

Molecular mass of purified pumAi is 35.3 and 36.6 kDa, estimated by gel filtration chromatography and SDS-PAGE, respectively, suggesting that the enzyme is a monomer. pumAi had a pI of 5.5 and was stable and active in an acid pH range from 3.0 to 6.0, with an optimum pH at 4.0. The purified enzyme was stable for 1 h in a range of 5-40°C; it was most active at 35-45°C, with an optimal temperature at 40°C. The enzyme activity was completely inhibited with pepstatin A. Pefabloc and EDTA-Na_2 had a slightly inhibito-

ry effect on the protease. No effect was observed with the other inhibitors tested. The purified enzyme degraded albumin, gelatin, and hemoglobin at pH values of 4.0, 5.0, and 7.0, while casein was better degraded at pH 5.0. No detectable degradation of collagen was observed.

pumAe proteinase has a molecular weight of 72 kDa and 74 kDa, estimated by gel filtration chromatography and SDS-PAGE respectively, suggesting that pumAe is composed of one subunit. The enzyme had a pI of 5.5, was stable in a broad range of pH from 2.0 to 8.0 for 12 h, and was active in an acid pH range from 2.0 to 5.0, with an optimum pH at 4.0. The enzyme was stable for 60 min in a range of 5-40^{o}C, was most active at 35-60 ˚C, with an optimal temperature of 45^{o}C. Results from protease specific inhibitors showed that this enzyme is a no aspartil protease.

The molecular mass of the purified pumAPE estimated by SDS-PAGE analysis was approximately 58 kDa. The relative molecular mass of the native enzyme estimated by gel filtration on Superose 12 column was around 110 kDa. These results suggest that the purified enzyme is a dimmer. Aminopeptidase pumAPE had a pI of 5.1 and showed activity against Lys-pNA in a narrow pH range (7.0 to 9.0), with an optimum at pH 7.0. The activity was stable in the pH range 7.0 to 9.0 for 12 h at 4 ^{o}C. The enzymatic activity was optimal at 35^{o}C. The activity of the enzyme was reduced by incubation for 1 h at high temperatures of 55.0 to 80.0^{o}C. The presence of chelating agents, such as EDTA-Na_2 and 1-10, phenanthroline, inhibited the aminopeptidase activity, indicating that the purified enzyme is a metalloprotease. Bestatin, a typical inhibitor of exopeptidases, caused strong inhibition of the purified enzyme. PMSF, Pefabloc (serine proteases inhibitors) and leupeptin (cysteine and serine proteases inhibitor) had an inhibitory effect on the enzyme. Presence of Cu^{2+}, Hg^{2+}, and Zn^{2+} caused complete inhibition of the purified enzyme at 1mM. Other cations such as Co^{2+}, Ni^{2+}, and Mn^{2+} had a strong inhibitory effect on the aminopeptidase, whereas Mg^{2+} and Ca^{2+} had a slight inhibitory effect on its activity. No stimulatory effect was observed with any of the metal cations tested. Maximal hydrolysis rates were obtained when lysine was in the N-position. Hydrolyses of substrates containing arginine and alanine were of 87.6% and 46.7%, respectively. The relative activities toward these substrates showed that the N-terminal residue presents, in order of preference: lysine, arginine, alanine, leucine, methionine, proline, and phenylalanine. No activity was observed against substrates that contained two amino acids which ones in the N-position.

3.3. *Determination of kinetic parameters*

The enzyme had a K_m value of 3.5 μM and a V_{max} value of 11430 μmol.h^{-1}.mg^{-1} on Suc-R-P-F-H-L-L-V-Y-MCA as substrate. The K_m and V_{max} values of the enzyme for Lys-pNA at pH 7.0 and 37ºC were 54.4 μM and 408 μmol.min^{-1}.mg^{-1}, respectively.

The aminopeptidase pumAPE from *U. maydis* is the first exopeptidase found in this fungus and was purified to homogeneity by ammonium precipitation and three consecutive chromatographic steps. Hydrophobic interaction and gel filtration chromatography were critical steps for separating the other protein contaminants. The purified enzyme is a dimmer with a mass of 110 kDa estimated by gel filtration and 58 kDa by SDS-PAGE as monomer.

No aspartyl proteinases have been reported in basidiomycetes. Intracellular aspartyl proteinase pumAi from *U. maydis* was purified. Molecular masses of ascomycete intracellular aspartyl acid proteinases from *Candida albicans*, *S. cerevisiae, Aspergillus niger, Neurospora crassa,* and *Coccidiodes immitis* (55-60, 41.7, 39.0, 42.9 and 45.0 kDa, respectively) are slightly higher than that of pumAi (Portillo and Gancedo 1986; Meussdoerffer *et al*., 1980; Reichard *et al*., 2000; Vazquez-Laslop *et al*., 1996; Johnson *et al*., 2000). Probably all these aspartyl proteases belong to the same orthologous family; however, no expression has been studied or associated to any dimorphic process.

To compare the pI from pumAi of *U. maydis* with the ascomycete aspartyl proteases aforementioned, proteinase A from *S. cerevisiae* (Meussdoerffer *et al*., 1980), and proteinases from *A. niger*, *N. crassa, C. immitis* (GenBank accession numbers: U03278, U36471 and AAF28186), we calculated the pI values from sequences deposited in GeneBank using bioinformatics software (http://www.expasy.org/tools/). *U. maydis* experimentally obtained pum Ai pI was 5.5, a slightly higher value than that of ascomycete proteases, which had pI values ranging from 4.4 to 5.1. No comparisons with other fungal aspartyl proteases were performed, since no basidiomycete sequences have been reported.

This is the first report on the purification and characterization of a basidiomycete aspartyl acid protease. Previous *in vitro* experiments using *U. maydis* in acidic conditions show that yeast-mycellium transition is inhibited by pepstatin A (Mercado-Flores et al. 2003), a high specific aspartyl proteases inhibitor that binds to the active site of the enzyme (Salvesen *et al*., 1989).

Probably, the enzyme has other physiological roles as it has been detected basically in yeast and mycelium during exponential growth (Mercado-Flores et al. 2003). Dimorphic transition plays an important role during the initial steps of tissue invasion; basically, a mycelial phase invades the corn seeds and other tissues (Banuett 1995). Thus, it is possible that the pumAi proteinase plays an important role, with other proteins and molecular signals, during the complex pathogenic phenomenon of *U. maydis*.

The purified enzyme pumAe is a monomer with a molecular mass of 72-74 kDa. Acid extracellular non-aspartyl proteases from other fungi (*Aspergillus niger* var. *macrosporus*, *Scytalidium lignicolum*, and *S. sclerotiorum*) have smaller molecular masses (20, 21.5 and 20.7 kDa, respectively); the same has been observed in the theoretical mass of the extracellular proteases encoded by *eapB* and *eapC* genes from *Cryphonectria parasitica* (GenBank accession No. S63630 and S63631) with a molecular mass of 28.23 and 28.25 kDa respectively, as well as in the acid protease (36.69 kDa) from *Neurospora crassa* (GenBank accession No. CAD36982), and in the pepstatin-insensitive protease from *Talaromyces emersonii* (GenBank accession No. AF439998.2) with a theoretical weight of 24.6 kDa.

In *U. maydis* the *rep1* gene is expressed abundantly in the filamentous stage, where it is required for the development of aerial hyphae. The *rep1* gene encodes a protein of 652 amino acids that is processed into 11 small peptides related in sequence. These peptides are secreted and located in the cell wall, where they mediate surface hydrophobicity and have been implicated in attachment of hyphae to hydrophobic surfaces, a function that might also be semi quantitative important for plant-fungus pathogen interaction. Specifically, peptides 1-4 lack a lysine residue expected at the N-terminus, indicating a proteolytic processing event that could be due to a lysine aminopeptidase (Wösten *et al.*, 1996). Probably, the pumAPE aminopeptidase plays an important role in the production of this hydrophobic peptide, and indirectly participates during plant-pathogen interactions.

Proteolysis plays an essential role in response to stress, which, probably gives since as, gives as result, a complete reorganization of the cellular metabolism; for example, the process of sporulation of the yeast *S. cerevisiae* is taken to end in conditions of nutritional stress, in this case there has been demonstrated that the activity of the enzymes for protein hydrolyses increases considerably, principally those that are of location vacuolar (Hilt *et al.*,

1992). The *PRAum* gene expresses in presence of corn infusion and ammonium sulphate, suggesting that its expression is regulated by an easy assimilation source of nitrogen and also by inductor molecules from the host (corn) when added as nitrogen source. On the other hand, *APEum* expresses constantly when cells are exposed to proline (not easy assimilation nitrogen source) and when cells are grown in a medium lacking of source of nitrogen. This suggests that the protein encoded by *APEum* may be involved in nitrogen metabolism, providing the cell the nutriensts it requeires when growing in a stressing medium. The aminopeptidase pumAPE could participate by supplying the cell with basic amino acids, as has been suggested for the peptide products of proteosomes (Hilt *et al*., 1995; Kisselev *et al*., 1999) or during activation and inactivation of peptides (Cadel *et al*., 1995; Hersh *et al*., 1987).

The presence of a bindign site for pacC protein in the *PRAum* promoter region suggests pH regulation. No pacC-binding site was found in *APEum* promoter region. In case of phytopathogen fungi it is possible to think that the structural proteins of the cellular wall corn, limitation of nitrogen or nutrients and the pH might regulate the production of the proteases during pathogenesis.

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Atypical laccases from the white-rot fungus *Pleurotus ostreatus* and their application for the treatment of industrial coloured effluents

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1. Introduction

1.1. *Laccases*

White-rot fungi are the most efficient decomposers of lignocellulose because of their capability to synthesize the relevant hydrolytic (cellulases and hemicellulases) and oxidative (laccases, lignin-peroxidases and Mn-peroxidases) extracellular enzymes required to degrade the major components of substrates (cellulose, hemicellulose, and lignin) into low-molecular-weight compounds that can be assimilated in fungal nutrition [1]. Recently, extensive research on these fungi has been conducted with the aim of isolating new organisms able to secrete new enzymes with capability to be used in industrial applications, such as bioremediation of polluted soils and industrial waste-waters, biobleaching and biopulping in pulp and paper industries, textile and food industries, *etc*.

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Fungal laccases (benzenediol: oxygen oxidoreductases; EC1.10.3.2) are ligninolytic enzymes that have been isolated from various fungi [2]. They belong to the class of the blue oxidases containing 4 copper atoms/molecule distributed in three different copper binding sites [3, 4]. The type-1 site is responsible for the intense blue colour of the enzyme due to a maximum absorbance at 605 nm; the type-2 site does not exhibit signals in the visible absorbance spectrum; and the type-3 site incorporates two copper centres and is responsible for a band near 330 nm. All these copper ions are involved in the catalytic mechanism. Laccases reduce oxygen to water and simultaneously perform a one electron oxidation of aromatic substrates (polyphenols, methoxysubstituted monophenols, aromatic amines, etc.). These enzymes are present in multiple isoforms, depending on the fungal species and environmental growth conditions [5, 6].

1.2. Pleurotus ostreatus *laccases*

Laccase isoenzymes produced by *Pleurotus ostreatus*, a white rot basidiomycete fungus, have been extensively studied. Five different isoenzymes have been purified and characterized: POXC, POXA1b, POXA1w, POXA3a and POXA3b; seven different genes and the corresponding cDNAs have been cloned and sequenced [7, 8, 9].

POXC is the most abundantly isoenzyme produced under all the growth conditions examined [7] (maximum production level 25 mg/l); it shows all typical laccase characteristics: four copper ions/molecule, acidic pI, and stability to proteolytic degradation. All the others *P. ostreatus* laccases (POXA1w, POXA1b, POXA3a and POXA3b) are atypical phenol-oxidases [10, 8, 11].

POXA1w shows a remarkable high stability with respect to both pH and temperature if compared with that of POXC and of other known laccases, and it exhibits a neutral pI (6.7). The most striking characteristic of this protein is the lacking of the typical blue colour and its unusual metal content. UV/visible spectrum, atomic absorption and polarography proved that this enzyme contains only 1 copper atom/molecule- instead of the usual 4 atoms- and furthermore, 2 zinc atoms and 1 iron atom. Nevertheless, the classification of this enzyme as laccase was based on: i) the high degree of identity of

the determined stretches of primary structure with the corresponding sequences of known laccases; ii) the use of O_2 as oxidative substrate and the lack of formation of H_2O_2 as a product in the catalysed reaction; iii) the almost standard pattern of substrate specificity.

POXC production is strongly increased by the presence of copper ions, whilst POXA1w production is substantially unaffected under this condition; furthermore a new neutral laccase isoenzyme (POXA1b) is produced in copper supplemented cultures. POXA1b shows the same characteristics of POXA1w concerning the stability with respect to pH and temperature. Furthermore POXA1b is only partly secreted [12].

In this study we review purification and characterisation of two closely related isoenzymes, POXA3a and POXa3b, and their role in decolourisation of Remazol Brilliant Blue R (RBBR), a molecule frequently used as starting material in the production of many polymeric commercial dyes [13]. We also describe optimal conditions for using selected purified laccases or immobilized crude laccase mixtures in the treatment of this model dye [14].

2. Materials and Methods

2.1. *Organism and culture conditions*

White-rot fungus, *P. ostreatus* (Jacq.:Fr.) Kummer (type: Florida) (ATCC no. MYA-2306) was maintained through periodic transfer at 4 °C on PDY agar plates (2.4% potato dextrose and 0.5 yeast extract, Difco Laboratories, Detroit, MI). Incubations were carried out as previously described [10] and laccase production was induced by addition of 150 μM copper sulphate.

2.2. *Enzyme purification*

Secreted proteins were precipitated from the filtered medium by addition of $(NH_4)_2SO_4$ up to 80% saturation and, after extensive dialysis, loaded onto a DEAE Sepharose Fast Flow (Pharmacia Biotech Inc.) column as previously described [10]. Two fractions containing laccase activity, recovered with the equilibrating buffer, were separately pooled, concentrated on an Amicon

PM-10 membrane and equilibrated in Tris-HCl 50mM pH 8.0. Each pool was loaded onto an anion exchange Mono Q HR5/5 (Pharmacia) column in a fast protein liquid chromatography system (FPLC, Pharmacia) equilibrated with the same buffer. The active fractions were pooled, concentrated and loaded onto a gel filtration Superdex 75 PC 3.2/30 column in a SMART System (Pharmacia); the active fractions were pooled and desalted.

2.3. *Enzyme assays*

Laccase activity was assayed at 25 ºC, using 2,2'-azinobis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS), DMP and syringaldazine as substrates as previously described [10].

2.4. *Remazol Brilliant Blue R transformation by laccase*

The decolourising experiments were performed using the dye RBBR 50 μM, and the purified laccase POXC or POXA3 (1 U/ml) in sodium acetate 20 mM, pH 4.5 buffer. The same incubation was performed using a laccase mixture of POXC and POXA3 (1:1, U:U ratio) from 1 to 10 U/ml final activity. Control samples without enzyme, were run in parallel under identical conditions. All reactions were incubated at 20 ºC for 100 min.

2.5. *Effect of temperature and pH on dye decolourisation*

The effect of temperature on dye decolourisation was studied incubating the reaction mixture, prepared as above described, at 20, 30 or 40 ºC. The effect of pH on dye decolourisation was studied performing the experiments in the pH range 4-7 using the Mc Ilvaine buffer. In all cases, dye decolourisation was monitored as above described.

2.6. *Immobilization method*

The immobilization procedure was carried out at 4 ˚C. Different amounts of crude laccase preparation (from 0.2 to 660 U) were mixed with 5 ml of 3% sodium alginate solution (low viscosity, Sigma), centrifuged at 4,000 rev

min-1 for 5 min to remove air bubbles, and extruded drop by drop through a needle (0.4 mm internal diameter) into a 0.15 M $CuSO_4$ aqueous solution (pH 4.0) under continuous agitation. The resulting spherical blue beads were left to solidify for at least 30 min in the copper solution and then washed exhaustively with distilled water until pH 5.0-5.5 was reached. The total wet weight of beads obtained from 5 ml of sodium alginate solution was about 3.5 g. The beads were stored wet at 4 °C. The immobilization yield was calculated as the fraction of laccase activity found after dissolution of alginate beads (by incubation for 3 h at 4 °C in 50 mM sodium phosphate buffer pH 7.0, 50 mM EDTA) with respect to the activity of the enzyme added to the soluble alginate preparation.

2.7. *Continuous packed-bed reactor*

A glass column (130 mm x 17 mm, working volume 25 ml) was filled with 24 g of copper alginate beads. The flow rate was 0.2 ml/min, and the feed solution was 50 μM RBBR in 20 mM sodium acetate buffer, pH 4.5. Samples were collected after the passage of at least 25 ml of feed solution to reach the concentration equilibration (time 0). All reactor systems were operated at room temperature (approximately 20 °C).

2.8. *Chitosan treatment of alginate beads*

A 24 g portion of copper alginate beads was transferred into a solution of 0.15% (w/v) chitosan, in 20mM sodium acetate buffer pH 4.5 containing 0.1 M CuSO4, and incubated for 20 h at 4 °C on a rotary shaker. After that, the alginate chitosan beads were extensively washed with 20 mM sodium acetate buffer pH 4.5 and used to fill the glass vessel reactor as described above.

3. Results

3.1. *Purification of laccase isoenzymes POXA3a and POXA3b*

Two active different protein fractions (POXA3a and POXA3b) were recovered after chromatography (fig 1) of the ammonium sulphate precipitate of

culture broth collected after 10 days. Both isoenzymes were purified to homogeneity (gel filtration chromatography, fig 2, native PAGE and isoelectrofocusing). Isoelectric points of POXA3a and POXA3b are 4.3 and 4.1, respectively, and the molecular mass, determined by gel filtration chromatography, is 56 kDa for both proteins. Specific activities towards ABTS of POXA3a and POXA3b are 2000 and 1050 U/mg, respectively. However purified isoenzymes displayed a more complex pattern when analysed by SDS-PAGE, in fact three bands (MW 67, 18 and 16 kDa, respectively) were observed for both proteins (fig 3).

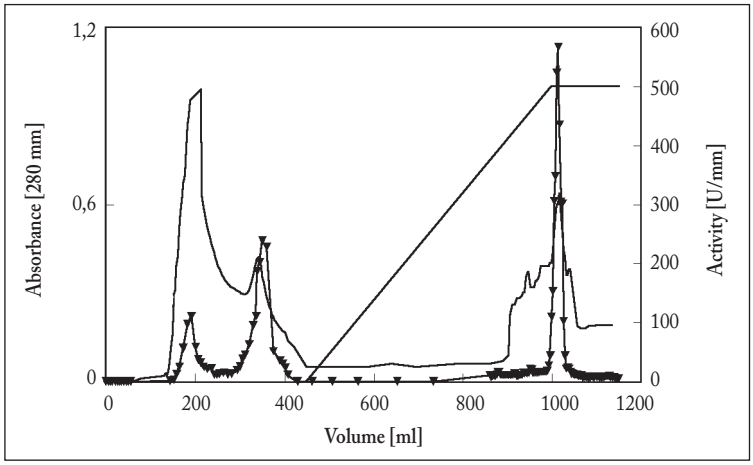

Figure 1. DEAE-Sepharose chromatography of P. ostreatus-secreted proteins. DEAE-Sepharose fast flow elution profile of proteins and laccase isoenzymes secreted by *P. ostreatus* is shown with three different active protein fractions (POXA3a, POXA3b, and POXC).

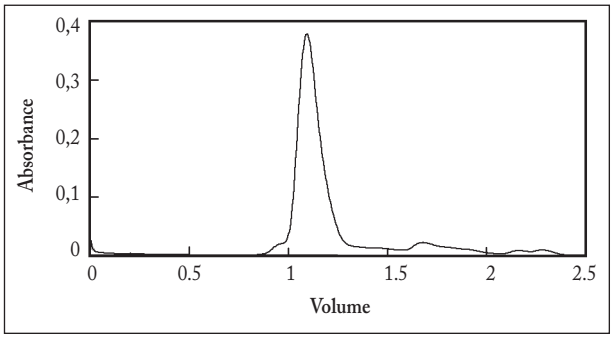

Figure 2. Gel filtration chromatography of POXA3a fraction from MONOQ: a single peak is detectable

3.2. *Metal content analyses*

UV-Vis absorption spectra of POXA3a and POXA3b display the characteristic absorbance peak at 600 nm, due to the presence of type I copper; copper content, determined by atomic absorption, results to be 3.3 copper/protein (mol/mol) for both proteins.

3.3. *Structural analyses*

The two isoenzymes were analysed by MALDI-MS: the mass spectra showed a peak exhibiting a molecular mass centred at about 60,860.4 Da for POXA3a and 60,700.6 for POXA3b; moreover the spectra showed the occurrence of two other components whose molecular masses were measured as 16,870.5 and 18,103.8 Da for POXA3a and 16,838.1 and 18,090.1 Da for POXA3b. The N-terminal sequences of the 60 kDa subunits are identical for both POXA3a and POXA3b, while the same analyses, carried out on the four 16 and 18 kDa subunits, revealed that all subunits have a blocked N-terminus.

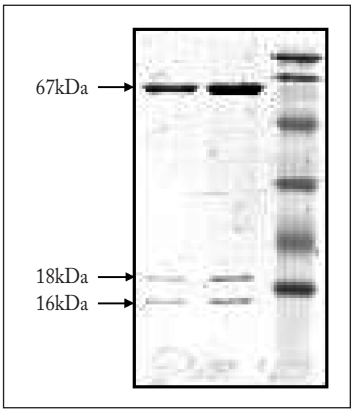

Figure 3. Coomassie stained SDS–PAGE of purified POXA3a and POXA3b laccase isoenzymes.

Mass spectrometric analyses (peptide fingerprints) and N-terminus sequencing did not reveal any significant differences between POXA3a and POXA3b large subunits. Furthermore these analyses show that differences between the 18 and 16 kDa subunits of both isoenzymes are only due to the presence of a glycosidic moiety on the 18 kDa subunits. None of the sequenced peptides from small subunits shows significant homology with proteins in data banks.

3.4. *Cloning and sequencing of poxa3 gene and cDNA*

Oligonucleotide-primer mixtures were designed on the basis of POXA3a tryptic peptide sequences. The 500 bp amplified fragment, homologous to

known laccase genes, was used to screen a *P. ostreatus* genomic library. Two oligonucleotides were designed using the predicted N and C termini of the protein and were used to amplify POXA3 encoding cDNA. The 1500 bp amplified fragment was cloned and sequenced allowing the determination of *poxa3* gene whole structure. The coding sequence is interrupted by 21 introns.

3.5. *POXA3a and POXA3b activation*

POXA3 specific activity increases during fungal growth, probably due to proteolytic activation. POXA3 isoenzymes, purified from broth supplemented with Phenylmethanesulfonyl fluoride (PMSF), were incubated with the serine protease from *P. ostreatus* (*Po*Sl) (fig 4). Under this condition, an increase of POXA3 activity was obtained (about 30%), confirming the generation of more active POXA3 isoform(s) due to *Po*Sl-induced proteolysis.

3.6. *Biotransformation of RBBR by* P. ostreatus *laccases*

Decolourisation experiments by *P. ostreatus* in solid and liquid media were performed using PDY broth in the presence of veratryl alcohol, added with RBBR at two different concentrations (5 and 50 μM). *P. ostreatus* is able to decolourise the dye in all cultural conditions examined, with a temporal relationship between laccase production and dye biotranformation (fig. 5).

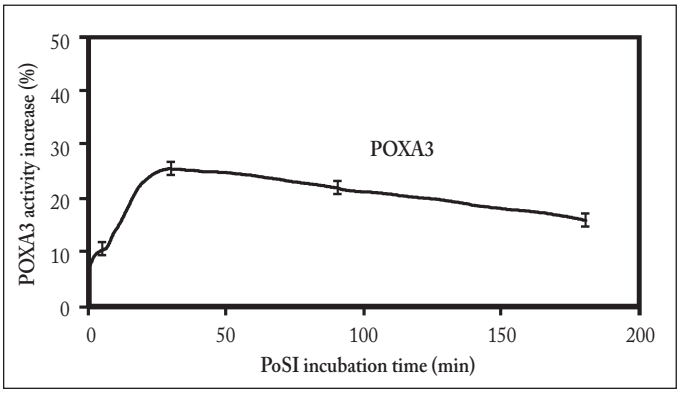

Figure 4. *In vitro* incubation of POXA3 with an extracellular subtilisin-like *Pleurotus ostreatus* protease (*Posl*).

In order to elucidate the role of the oxidative activities produced during biodegradation of RBBR by *P. ostreatus*, purified POXA3 (1 U/ml) or POXC (1 U/ml) isoenzymes were incubated at 20°C with RBBR: after 100 min of incubation, 30 and 50% of decolourisation was obtained by POXC and POXA3, respectively. No more than 60% of decolourisation was obtained even when the enzyme concentration or the incubation temperature was increased. Kinetic parameters of the two laccases were also determined using RBBR as substrate: the *K*m values (0.054 and 0.051 mM for POXA3 and POXC, respectively) are very similar whilst the catalytic efficiency (*k*cat/*K*m) of POXA3 ($9.0x10^6$ mM^{-1} min^{-1}) is six-fold higher than that of POXC ($1.5x10^6$ mM^{-1} min^{-1}). Therefore, the difference in decolourisation efficiency of the two laccases could be due to a variation in enzymatic turnover rather than in substrate affinity.

The effect of a 1 U/ml (final laccase activity) POXC/POXA3 mixture (1:1; U:U) on RBBR was tested. As shown in figure 6, the enzymatic mixture determines an increase both in the rate and in the final level of dye decolourisation (74%) with respect to each isoenzyme working separately. Also enzyme concentration and incubation temperature affected dye decolourisation.

3.7. *RBBR decolourisation by crude laccase complex mixture.*

Crude enzymatic mixture obtained after ammonium sulphate precipitation of culture broth supplemented with $CuSO_4$ and ferulic acid was found to be more efficient in RBBR decolourisation than any single component.

The effect of enzyme concentration on dye degradation was studied: increasing the amount of the crude preparation used, from 2 to 100 U/ml of laccase activity a more efficient dye decolourisation (up to 70%) was observed (fig 7).

3.8. *Immobilization of crude laccase mixture in copper alginate beads and its performance in dye decolourisation*

Optimal conditions for crude laccase mixture immobilization by entrapment in copper alginate beads were set up. Immobilization yield was 65% when laccase concentrations ranging from 15 to 120 U/ml of sodium alginate solution were used, obtaining beads whose laccase activity ranged from 20 to 100 units per gram of beads (U/g). Immobilized protein mixture in copper

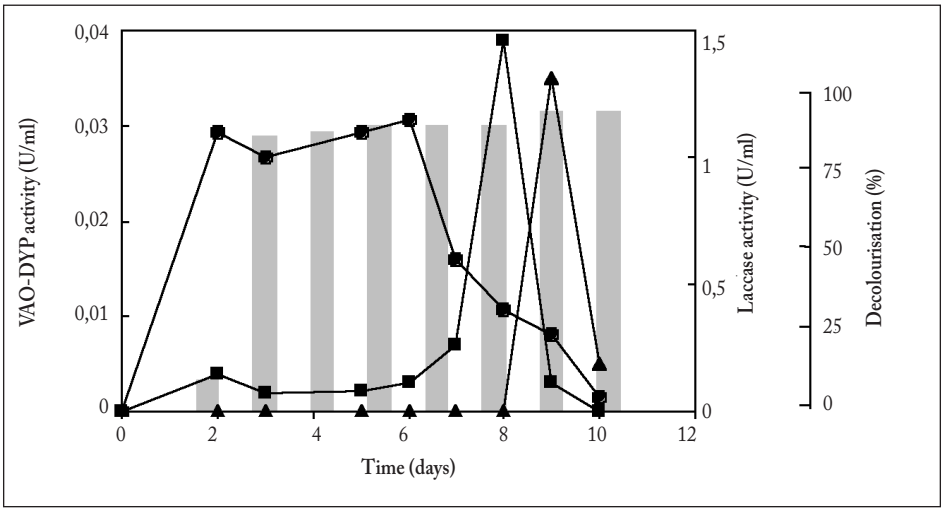

Figure 5. Decolourisation of RBBR in *P. ostreatus* culture supplemented by 50 μM of Remazol Brilliant Blue R. Symbols: laccase activity (•); DYP activity (▲); AAO activity (+); percentage of decolourisation ( ).

alginate beads is more stable if compared with free enzyme mixtures. Optimal conditions for batch decolourisation process were determined (pH 4.5, temperature 20°C, 100 Units per gram of beads).

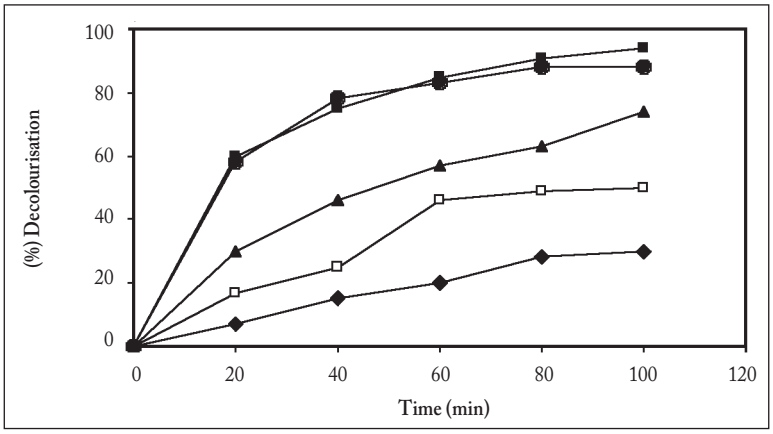

Figure 6. Remazol Brilliant Blue R decolourisation after treatment with laccase isoenzymes in different reaction conditions; POXC (1 U/ml; 20 °C) (❑); POXA3 (1 U/ml; 20 °C) (◆); POXC/POXA3 mixture (1:1, U:U; 1 U/ml; 20 °C) (▲); POXC/POXA3 mixture (1:1; U:U; 10 U/ml; 20 °C) (■); POXC/POXA3 mixture (1:1, U:U; 1 U/ml; 30 °C) (•).

3.9. *Performance of immobilized laccase mixture in a fixed-bed reactor*

The immobilized enzyme mixture was used over 7 days in a fixed-bed reactor, under the optimal conditions set up in batch experiments, operating at a RBBR loading rate of 20.9 mg L^{-1} h^{-1} (fig 8). In these conditions (bioreactor 1) the final decolourisation percentage was 20% after elution of 100 V/V_R (volumes of dye solution respect to the reactor retention volume, V_R=18 ml). The leached laccase activity, determined during this continuous operation, quickly increased at the beginning of the decolourisation process, and the total activity washed out amounted to 1000 U. Attempts were performed to decrease enzyme release.

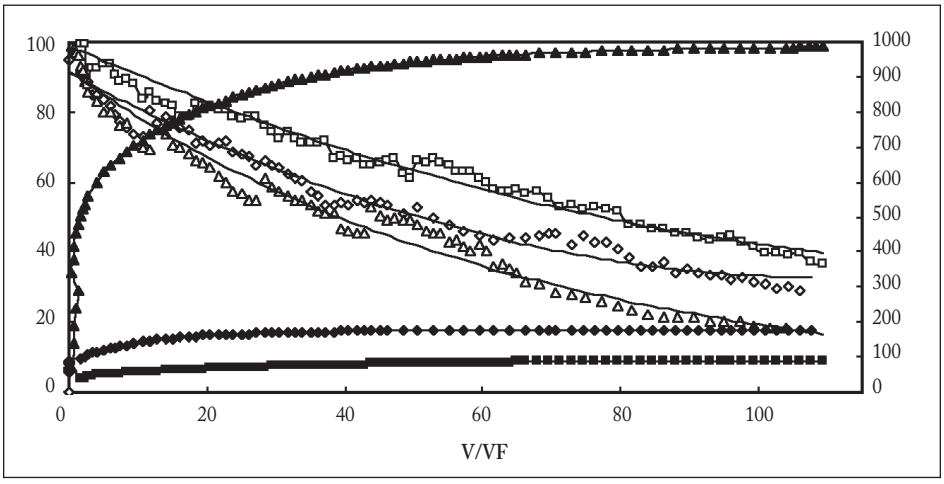

Figure 7. RBBR decolorization in continuous experiments by packed bed bioreactors. Bioreactor 1 (∅): 100 U laccase activity/g copper alginate beads. Bioreactor 2 (◊): 50 U/g. Bioreactor 3 (❑): 50 U laccase activity/g copper alginate beads treated with chitosan. Laccase leaching was measured (▲, bioreactor 1; ◆ bioreactor 2; ■ bioreactor 3).

An experiment (bioreactor 2) was performed using a lower amount of immobilized enzyme mixture (50 U/g). As shown in figure 8, the final decolourisation percentage obtained by this bioreactor was enhanced (about 30%) and laccase leaching was 5-fold decreased with respect to the previously mentioned

continuous experiment (bioreactor 1). Further improvements in the final decolourisation level (40%) and in laccase retention were obtained using chitosan treated beads (bioreactor 3). As a fact, chitosan is known to improve beads mechanical resistance and significantly to reduce leaching [15, 16].

4. Discussion

POXA3a and POXA3b, two closely related laccase isoenzymes, are produced in *P. ostreatus* copper supplemented cultures. SDS-PAGE and MALDI-MS analyses of purified POXA3a and POXA3b reveal the presence of three different polypeptides of 67, 18 and 16 kDa, whereas the native proteins behave homogeneously (as demonstrated by gel filtration chromatography, isoelectrofocusing and native-PAGE analysis). None of the previously characterised *P. ostreatus* laccase isoenzymes shows similar behaviour, and all of them are monomeric proteins. On the other hand, it has been reported that some laccase enzymes from *Phellinus ribis* [17], *Trametes villosa* [18] and *Rhizoctonia solani* [19] show homodimeric structure.

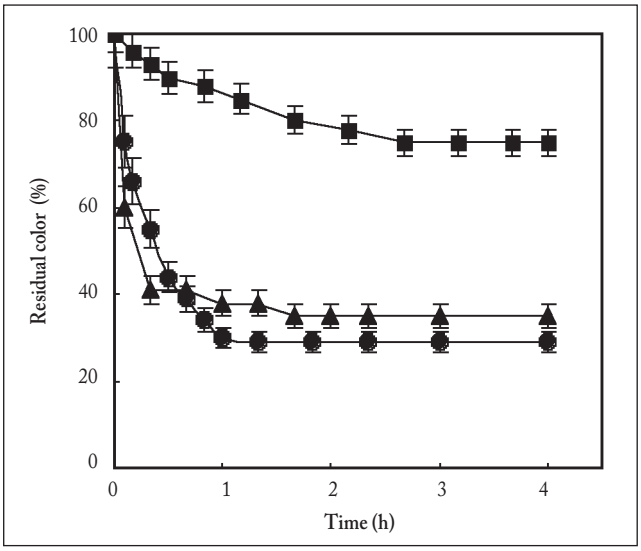

Figure 8. RBBR decolourisation obtained by incubation with different amounts of crude laccase mixture. •º, 2 U/ml laccase activity, 20 ˚C; ■ 20 U/ml laccase activity, 20 ˚C; ▲100 U/ml laccase activity , 20 ˚C.

An unique *poxa3* gene has been identified and protein sequence deduced by cDNA has been verified by means of MALDI-MS mapping against the POXA3a and POXA3b large subunits. It is not possible to univocally associate this gene to either POXA3a and/or POXA3b, or to exclude the existence of another *poxa3* gene. Deduced amino acidic sequence contains all putative copper-binding residues, as well as the five Cys residues found in all the known laccase sequences.

Sequence data from the 18 kDa POXA3a subunit, accounting for about 50% of entire sequence, did not give information on the nature of this subunit because of the absence of significant homology with other known proteins. Furthermore, no sequence encoding these peptides have been recognised in the 3' and 5' flanking region of the *poxa3* gene, thus excluding that small subunit could be originated from maturation of a single polypeptide chain containing the largest one.

Due to their enzymatic properties and relatively low production cost, laccases represent a promising tool for applications in the textile industry effluent bioremediation.

POXC and POXA3 laccases are able to perform RBBR transformation *in vitro*. In particular, POXA3 shows higher decolourisation efficiency with respect to POXC, as also confirmed by kinetics constants determined using RBBR as substrate. It has been also verified that a more efficient process take place in the presence of a mixture of POXC and POXA3 suggesting that RBBR degradation *in vivo* could be due to a concerted action of the two isoenzymes. Hence, we demonstrated that a complete RBBR transformation can be obtained using a simple mixture of two laccase isoenzymes in the absence of any redox mediators and selecting optimal enzyme concentration, temperature and pH values. These findings differ from those reported for other laccases, which also transform RBBR in the absence of redox mediators, but in this case only an incomplete decolourisation is obtained [20, 21, 22]. On the other hand, other authors [23, 24] reported that RBBR is decolourised only when small molecular weight redox mediators are added to the laccase enzymes. These results suggest a strict correlation between decolourisation and detoxification. Reasonable basis for development of a cheaper biotechnological colour reduction process have been provided: a crude laccase mixture preparation was used to decolourise RBBR. Once the potentiality of the enzymatic system had been assessed, the mixture was im-

mobilized by entrapment in copper alginate beads. RBBR decolourisation efficiency was about 70% even after 20 cycles of stepwise dye additions in batch operations. Different strategies for continuous decolourisation in a fixed-bed bioreactor were analysed. The best performances were obtained by decreasing enzyme loading and improving laccase retention by coating the alginate beads with chitosan.

5. Acknowledgement

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Section 2

Lecture abstracts

Natural and Artificial Hybridization of *Agaricus subrufescens* Peck (= A. Blazei Murrill Sensu Heinemann): Lessons from the Quasi-Alleles of the rDNA ITSI+2 Region.R.

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Agaricus subrufescens Peck was described from both wild and cultivated specimens in 1893. It has been sporadically cultivated in various countries since that time, and is presently an economically important "nutriceutical" food. It is known by several names, including *A. rufotegulis* Nauta, *A. brasiliensis* Wasser et al., and *A. blazei* Murrill *sensu* Heinemann. A long-term study of diverse isolates and specimens, emphasizing cultural studies and analysis of rDNA ITS1+2 sequences, strongly indicates that a single phylogenetic entity exists. Some interpopulational interfertility has also been demonstrated. Yet the picture is not simple. The species is amphithallic, with complementary reproductive routes, producing recombinant spores with cryptic karyotic states and some self-fertility. Sequences from the Americas were always highly heteromorphic, while those from Hawaii and the UK were homomorphic. This implies that American isolates may be hybrids between (at least) two formerly isolated populations. To test that idea, ITS1+2 sequences from isolate SBS1, an SSI from a California strain, were amplified, cloned and sequenced. Both allelism and recombination are evident in these 711-713 nt sequences: 4 (3+1) parental and 11 recombinant sequences were recovered. The mechanism of fine-scale recombination is unknown (PCR artifacts have not been ruled out). Recombination events exceeded 1.0 per 700 nt. Physical linkage was apparent among 11 polymorphic characters distributed along the ITS1+2. On this basis the parental allelic sequences were deduced, and a comparison with the homomorphic UK sequence was made. The evidence suggests that a European-like strain may have contributed one ITS1+2 allele to an ancestor of the isolate from California. However, if true, "crossovers" must then occurred prior to the origin of the SBS1 SSI, possibly in the SBRF progenitor (or its progenitor(s)).

Genetics and Cellular Biology of Basidiomycetes-VI. A.G. Pisabarro and L. Ramírez (eds.)
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Expression of Mating Type Genes in Heterologous Basidiomycetes

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Mating processes in basidiomycetes are controlled by genes encoding two types of transcription factors (HD1 and HD2) and by genes encoding pheromones and pheromone receptors. For a successful mating reaction, an HD1 protein and an HD2 protein of different specificity have to interact and a pheromone with a pheromone receptor of different specificity. With now having cloned mating type loci from several different basidiomycetes, evolution of these loci and their genes can be addressed by sequence analysis as well as by transformation of genes into heterologous species.

Transformation of cloned mating type genes into strains of the same species with different mating type genes can activate mating type controlled development. The *A* mating type genes of *Coprinopsis scobicola* and of *Coprinellus disseminatus* were found to be functional in *Coprinopsis cinerea* in combination with the endogenous *A* mating type genes. Moreover, *B* genes of *C. disseminatus* in *C. cinerea* cause peg formation subapical to septa with *A*-induced clamps cells and fusion of clamp cells with the subapical peg. In several *C. cinerea* monokaryons, transformed *A* genes of *Schizophyllum commune* were not observed to induce clamp cells by interaction with endogenous *A* genes. However, in crosses of *C. cinerea* transformants carrying compatible *S. commune A* genes, clamp cell production has occasionally been observed. To our surprise, when using *S. commune A* genes or the homologous *b* genes of *Ustilago maydis* to transform a specific *C. cinerea* monokaryon, colonies of transformants may develop faster growing sectors with hyphae having clamp cells.

Our laboratory is supported by Deutsche Bundesstiftung Umwelt (DBU) and scholarships by the Mahasarakham University (to PS) and the Rajamangala Institute of Technology (to WC).

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Development of a Sporeless Strain of Oyster Mushroom (*Pleurotus ostreatus*)

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The enormous amounts of spores produced by oyster mushroom (*Pleurotus ostreatus*) cause lung-related health problems among employees working in oyster mushroom cultivation. If sporeless varieties are used for large-scale cultivation, these lung problems can be avoided.

For development of a commercially attractive strain of sporeless oyster mushroom, strain ATCC 58937, a sporeless strain of oyster mushroom was used as a donor of the trait. Microscopic analysis of basidia showed that meiosis was aborted at an early stage. Both nuclear types that constitute strain ATCC 58937 could be retrieved by protoplasting. Protoplasting commercial strain HK35 yielded only one of its nuclear types. Crosses between the ATCC nuclear types and the HK35 nuclear types (either directly or using the Buller phenomenon) yielded normal sporulating strains, indicating that sporelessness was caused by a recessive trait. Among the offspring of crosses between the ATCC nuclear types and the HK35 nuclear types the sporeless trait segregated in a 1 to 1 ratio. The sporeless trait could be mapped and strongly linked genomic markers were developed.

The breeding strategy was to successfully introduce sporelessness into both nuclear types of a commercial variety, to achieve a sporeless variety. In a first cross between a sporeless culture and a commercial strain, not only sporelessness was transferred to the commercial variety. Therefore, repeatedly backcrossing the progeny of the first cross with the commercial variety is used to try to restore the original genetic material from the commercial strain as much as possible. Performance of a number of "prototypes" of a sporeless oyster mushroom was tested on commercial mushrooms farms and proved to be satisfactory.

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Qtl Mapping of Pathogenicity in *Heterobasidion annosum sensu lato*

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Heterobasidion annosum (Fr.) Bref. s*ensu lato*, casual agent of annosum root rot in conifers, is the economical most devastating forest pathogen in the northern hemisphere. A genetic linkage map of *H. annosum*, was constructed from a compatible mating between isolates from the North American S and P intersterility groups. The linkage analysis of 358 AFLP markers in 102 progeny isolates generated 19 linkage groups containing 6 or more markers that covered 1468 cM.

Two distinct methods were used to analyse the segregation of pathogenicity. 1 year old pine seedlings grown in the greenhouse were infected with *H. annosum* progeny isolates. Pathogenicity was measured as mean necrosis lengths caused in 10 pine plants. One QTL for pathogenicity was found on linkage group 15 with a LOD peak of 2.95, spanning a 31.2 cM large area. This QTL explained 14.3% of the variation. Another QTL were found in a small linkage group containing 5 markers and spanning 36.8 cM, with a LOD peak of 4.40 at marker *paacts02*. 19.1% of the variation could be explained by this QTL. The heritability of pathogenicity on pine was estimated to be 0.21 in this study.

The disease increase rate values from an *in vitro* test were used as another estimate of pathogenicity and used for the QTL-analysis. From the *in vitro* pathogenicity test, a large area of 26.5 cM on linkage group 11 between the markers *acgcs5* and *paacgp08* contains a high QTL probability of LOD 3.09. This QTL explained 16.4% of the total variation in this experiment. The heritability of pathogenicity on pine was estimated to be 0.088 in this study.

Successfully localization of the two intersterility genes (S and P) on the map were carried out through mating of the progeny isolates with three tester strains carrying known intersterility genotypes.

Genetics and Cellular Biology of Basidiomycetes-VI. A.G. Pisabarro and L. Ramírez (eds.)
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Gene Expression During Symbiosis in the Ectomycorrhizal Fungus *Tricholoma vaccinum*

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We have identified genes specifically expressed in host interaction using an RNA fingerprinting technique. These genes can subsequently be used to identify host signals for induction of genes with function in host specificity. The easy method allowed to screen RNA from mycorrhiza of different age (2 weeks to 13 months) synthesized *in vitro* in comparison to RNA from both partners, *Tricholoma vaccinum* and *Picea abies*, grown in single culture. The resulting cDNA was used in an arbitrarily primed PCR using 5 primers which had been selected out of 20 to give more than 20 bands of a size up to 1.9 kb. From 145 bands separated on agarose gels 52% were differentially expressed. The fungal genes were identified by Southern blot analyses and subsequenly differential expression was verifie by reversed Northern blots. Since the bands were, in many cases, still containing more than a single fragment, 130 fragmetns were cloned, of which 57 were mykorrhiza-specific.

Of 23 fungal genes with mycorrhiza-specific expression, sequence analyses were performed in order to identify the nature of the encoded protein *in silico*. Among them different classes of function were defined with respect to putative function of the respective gene in the symbiosis. Examples with possible ecological function include aldehyde and alcohol dehydrogenases, ubiquitin binding protein, phospholipase B, ß-1,4 glucosidase, a binding protein for basic amino acids, an APS kinase, two MATE transporters and Ras. The possible roles of these proteins will be discussed. Additionally, two different classes of retrotransposon were identified which is the first identificaiton of actively expressed trnasposons in ectomycorrhizal fungi which might be the reason for high morphological diversity observed with *T. vaccinum*.

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Soybean Rust, a Rising Star in Phytopathology

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Phakopsora pachyrhizi, an obligate parasite, causes soybean rust, the most devastating foliar disease on soybeans. It is responsible for significant losses of soybean crop in Africa, Asia, Australia and South America, becoming a major threat to world soybean production. Recent findings of this pathogen in the Southeast continental U.S.A. have triggered an increasing interest in the scientific community. Here, we present strategies and preliminary results from the *P. pachyrhizi* Genome Sequencing Project.

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Evidence for a 200 gene Ribosome and rRNA Biosynthesis (rrb) Regulon in Fungi

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Two challenges of the post genomic era are 1) the need to assign functions to as yet uncharacterized gene products, and 2) the requirement to understand how the expression profiles of large sets of genes are regulated in response to changing environments. Towards these aims, we have used transcriptional profiling analysis to identify and characterize a large set (over 200 genes) of transcriptionally co-regulated genes whose products are involved in rRNA and ribosome biosynthesis. Many of the genes within this set were previously unknown with regards to their function. This RRB regulon is distinct from the ribosomal protein (RP) regulon, and is characterized by a unique pair of conserved promoter motifs. The organization of the RRB regulon appears to be evolutionarily conserved at least from *S. cerevisiae* to *S. pombe*. The strategies used to identify and characterize this gene set can be widely used in other organisms to help fulfill the two needs outlined above.

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Ectopic Expression of constitutively Activated Small GTPase Cdc42 Alters the Morphology of Haploid and Dikaryotic Hyphae in the Filamentous Homobasidiomycete *Schizophyllum commune*

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Cloning of the *Cdc42* gene from *Schizophyllum commune* enabled investigation of the role of *ScCdc42* in the regulation of vegetative growth and sexual reproduction in this fungus, which has a well-characterized hyphal cell structure, cytoskeleton and mating system. Ectopic expression of the constitutively active *Sccdc42*^{G12V} or *Sccdc42*^{Q61L} allele from native or inducible *ScCel1* promoters had dramatic effects on hyphal morphology, cytoskeletal structure and Cdc42 localization, while ectopic over-expression of the wt or the dominant negative *ScCdc42*^{D118A} allele had no detectable effect. For transformants with constitutively active *Sccdc42* tip growth of apical cells in the leading hyphae was normal but polar tip growth in side branches was altered implying different regulation of polarity establishment in the two groups of apical cells.

The *S. commune* genome also contains a gene encoding RacGTPase. Rac1 might regulate the polarized growth of leading hyphae while ScCdc42 regulates the development of side branches in *S. commune*. In transformants with constitutively active *Sccdc42* branch emergence at exceptional sites and isotropic growth next to the septum proved that the branch site selection and subsequent hyphal development are under ScCdc42 control. Poor dikaryotization along with irregular clamp connections in mates with *Sccdc42*^{G12V} or *Sccdc42*^{Q61L} allele suggested that Cdc42 also contributes to efficient mating in *S. commune*. Our results provide strong evidence that Cdc42 is involved in the control of hyphal morphogenesis in filamentous homobasidiomycetes.

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Biochemistry of Volatile Compounds Synthesis in *Agaricus bisporus*

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Agaricus bisporus unique flavour is due to the release of a set of eight-carbon volatile compounds, which biosynthetic pathway has not been elucidated yet, despite of the numerous implications of those volatile compounds. Beside their influence on crop quality, they are also important for insect perception and play a part in triggering the switch from vegetative to reproductive growth in mushrooms.

8-carbon volatiles are derived from the oxygenation and the cleavage of the polyunsaturated fatty acid linoleic acid. This reaction has similarities to the plant system, but also major differences. Examination of the enzymic mechanisms and the fatty acid chemistry suggested that the enzyme involved in the oxygenation step could be a lipoxygenase (as found in plants) or a heme-dioxygenase, similar to the recently isolated linoleate diol synthase from *Gaeumannomyces graminis*.

In order to characterise the biochemical pathway leading to eight-carbon volatile production, we investigated fatty acid and lipids distribution in *Agaricus bisporus*, as well as hydroperoxide and volatile compounds levels. In parallel, we searched for candidate genes susceptible to encode the enzyme responsible for this novel oxidation route in fungi.

The combination of analytical methods, such as GC-MS, with a molecular approach based on degenerate PCR and library screening provided us with a broad range of results. These results establish the relation between fatty acids and volatile compounds and enabled us to gain a better understanding of mushroom volatiles biosynthesis and lipid metabolism.

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Expression of GFP and DsRed in the Homobasidiomycete *Schizophyllum commune*

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Expression of GFP and DsRed was studied in the homobasidiomycete *Schizophyllum commune*. *CGFP* or *sGFP* fused to the *SC3* promoter resulted in similar steady state mRNA levels. These levels were considerably lower than that of *SC3*. Despite the low mRNA levels, both *GFP* variants resulted in fluorescent hyphae. The *sGFP* expressing strains showed stronger fluorescence than the *CGFP* expressers. When *CGFP* was fused to the N-terminal or C-terminal part of the mature SC3 protein, no fluorescence was observed. However, accumulation of *GFP::SC3* mRNA was almost similar to that of endogenous *SC3*. Moreover, secreted proteins of transformants (ΔSC3 background) secreting the N-terminal fusion reacted with an SC3 antiserum. A C-terminal fusion of GFP to the SC15 protein did result in fluorescence. However, the protein was found in the cytoplasm instead of being secreted into the medium. *DsRed2* was successfully expressed behind the *SC3* or *GPD* promoter. Since only a low background fluorescence was observed, DsRed2 is preferred over GFP as a reporter protein.

Genetics and Cellular Biology of Basidiomycetes-VI. A.G. Pisabarro and L. Ramírez (eds.)
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The Roles of SC3 and SC15 of Schizophyllum commune during Growth on Wood

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The SC3 hydrophobin plays several roles in growth and development of *S. commune*. It lowers the surface tension of the aqueous substrate, enabling aerial growth, and it coats the aerial hyphae rendering them hydrophobic. SC3 also allows hyphae to attach to hydrophobic surfaces. Moreover, SC3 has a role in the cell wall architecture. In the absence of the hydrophobin, *S. commune* produces more mucilage. The SC15 protein mediates aerial hyphae formation and attachment in the absence of SC3. Besides being secreted into the medium, the protein can be found in the mucilage that binds aerial hyphae together. So far, studies were performed on minimal media. We here assessed the roles of SC3 and SC15 during growth on wood. SC3 and SC15 were shown not to play a role in colonization of wood. Biomass formation and radial extension was similar in the wild-type and in strains in which either or both *SC3* and *SC15* were deleted. Interestingly, in contrast to growth on minimal medium deletion of *SC15* alone affected formation of aerial hyphae. A similar reduction was observed for the *SC3* mutant while reduction in the ∅*SC3*∅*SC15* was most dramatic. At the moment we assess the role of SC15 in the fruiting body. Preliminary data using GFP as a reporter indicate that *SC15* is expressed within the fruiting body. Here it may play a role in the mucilage surrounding the hyphae.

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Fungal Laccase: Propierties and Aplications

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Laccase (EC 1.10.3.2; benzenediol:oxygen oxidoreductase) was first discovered at the end of the 19th century in the sap of Oriental lacquer trees. Later on, the laccase from the white-rot basidiomycete *Trametes versicolor* was thoroughly characterized using biochemical and biophysical methods. It is an extracellular blue multicopper glycoprotein. The copper ions are involved in the catalytic process, in which a reducing substrate, typically a phenol, is oxidized and molecular oxygen is reduced to water. Today, a multitude of different laccases and laccase genes from various sources have been characterized. The enzyme seems to have different physiological roles in different types of organisms. Several of the best characterized laccases come from basidiomycete fungi causing white-rot decay of wood. These laccases are generally regarded to be associated with the biodegradation of lignin, although more research is needed to shed light on the fundamental molecular mechanisms. Recent advances with regard to the structural and functional diversity of laccases will be discussed in relation to efforts to clarify the physiological roles of the enzyme and to elucidate its potential in various applications, including detoxification, bleaching, and analysis.

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Expression Profiling of Natural Antisense Transcripts in *Agaricus bisporus*

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Our view of the role of RNA has changed from being a passive intermediary of genetic information to acting as a regulator of gene expression in the form of short-interfering RNA, micro RNA, riboswitches and natural antisense. Long length natural antisense transcripts (NATs) have been identified for genes which are up-regulated after harvest in the fruitbody of the mushroom *Agaricus bisporus*. These NATs therefore are likely to be involved in the regulation of postharvest events such as development and senescence. A novel quantitative reverse transcriptase PCR technique has been developed. The data have been statistically analysed to produce expression profiles of NATs for six postharvest genes. The average antisense/sense ratios varied by three orders of magnitude, from 8.0 for *shs13* to 6 x 10^{-3} for cruciform DNA binding protein. The expression profiles were found to be highly specific for individual genes, to be dynamic over time and highly variable between neighbouring tissues. This latter characteristic has led to the speculation that NATs may be involved in tissue differentiation. Sequence information of natural antisense transcripts form *A. bisporus* suggests that they are synthesized from messenger (sense) RNA by RNA-dependent RNA-polymerase. Evidence will be presented that to support the hypothesis that the level of antisense may be controlled by the 3' processing of sense RNA.

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Molecular Toolkit Development for Gene Expression and Gene Silencing Technologies in the Homobasidiomycete Fungi *Agaricus bisporus* and *Coprinus cinereus*.

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We have developed a "Molecular Toolkit" comprising interchangeable promoters and marker genes to facilitate transformation of homobasidiomycete mushrooms and subsequent analysis of gene expression. We will describe the testing of a wide range of promoters in both *Agaricus bisporus* and *Coprinus cinereus* when linked to a range of selectable and visual marker genes, along with the parameters required to successfully achieve foreign gene expression within these organisms. It has been previously demonstrated that a prerequisite for GFP expression in *A. bisporus* and *C. cinereus* is an intron. We describe the construction of an expression vector containing a multiple cloning site linked to an intron thus allowing different genes to be easily expressed in *A. bisporus* and *C. cinereus*. We report on the development of gene silencing technologies within *A. bisporus* and *C. cinereus*. In particular the serine protease has been targeted for gene silencing in *A. bisporus*. Serine protease has been implicated in post-harvest and age-related senescence of sporophores. On harvesting, mushrooms degenerate rapidly to give browned caps and loss of texture in the fruit body, and such problems can dramatically reduce sale ability of the mushrooms. Suppression of genes involved in these pathways could increase mushroom shelf-life and profitability for mushroom growers, or help to further elucidate the complex biochemical pathways involved in post-harvest degradation. Progress will also be reported on gene silencing in *C. cinereus*.

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Investigation into the Fungal-Fungal Interaction Between *Verticillum fungicola* and *Agaricus bisporus*

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Plant and animal pathogens section

The cultivated button mushroom, *Agaricus bisporus*, is amenable to number of pathogenic threats including bacteria, viruses, mites, insects and fungi. Currently, the most significant threat to the commercial mushroom industry is the mycoparasite, *Verticillium fungicola*. Infection by *V. fungicola* can drastically reduce the yield and value of mushroom crops. The severity of this disease is dependent on the developmental stage of *A. bisporus* at the time of infection and is manifested in three types of symptoms: spotty cap, stipe blowout and dry bubble. An aim of our research has been to develop molecular tools for *V. fungicola* that will allow us to study the interaction between this pathogen and *A. bisporus*. These tools have included transformation methods, marker gene techniques as well as gene-knockout technologies. This has involved the use of Agrobacterium and T-DNA to introduce disruption constructs into *V. fungicola* as part of a molecular investigation into this fungal-fungal interaction. We have developed an efficient transformation system for *V. fungicola* that we have now adapted to give high levels of targeted mutagenesis. This technique has successfully generated targeted mutants of a β-1-6 glucanase homologue from *Trichoderma harzianum* and a Mitogen Activated Protein Kinase homologue (*PMK1*) from *Magnaporthe grisea* identified using degenerate PCR primers. We have also developed T-DNA tagging technology in a mycology context for random mutagenesis in *V. fungicola*.

Genetics and Cellular Biology of Basidiomycetes-VI. A.G. Pisabarro and L. Ramírez (eds.)
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Characterisation of *Agaricus bisporus* Response Genes to *Verticillium fungicola* infection

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The mycoparasite *Verticillium fungicola* is a persistent threat to the cultivation of the mushroom *Agaricus bisporus*. Mushroom "dry bubble" is characterised by an undifferentiated mass of cells and can result in major crop losses. During the establishment of "dry bubble" substantial changes occur in the biochemistry and physiology of both partners. To enable new insights to be made into the molecular events underlying the disease, work is in progress to identify genes expressed during pathogen infection. Subtractive Suppressive Hybridisation (SSH) has enabled recovery of 65 expressed sequenced tags (ESTs) differentially expressed during infection. After database searches 27 of the genes were identified as most likely from *V. fungicola*, 25 from *A. bisporus* and 13 unknown. Bioinformatic analysis suggested that the response genes identified were involved in a range of biological functions that included stress, signalling, protein synthesis and cell wall structure and function.

Specific full-length genes will be recovered using cDNA library constructed from lesions of *A. bisporus* infected with *V. fungicola*, enabling silencing approaches to be used to further investigate the role of the identified genes in disease. An alternative higher-throughput method of gene function analysis, RNA interference (RNAi) using *A. bisporus* model genes (*URA3*, *CBX*), is also being developed. Silencing constructs expressing RNAi hairpin were transformed into *A. bisporus* using *Agrobacterium tumefaciens* and hygromycin resistance. Screening of the transformants by PCR confirmed integration of the silencing construct in 24 transformants. RT-PCR is being used to confirm transcription of the RNAi hairpin. Quantitative PCR will be used to analyse levels of target gene transcripts post RNAi transformation. The role of *A. bisporus* genes identified, in the infection process, will be determined through infection trails with *A. bisporus* silenced lines.

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Pathogenicity in *Heterobasidion annosum*

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Heterobasidion annosum is one of the major pathogens to conifers in the Northern Hemisphere. Research has been carried out to characterise the molecular mechanism of the pathogenicity in this basidiomycete. One factor that has limited the research about *H. annosum* pathogenesis is the lack of coding sequence information. Therefore, a project on producing sequence data from *H. annosum* by generating ESTs has been carried out. The collection of sequence data will assist future research on *H. annosum* together with the high density cDNA arrays that were also constructed in this work. It is interesting that 30% of the genes identified did not have any similarity to any known proteins and 16% had similarity only with proteins with unknown functions. Recently, progress has been made in work on mapping the pathogenicity factors in *Heterobasidion* using a hybrid between North American P and S homokaryons. Based on AFLP markers, a genetic linkage map was established that allowed for mapping QTLs for pathogenic growth towards seedling roots and pine innerbark. The next step underway is to verify the identity of candidate genes located within the established region of the genome. Future functional analysis of both QTL and EST-derived candidate genes should be aided by the recently established *Agrobacterium*-mediated transformation system in *Heterobasidion*.

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Virus Related Symptoms in Crops of the button Mushroom *Agaricus bisporus*

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Since more than five years symptoms are seen in Dutch mushroom crops that correlate with the presence of a set of dsRNAs. These dsRNAs are indicative for the presence of viruses. Symptoms are discoloration of white mushrooms varying from cream to brown. Crops can be heavily affected with more than 50% of the mushrooms showing discoloration. Mostly, however, crops show mild symptoms with a few percentages of mushrooms showing an off-white color. Since traiders associate this discoloration with low quality economical damage can be substantial for individual farmers.

In affected crops up to 15 dsRNAs are observed with varying intensity. Only the five shortest dsRNAs show a perfect quantitative and qualitative correlation with the symptom. They are always present in discolored mushrooms and the intensity of the bands seen in agarose gels vary with the intensity of the discoloration. Most of the other dsRNAs are also present in affected crops but are also seen in crops without any visual symptoms. We have started a molecular analysis of the dsRNAs in order to design sensitive tests for each dsRNA. This will reveal if all or some dsRNAs are always present in mushrooms or are derived from an unknown infectious source.

From a number of dsRNAs 60 to 80% of the sequence has been determined. RT-PCR tests have been designed recently for these dsRNAs and the first result of tests of several crops will be presented.

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Double Stranded RNAs in the Mushroom Virus X Complex of *Agaricus bisporus*

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Mushroom virus X (MVX) is a new disease of the commercial mushroom, *Agaricus bisporus*. When first recorded in 1996 it was associated with localised areas of pin suppression. It is now thought to be responsible for a range of symptoms. It seriously affected the UK mushroom industry in 2000/01 and has now been reported in Ireland, Holland and a number of other mushroom growing countries. The objectives of the work programme put in place to address this problem were to determine the relationship between major dsRNA bands present in the MVX complex and to sequence characterise major components; to develop an RT-PCR diagnostic test for separate viruses within the MVX complex and test commercial spawns and ARP cultures for virus presence; to characterise transmission of dsRNA elements between strains, assess effects of cross infection and determine significance of dsRNA partitioning during transmission through spores; and to determine the rate of spread of MVX into healthy compost from a point source

Analysis of diseased mushrooms has identified 26 double stranded RNA (dsRNA) molecules associated with MVX symptoms. Evidence collected to date suggests that MVX might be a complex of viruses, rather than a single virus. Three of these dsRNAs have also been shown to be present in asymptomatic mushrooms. Vertical transmission was shown to occur through production of single spore progeny isolates from infected sporophores. Partitioning of dsRNAs was shown to occur. To determine whether horizontal transmission occurred, dual-culture *in vitro* tests were performed using donor and acceptor strains. Cultures produced from these transmission lines contained a reduced number of dsRNAs compared with the original donor strains.

Sequence data has been generated for several dsRNAs.

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Fungal Diversity Adds Value to Biotecnology and Agriculture

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Mediterranean countries host rich biological diversity (genetic, population, species, habitats, communities, ecosystems). Until recently research on the fungal diversity was focusing relatively more on phytopathogenic fungi, invertebrate parasites, and saprotrophic and ectomycorrhizal mushrooms (Pezizales, higher Basidiomycetes). For higher Basidiomycetes in particular, detailed inventories and check-lists have been compiled in many western European countries. In the Mediterranean region, however, pertinent data are limited and fragmentary; only recently new information has started to accumulate. Indicative is the case of Greece, where selected ecosystems are studied in respect to their macromycetes diversity, revealing the existence of taxa with significant ecological and economic interest. Prerequisites for the exploitation of biological resources (incl. fungi) is the availability of a large number of individuals with a wide genetic basis, which are correctly identified and suitably evaluated. For example, elucidating taxonomy and clarifying phylogenetic relationships among *Pleurotus* species has contributed significantly to their widespread use. Large-scale applications related directly (or indirectly) with mushroom resources and their exploitation include the edible mushroom industry, production of medicinal and health-promoting factors, improvement of soil fertility, remediation of soils, enhanced plant growth, suppressiveness of soil-borne pathogens of plants, animal feed, transformation of xenobiotics and antibiotics, biosorption of toxic elements, decolorization of organic pollutants, degradation of industrial and agroforestry wastes, etc. Particular emphasis is given to the upgrade of lignocellulosic wastes and residues through their detoxification and biotransformation into value-added products; among them, soil conditioners and fertilizers generated from spent mushroom substrates conform with the much-sought notion of sustainability in agriculture.

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Agaricus devoniensis Complex Comprises a Group of Heterothallic Isolates Constituting a Basis for Breeding

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A recent phylogenetic reconstruction of *Agaricus* section *Duploannulati* revealed that *A. devoniensis* and *A. subfloccosus* are two complexes of species close to *A. bisporus*. The *A. subfloccosus* complex comprises two homothallic entities, while the *A. devoniensis* complex was never studied until now. A sample of 26 isolates, some being unreliably determined, were examined to (i) confirm their identity using a PCR-RFLP marker revealing a characteristic *A. devoniensis* ITS polymorphism, and (ii) for their ability to fruit in standard conditions used for *A. bisporus* cultivation. Twenty one isolates were confirmed as *A. devoniensis*, and only two collections from USA were unable to fruit. The five remaining isolates were excluded from the complex and were unable to fruit; their ITS1+2 regions were sequenced and alignments indicated that four of them were similar to *A. campestris* and that one belonged to a new entity close to *A. bitorquis* and *A. cappellianus*. For the 19 fructifying isolates of the complex, we attempted intrastock and interstock mating tests with single spore isolates: for three isolates, we did not get spore germination; and for seven isolates, we observed partial to complete intersterility between strains. The nine remaining isolates exhibited a unifactorial system of sexual incompatibility for which eight different mating type alleles were detected. Within this group, the heterothallic and presumably interfertile isolates differed in their origin (Greece, France), their habitat (dune, coniferous trees), and their morphology (mean spore length: 5.6 to 6.6 μm); they constitute a diversified genetic basis usable to select smooth white and attractive cultivars for this tasteful edible and cultivable species.

Genetics and Cellular Biology of Basidiomycetes-VI. A.G. Pisabarro and L. Ramírez (eds.)
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Section 3

Poster abstracts

The *Coprinus Cinereus* Genome Project

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Coprinus cinereus is an increasingly attractive basidiomycete model system. Its genome has been sequenced and is publicly available; it is readily cultured in the laboratory on defined media, it has highly synchronous meiosis, and numerous laboratory techniques have been adapted for use with it. The 10X shotgun sequence released by the Whitehead Institute comprises 36 Mb of the 37 Mb of the genome, which have been assembled into 106 supercontigs containing 431 contigs. cDNA libraries have been constructed from two meiotic stages, and 1432 candidate genes have been identified from them. Another set of cDNA libraries has been constructed from vegetative *Coprinus cinereus* Okayama 7 grown under different environmental conditions, including heat shock, rapamycin treatment, minimal medium, rich medium, and complex carbon and nitrogen sources. 5000 ESTs are being sequenced from these libraries. The EST sequences have been aligned with the genomic sequence, as have known *C. cinereus* genes from GenBank. Data from known ascomycete gene sequences have been used to train SNAP software to predict a total of 11,340 genes from the remainder of the genome. BlastX and Pfam have been used to assign tentative functions to predicted genes as well as ESTs. tRNA genes have also been identified in the genome. All genomic information is available online via our Gbrowse server.

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Expression Profiling of Natural Antisense Transcripts in *Agaricus bisporus*

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Our view of the role of RNA has changed from being a passive intermediary of genetic information to acting as a regulator of gene expression in the form of short-interfering RNA, micro RNA, riboswitches and natural antisense. Long length natural antisense transcripts (NATs) have been identified for genes which are up-regulated after harvest in the fruitbody of the mushroom *Agaricus bisporus*. These NATs therefore are likely to be involved in the regulation of postharvest events such as development and senescence. A novel quantitative reverse transcriptase PCR technique has been developed. The data have been statistically analysed to produce expression profiles of NATs for six postharvest genes. The average antisense/sense ratios varied by three orders of magnitude, from 8.0 for *shs13* to 6 x 10^{-3} for cruciform DNA binding protein. The expression profiles were found to be highly specific for individual genes, to be dynamic over time and highly variable between neighbouring tissues. This latter characteristic has led to the speculation that NATs may be involved in tissue differentiation. Sequence information of natural antisense transcripts form *A. bisporus* suggests that they are synthesized from messenger (sense) RNA by RNA-dependent RNA-polymerase. Evidence will be presented that to support the hypothesis that the level of antisense may be controlled by the 3' processing of sense RNA.

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Molecular Characterization of A Cellobiohydrolase Gene Family in the Fungus *Pleurotus ostreatus*

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Cellulose is the most abundant biological polymer on Earth. Its chemical composition consists of D-glucose units linked by β-1,4- glycosidic bonds forming linear polymeric chains with a reducing and a non-reducing end. Cellulose chains may either adhere to each other, via hydrophobic and van der Waals interactions, forming crystalline structures or remain more loosely packaged (amorphous cellulose). Consequently, the physical structure and morphology of native cellulose is complex and not uniform.

Biological degradation of cellulose depends on the action of three types of enzymes: endoglucanases (E.C.3.2.1.4), cellobiohydrolases (E.C.3.2.1.91) and β-glucosidases (E.C.3.2.1.21). All them hydrolyse β-1,4-glycosidic bonds but they differ on the substrate specificity. Endoglucanases hydrolyse the amorphous regions of the cellulose fibbers generating new reducing and non-reducing ends, cellobiohydrolases attack the molecule ends yielding cellobiose units, and β-glucosidases hydrolyse cellobiose molecules yielding glucose.

Cellobiohydrolases can be classified into two groups: type I (CBHI) and type II (CBHII), each having opposite chain-end specificities. CBHI prefer the reducing ends while CBHII act at non-reducing ends.

By the screening of a genomic library from the basidiomycete *Pleurotus ostreatus* var. *florida*, we have isolated five *cbhI* genes, named *cbhI1, cbhI2, cbhI3, cbhI4* and *cbhI5*, proving the occurrence of a multigenic family coding for this enzymatic activity. Using this sequences as probe, it has been possible to know the conditions in which are expressed those genes. This has allowed the synthesis of the each gene cDNA and, by comparison of this sequence with the corresponding genomic sequence, the characterization of their structure.

On the other hand, using the RFLP technique and a progeny of 80 monokaryons derived from the dikaryon N001, the five genes have been mapped on the linkage map of *P. ostreatus* var. *florida* mapping the *cbhI1* to the chromosome IV and the others to the chromosome VI.

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Molecular Characterisation and Expression analysis of Developmentally Regulated Genes in *Agaricus bisporus*

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Analysis of cDNA transcripts, PCR based methods and genomic library screening have been used to clone and characterise developmentally regulated genes in the cultivated white button mushroom *Agaricus bisporus*. Up-regulated genes identified during the rapid expansion phase of the sporophore include sugar transporter gene *sut1*, putative riboflavin-aldehyde-forming-enzyme gene (*raf*) and three novel morphogenes *mag2-mag4*. Further, a hexose transporter gene *sut2* and lectin genes *abl1* and *abl2*, among others have been cloned from *A. bisporus* using PCR based strategies. Northern analysis indicated their up-regulation during sporophore differentiation and development. Sugar transporter gene *sut1* transcripts increased abundantly during sporophore development and although *sut1* showed varying levels of homology to other sugar transporters, its substrate preference could not be identified based on homology. Interestingly, analysis of basidiomycete genome sequences revealed the presence of a putative *sut1* homolog in the white rot fungus *Phanerocheate chrysosporium*. On the other hand, *Ab sut2* showed strong homology to fungal glucose/hexose transporters and its homologs also appear to be present in *A. bitorquis* and *Coprinus cinereus* suggesting a generic role. Analysis of the genomic cosmid clones revealed that the lectin genes *abl1* and *abl2* are present in close proximity to each other and further characterisation is on-going.

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Ras Module Function is Involved in Regulation of Sexual Development *Schizophyllum commune*

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The white rot fungus *Schizophyllum commune* is used as a model to investigate sexual development in hymenomycetes. We isolated the gene *gap1* encoding a GTPase-activating protein for Ras. Disruption of *gap1* should therefore lead to strains accumulating Ras in its activated, GTP-bound state and to constitutive Ras signaling. Mating behavior was not altered in *Δgap1* monokaryons whereas growth rate in *Δgap1* monokaryons was reduced about 25%. Dikaryotic *Δgap1*/*Δgap1* strains displayed 50% growth reduction. Hyphal growth was disturbed showing a wavy growth pattern. In dikaryons, clamp formation was severly disturbed as hook cells failed to fuse with the penultimate cell at the site that in wildtype cells is marked by a peg formed from the mother cell. Instead, the dikaryotic character of the hyphae was rescued by fusion of the hooks with nearby developing branches. The mating type genes of the *B* factors encoding a pheromone receptor system are known to be required for clamp cell fusion. A role for Ras in the same process in discussed. Fruitbody formation was observed in homozygous *Δgap1*/*Δgap1* dikaryons which, however, formed increased numbers of fruit body primordia, whereas the amount of fruit bodies was not raised. Mature fruit bodies formed no or abnormal gills. No production of spores could be observed. Similar phenotypes in fruitbody development had been previously described for elevated intracellular cAMP levels. Thus, the signalling of Ras is discussed with respect to cAMP signalling.

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Study of Two Acidic Proteinases, Probably Involved in the Dimorphism and Pathogenicity of *Ustilago maydis*, Basidiomycete of the Corn Smut Disease

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Ustilago maydis is a dimorphic phythopathogenic fungus and the causal agent of the corn smut disease. In this work, the purification and biochemical characterization of the acid proteinases pumAe (extracellular) and pumAi (intracellular) of *U. maydis* were performed. Also, identity of the gene that encodes for pumAi (*PRAum*) was explored in the genome of the fungus. The proteases were purified and biochemically characterized. The molecular masses of pumAe and pumAi were 72 and 35.3 kDa respectively. The optimal pH of activity of proteinases was 4.0. The pumAe K_m value was of 3.5 μM and a V_{max} of 11430 μmol h^{-1} mg^{-1} when Suc-R-P-F-H-L-L-V-Y-MCA was used as substrate. The protease pumAi was inhibited by pepstatine A. Yeast-to-mycelium transition was inhibited by Pepstatine A in the culture medium. The hypothetical gene that encodes for protease pumAi (*PRAum* gene) was located in the *U. maydis* genome project and was amplified by PCR and cloned into TOPO-TA 2.1 plasmid and pNMT-1, a *Schizosaccharomyces pombe* expression vector. In the *U. maydis* genome one copy of the gene by Southern blot analyses was detected. In brief, the expression of this gene (*PRAum*), performed by RT-PCR assays, was regulated by the source of nitrogen. The heterologous expression experiments in *S. pombe* allowed a fast purification and confirmed that pumAi enzymatic activity was encoded by *PRAum* gene.

Genetics and Cellular Biology of Basidiomycetes-VI. A.G. Pisabarro and L. Ramírez (eds.)
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Molecular Analysis of Aminopeptidase PumAPE from *Ustilago maydis* Encoded by *APEum* Gene: Enzyme Purification and Differential Expression

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Heterobasidiomycete *Ustilago maydis* is a dimorphic phytopathogenic fungus, causal agent of corn smut, a widespread disease. Recently, proteolytic system of this fungus was described and an aminopeptidase activity, probably involved in pathogenicity, was detected. The aminopeptidase pumAPE was purified from the haploid phase of *U. maydis* FB1 strain. The purification procedure consisted of ammonium sulphate fractionation and three chromatographic steps, resulting in a 23% recovery. The molecular mass of the dimeric enzyme was estimated to be 110 kDa and 58 kDa by gel filtration chromatography and SDS-PAGE respectively. Enzymatic activity was optimal at pH 7.0 and 35 ºC toward Lys-*pNA* and the p*I* was determined to be 5.1. The enzyme was inhibited by EDTA-Na_2, 1,10-phenanthroline, bestantin, PMSF and several divalent cations (Cu^{2+}, Hg^{2+} and Zn^{2+}). The aminopeptidase exhibited a higher specificity for substrates with lysine and arginine in the N-position. The K_m value was 54.4 μM and the V_{max} value was 408 μmol min^{-1} mg^{-1} for Lys-*pNA*. A pair of primers was designed in order to amplify the gene *APEum* encoding this activity. In order to determine the number of copies in the genome, a *APEum* gene fragment was used as probe in a Southern blot. Only one copy of the gene by genome was detected. Also, differential expression of *APEum* was assessed under different physiological conditions. In brief, high expression levels were detected on media supplemented with corn infusion, proline, and ammonium.

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Mitogenic Activated Protein kinase Kpp6 Signaling in the Phytopathogenic Fungus *Ustilago maydis*: Identification of Downstreams Elements

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Pathogenicity of *U.maydis* is dependent on its ability to mate. Mating requires active cAMP-PKA and MAP kinase cascades and except for the signaling inputs the downstream components are also required during pathogenesis. In addition a MAP kinase, called Kpp6 was described, that works in pathogenicty but not in mating. Kpp6 displays high similarity to Kpp2, the MAP kinase that works in mating. *kpp6* mutants were morphologically indistinguishable from wild type but were unable to induce anthocyanin production and were unable to penetrate into the plant, despite the fact that they did produce appressoria. In this work we are studying the downstream elements of Kpp6 and determine their function during the penetration process. To obtain this information we used microarray assays with RNA from *Ustilago* growing on plant surface. We identified 29 genes which are reduced in expression in Kpp6 mutant with respect the wild type strain. We generated knockout strains for some of these genes in compatible strains of *U. maydis* and analyzed the phenotypes in pathogenicity. Our preliminary results suggest that Kpp6 regulates the penetration of *Ustilago maydis* at the stage where lytic enzymes expression is no longer required.

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Formation of Hyphal Loops in Xylotrophic Cophinoid Mushrooms

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Recent molecular analysis split the traditional genus *Coprinus* (Homobasidiomycetes) into four distinct genera: *Coprinus*, *Coprinopsis*, *Coprinellus* and *Parasola.* Coprinoid mushrooms are usually saprotrophic on soil and/or dung of herbivores. However, more than 60 species are able to grow on wood and straw.

Xylotrophic mushrooms are forcing a relatively short supply of nitrogen and phosphorous nutrients. *Coprinus comatus* has been reported to produce specialized structures ("spiny balls") to penetrate nematodes for nutrient supply (Luo et al. 2004, Mycologia 96, 1218-1224). Nematode traps of other fungi involve adhesive hyphal network and knobs, hyphal loops and snares. Toxin production may support in nematode immobilisation.

Nematode-trapping species belong mainly to the mitosporic Deuteromycetes, but some are also found amongst Zygomycetes and Basidiomycetes.

We have observed hyphal loops in several wood-decaying basidiomycetes, such as *Daedalea quercina, Ganoderma lucidum, Lentinula edodes, Piptoporus betulinus* and *Pleurotus ostreatus*. Furthermore, regular and irregular hyphal loops and/or rings were observed in the four clades of Coprinoid species (*Coprinus comatus, Coprinellus angulatus*, *C. bisporus*, *C. curtus, C. domesticus, C. disseminatus*, *C. ellissi, C. micaceus, C. xanthothrix, Coprinopsis cinerea, C. gonophylla, C. radians, C. strossmayeri, C. scobicola,* and *P. plicatilis*). Hyphal loops were particularly often formed in *Coprinellus* species. Such structures were rare in *Coprinopsis atramentaria, C. cothurnata, C. romagnesiana, C. psychromorbida* and *Coprinus patouillardii* (an unclassified isolate).

It is not clear yet why Basidiomycetes fungi have these structures. Is it that many species have nematode trapping abilities by formation of such structures?

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Copper in Fruiting Body Development of *Coprinus cinereus*

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The model homobasidiomycete *Coprinopsis cinerea* grows best at 37˚C, but, normally, it produces fruiting bodies only at moderate temperatures around 25-28˚C. Light is needed to induce fruiting and also for fruiting body maturation. Cultures kept after fruiting induction predominantly in the dark form structures with an extended stipe and an underdeveloped cap (so-called "etiolated stipes"). In a day/night rhythm, caps develop further, basidia are formed, in which karyogamy and meiosis occurs and of which the basidiospores bud off. Besides light, fruiting body development in basidiomycetes has been repeatedly linked to enzymes belonging to the group of phenoloxidases, in particular the multi-copper containing laccases. However, their roles in fruiting remain unclear.

In attempts to induce laccase production in liquid standing cultures at 37˚C, to our surprise we found unusual inititation of fruiting body development. However, the abundantly formed primordia did never develop into mature fruiting bodies but into large-sized etiolated stipes, both in dark and in light. Laccase under these conditions was not detected in the medium but bound to the fruiting initiating mycelium. Moreover, enzyme production and etiolated stipe formation correlated with an increase from pH 5.5 to a slightly alkaline pH. Ammonium was found to be produced and nitrate reductase activity has enzymatically been shown. Under normal fruiting conditions, addition of copper to cultures enhances fruiting initiation in time and number.

To further unravel the potential involvement of laccases in fruiting as well as of proteins influencing ammonia secretion, we are studying expression of corresponding genes during vegetative growth and fruiting body development.

Work in our laboratory is supported by DBU (Deutsche Bundesstiftung Umwelt). MNG holds a CONACYT (Mexico) PhD studentship.

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Species Identification and Detection of Fungi in Biological Materials by FTIR Microscopy

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FTIR spectroscopy provides the opportunity to simultaneously detect many molecular bonds or functional groups of different polysaccharides, proteins, lipids, aromatic and other compounds. The measurement principle is based on the absorption of infrared light by dipolar molecular bonds. In combination with microscopy, local resolution of the chemical composition is possible. Each absorption point or peak in the spectrum can be integrated to create an image of the distribution of the corresponding compound. We use FTIR-microscopy in order to detect fungi in plant tissues such as in infected wood and in mycorhizal roots. For the development of a fast and inexpensive method for localisation and identification of fungi, differences between FTIR measurements of fungi and plant cells are characterized. In addition, FTIR spectra of different fungi are compared. Beech wood blocks were infected with *Trametes versicolor* and with *Schizophyllum commune* and FTIR spectra in sections of the infected wood determined. Cluster analysis revealed major differences between FTIR spectra recorded from wood fibres and empty vessel lumina and spectra from fungal mycelium, irrespectively of whether grown on the surface of wood or inside vessel lumina. Species specific clustering of spectra of fungal mycelium grown on the wood surface and inside vessel lumina demonstrated the potential of FTIR microscopy to identify fungal mycelium in wood. Currently, we are sampling FTIR spectra from various basidiomycetes in order to define species according to their specific FTIR spectra.

The work is supported in frame of the Lower Saxony Competence Network for Sustainable Timber Utilisation (NHN) by the Ministry of Culture of Lower Saxony and EFRE. The group of UK is funded by the DBU. MNG holds a PhD studentship from CONACYT, Mexico.

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Secondary Structure of the Ribosomal PreRNA ITS2 region as a Tool in Studies of Fungal Diversity and Phylogeny

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The ribosomal RNA gene, and particularly its 18S and 25S sequences, have proven valuable in large-scale phylogenetic analyses. The ITS region of the ribosomal RNA gene, instead, has not been regarded so useful in this respect. The main problem has been that these sequences show significant sequence and length variation and that they have been more or less unalignable beyond small closely related groups.

We have determined the common secondary structure of the ITS2 region and used it to align the sequences over the whole fungal kingdom. Significant properties of this structure include a central ring structure and three or four conserved loops – the presence of the ring being the most conserved feature. The core structure has also revealed the most conserved sites that are usable in kingdom-wide phylogenetic analyses. Surprisingly, the tree that is calculated with only the 5.8S ribosomal RNA and the conserved ITS2 sites has a very high correlation with the Fungal Tree of Life that has been calculated with four markers and much longer sequences. Furthermore, finding of the ITS2 secondary structure has revealed a number of group specific sequence signatures and structural RNA elements that can be used for more detailed analyses of different subgroups and their phylogeny.

Currently we are (mainly) examining the variation of the loop structures among the basidiomycetes and linking that to the taxonomy of fungi. Some examples of current findings will be shown.

Genetics and Cellular Biology of Basidiomycetes-VI. A.G. Pisabarro and L. Ramírez (eds.)
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A Novel Thaumatin-Like Protein-Encoding Gene from *Lentinula edodes*, *tlg1*, is Involved in Lentinan Degradation During Post-Harvest Preservation.

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Lentinan, which is a β-1, 3-linked-D-glucan with β-1, 6 branches isolated as anti-tumor active-substance from *Lentinula edodes*, is purified from fresh fruiting bodies and marketed for clinical use. However, it is known that lentinan content decreases during post-harvest preservation as a result of increased β-1, 3-glucanase activity. We isolated two exo-glucanase encoding genes, *exg1* and *exg2* from *L. edodes*. Transcription level of the *exg1* and *exg2* gene was higher in the stipe than in the pileus of young fruiting bodies. This suggests that the *exg1* and *exg2* are involved in stipe elongation in *L. edodes*. We also isolated one endo-glucanase encoding gene, *tlg1*, from *L. edodes*. The *tlg1* gene had 1.0 kbp cDNA length, and encoded protein was estimated as M.W. of 25 kDa and pI value of 3.48. Putative amino acid sequence of the *tlg1* displayed 43% identity to thaumatin-like (TL) proteins from *Arabidopsis thaliana*. TLG1 had 16 cysteins conserved in TL-proteins. TL-protein is pathogen related protein 5 in plant, and several TL-protein had endo-glucanase activity. Previously, it is considered that TL-protein is unique in plant, however, this research and recent genome sequence project revealed that similar sequences to TL-proteins are conserved in filamentous fungi. We measured β-1, 3-glucanase activity of *L. edodes* fruiting bodies after harvesting by somogyi-melson method using laminarin as a substrate, and endo-β-1, 3-glucanase activity by using AZCL-pachyman as a substrate. These revealed that glucanase activity increased during post-harvest preservation. Transcription level of the *exg1* gene decreased, but the exg2 and *tlg1* genes increased during post-harvest preservation. Western blot analysis showed that EXG2 and TLG1 expression increased after harvesting. Purified EXG1 did not degrade lentinan, but EXG2 and TLG1 degraded lentinan, therefore, we concluded that the *exg2* and *tlg1* genes are involved in lentinan degradation during post-harvest preservation.

Genetics and Cellular Biology of Basidiomycetes-VI. A.G. Pisabarro and L. Ramírez (eds.)
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Nutritional Value of Protein from Vegetative Mycelia of Edible Mushroom *Pleurotus ostreatus*

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Protein represents an essential part of our daily nutrient intake; wich has to cover our requirements for growth, maintenance and metabolic activity of cells and organs during all stages of life.

The use of fungi as food is not new. Higher fungi (mushrooms), have been used as food flavouring for centuries. Early man picked wild mushrooms from natural habitats, largely as a supplement to an otherwise monotonous diet, but probably did no use mushrooms as a primary source of protein. *Pleurotus ostreatus* (oyster mushroom) is an edible basidiomycete and it a wood destroying saprophytic fungus. This fungus is object of increasing biotechnological interest due to its chemicals related to lignin degradation products and become it produces secondary metabolites with pharmaceutical applications and some proteins of industrial potential.

The present work was designed to study the effects of supplementation a control diet with *P. ostreatus* mycelium for evaluation a nutritional value of mycoprotein and possible cholesterol lowering.

Forty-four male Wistar rats were divided into six groups that were fed during thirty-one days with diets supplemented with 2,5% and 5% *P. ostreatus* mycelia and/or 1% cholesterol (Control; C + M 2,5%; C + M 5%; C + Ch 1%; C + M 2,5% + 1% Ch; C + M 5% + 1% Ch) respectively. All the diets were isoenergetic and isonitrogenous and meet the requirements of growing animals (AIN-93). Body weight, food and water intake, faecal and urine excreted were registered daily, after decapitation fresh weight of main organs was registered and parameter haematics were analysed. An inter laboratory study involving all nutritional parameters were carried out.

The nutritional analysis of mycelia and mushrooms showed higher nitrogen content expressed as protein percentage in the vegetative mycelia (23-30%) than in oyster mushrooms (20%). This percentage was determined us-

Genetics and Cellular Biology of Basidiomycetes-VI. A.G. Pisabarro and L. Ramírez (eds.)
 ISBN 84-9769-107-5

ing Kjeldhal method and amino acid profiles using High Performance Liquid Chromatography (HPLC). No differences in food intake, final body weight, weight gain, and daily weight gain were founded among the six groups. Cholesterol reduction in animals feed with Ch 1% + M2, 5% and Ch 1% + M5% was 26% and 30% ($P < 0.050$) respectively, compared to control group Ch 1%. Also Cholesterol LDL was diminished in same groups 32% ($P < 0.050$) and 42% ($P < 0.010$), respectively.

Isolation, Molecular Characterization and Location of Telomeric Sequences of Basidiomycete *Pleurotus ostreatus* var. *florida*

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The white rot fungus *Pleurotus ostreatus* is an edible basidiomycete of increasing biotechnological interest due to its ability to degrade both wood and chemicals related to lignin degradation products.

Telomeres are specialized structures at the end of all eukaryotic chromosomes. Ensure chromosome stability and protect the ends from degradation and from fusing with other chromosomes. Telomeres sequences are extraordinary highly conserved in evolution. The loss of telomeric repeats triggers replicative senescence in cells.

For identification of restriction telomeric fragments in a previously described linkage map of *Pleurotus ostreatus* var. *florida* (Larraya et al., 2000), dikaryotic and eighty monokaryotic genomic DNAs were digested with diferents restriction enzymes (*Bam*HI, *Bgl*II, *Hind*III, *Eco*RI, *Pst*I, *Sal*I, *Xba*I and *Xho*I) electrophoresed and transferred to nylon membranes. Numerous polymorphic bands were observed when membranes were hibridized with human telomericd probe $(TTAGGG)_{132}$ (heterologous probe).

Telomeric restriction fragments were genetically mapped to a previously described linkage map of *Pleurotus ostreatus* var.*florida*, using RFLPs identified by a human telomeric probe (tandemly repeating TTAGGG hexanucleotide).

Segregation of each telomeric restriction fragment was recorded as the presence vs. absence of a hibridizing band. Segregation data for seventy three telomeric restriction fragments was used as an input table to be analysed as described by Ritter et al. (1990) and by Ritter and Salamini (1996) by using the MAPRF program software. Seventeen out of twenty two telomeres were identified.

Telomere and telomere-associated (TA) DNA sequences of the basidiomycete *Pleurotus ostreatus* were isolated by using a modified version of single-specific-primer polymerase chain reaction (SSP-PCR) technique (Sohapal et al., 2000). Telomeres of Pleurotus ostreatus contain at least twenty five copies of non-coding tandemly repeated sequence (TTAGGG).

Genetics and Cellular Biology of Basidiomycetes-VI. A.G. Pisabarro and L. Ramírez (eds.)
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Computacional Prediction of Protein-Coding Gene and Annotation of DNA Sequences with Agronomic Interest in *Pleurotus ostreatus* (Oyster Mushroom)

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Pleurotus ostreatus, commonly known as oyster mushroom, is a commercially important edible fungus with interesting biotechnological properties. Quantitative trait loci (QTL) analyses are rare in fungi and little is known about their number, position, and genetic structure. Previous studies of our group have allowed the construction of a genetic linkage map of *P. ostreatus* var. *florida*, which has provided the basis for performing an efficient QTL analysis. In fact, there is a region of the chromosome VII of *P. ostreatus* where the most QTLs related to the production and precocity characters have been mapped. These quantitative traits are presumably under the control of a polygenic genetic system and could be associated with some chromosomal regions. The hypothesis of this work is that there is a region in the chromosome VII of protoclon PC15 (monokaryotic parental of the N001 dikaryotic strain) where exist genes which are responsible for the QTLs mentioned above. In order to test this hypothesis, we are developing a molecular QTL analysis through the sequencing of a region with an approximated size of 320 Kbp in chromosome VII (protoclon PC15). For this purpose, a BAC genomic library was constructed and two BAC clones spanning the region of interest are being sequenced. To carry out an efficient computational prediction of protein-coding genes and its annotation on the partial sequences obtained up to date, we have used different Internet resources such as BLASTx, BLASTp, BLASTn, and FGENESH trained on some basidiomycetes genomic data like *Phanerochaete chrysosporium* and *Cryptococcus neoformans* (SoftBerry). To our knowledge, this is the firs molecular QTL analysis performed on this edible mushroom.

Genetics and Cellular Biology of Basidiomycetes-VI. A.G. Pisabarro and L. Ramírez (eds.)
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Mapping the *Pleurotus ostreatus* Genome

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Pleurotus ostreatus is a commercially important edible mushroom commonly known as oyster mushroom which has also important biotechnical applications. Industrial production of *P.ostreatus* is based on a solid fermentation process in which a limited number of selected strains are used. Optimization of industrial mushroom production depends on improving the culture process and breeding new strains with higher yields and productivities. In a previous study a linkage map of *P. ostreatus* strain N001 was constructed, which provided a basis for performing an efficient QTL (Quantitative trait loci) analysis based in a population of 80 sibling monokaryons. The map is based on the segregation of RAPD markers, RFLP markers, phenotypic characters and cloned genes. Nevertheless the linkage map is just a first step towards the selection of the appropiate parentals for new breeds.

In order to organize and improve the access to the data and information accumulated in the previous works mentioned above, a Microsoft® Excel Linkage Map Matrix (MELMM) was designed and created. On this linkage map matrix we could have an easy and functional view of the *P. ostreatus* linkage map data, such as, recombination frequencies, genotypes information and degree of similarity between monokaryons that will help us in the design of breeding crosses aimed at improving QTLs of agronomic interest of new commercial strains.

Genetics and Cellular Biology of Basidiomycetes-VI. A.G. Pisabarro and L. Ramírez (eds.)
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Wild Strains of Agaricus bisporus: A Source of Tolerance to Dry Bubble Disease

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Agaricus bisporus is susceptible to various pests and diseases. Dry bubble, caused by *Verticillium fungicola*, is currently the most serious disease and is distributed worldwide. All cultivars are susceptible and the pathogen develops resistance towards the very few fungicides admitted. Breeding for resistance is necessary and wild strains of *A. bisporus* are putative sources of tolerance. We present results on the susceptibility (severity of the disease, ability to develop the various symptoms) of some wild strains of the INRA-CTC and the PPO MRU collections.

A commercial strain revealed significant variability in agressiveness among isolates of *V. fungicola* var. *fungicola* responsible for the disease in Europe at present. Isolate VCTC, which induced severe symptoms revealed interesting tolerance among five wild *A. bisporus* strains and hybrids between wild strains. A cross test was performed with two cultivars and seven wild stains of *A. bisporus* contaminated with five isolates, two of var. *fungicola* and three of var. *aleophilum*, the latter responsible for the disease in USA and Canada. The wild strains screened were far more tolerant (3-9% of diseased mushrooms) than the cultivars (20-22%). All the strains were more susceptible to the var. *aleophilum* than to the var. *fungicola* isolates.

These experiments showed that very tolerant material exists in collection and can be used as parents to breed for resistance. The greater susceptibility of *A. bisporus* to *V. fungicola* var. a*leophilum* must be taken into consideration in breeding programmes, this variety being present in North America and being isolated in Europe in the past.

Genetics and Cellular Biology of Basidiomycetes-VI. A.G. Pisabarro and L. Ramírez (eds.)
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Identification and functional Characterisation of ctr1, a *Pleurotus ostreatus* Gene Coding for a Copper Ttransporter

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Copper homeostasis is primordial for life maintenance and especially relevant for ligning-degrading fungi whose phenol-oxidase enzymes depend on this micronutrient for their activity. In this paper we report the identification of a gene (*ctr1*), coding for a copper transporter in the white rot fungus *Pleurotus ostreatus*, in a cDNA library constructed from four-days old vegetative mycelium growing in submerged culture. The results presented here indicate that: (1) *ctr1* functionally complements the respiratory deficiency of a yeast mutant defective in copper transport supporting the transport activity of the Ctr1 protein; (2) *ctr1* transcription is detected in all *P. ostreatus* developmental stages (with exception of lamellae) and is negatively regulated by the presence of copper in the culture media; (3) *ctr1* is a single copy gene that maps to *P. ostreatus* linkage group III; and (4) the regulatory sequence elements found in the promoter of *ctr1* agree with those found in other copper related genes described in other systems. These results provide the first description of a copper transporter in this white rot fungus and open the possibility of further studies on copper metabolism in higher basidiomyetes.

Genetics and Cellular Biology of Basidiomycetes-VI. A.G. Pisabarro and L. Ramírez (eds.)
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Enzymatic Characterization of a Monokaryon Population of the edible Mushroom, *Pleurotus ostreatus* with a View to Genetic Improvement

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In this work the lignocellulolytic enzymes produced by the edible mushroom *Pleurotus ostreatus* var. *florida* were studied. The objective was to know their relationship with the degradation of the biopolymers present in the cell wall of wheat-straw for the purpose of explaining their influence on the production and quality characters of the fruiting bodies. The following enzymatic activities were studied both in solid and submerged culture: Ligninases (Lignin Peroxidase, Manganese Peroxidase (MnP) and Laccase), Cellulases (Glucohydrolases, Glucosidases) and Hemicellulases from the group Arabinofuran-Xylanases (Xylanase, Xilosidase, Glucoronidase, Arabinofuran-Oxidase and Acetylesterase), cooperating enzymes (Glyoxal Oxidase) and feedback enzymes (Glucose Oxidase (GOD), Aryl Alcohol Oxidase (AAO), Tyrosinase (TYR), Veratryl Alcohol Oxydase (VAO), Cellobiose Dehydrogenase (CDH)). The first studies regarding all the mentioned enzymes were performed using the dikayon (N001) and the parental monokarion strains "*fast*" (PC9) and "*slow*" (PC15).

The studies on all this whole group of enzymes, which are enough representative of the lignocellulolytic complex, let to conclude that (both in solid or submerged culture) the enzymes of major influence in colonizing the natural substrate and also those whose activity-determination better guarantees their further mapping were Laccases, MnP, AAO and TYR. Subsequently these four activities were measured in the monokaryon population being Laccases and MnP, those yielding the best levels in medium-7 (rich in nitrogen). In addition both enzymes allow the discrimination between "*fast*-" or "*slow*-" monokaryon strains both in solid medium with several dyes, or in liquid culture in agitation. The analysis of the enzymatic activities detected in the assayed conditions, in the population of "*fast*" or "*slow*" strains let to the

Genetics and Cellular Biology of Basidiomycetes-VI. A.G. Pisabarro and L. Ramírez (eds.)
 ISBN 84-9769-107-5

observation that they map in different places where the *loci* corresponding to Laccase (*pox*) and *mnp* genes are located. These results open the possibility to design more precise studies that could help to establish a correlation between the contribution of the genes already described and the activity of the different ligninolytic enzymes. In addition the results will contribute to know whether in *P. ostreatus* genome there are new genes or if they correspond with locations that regulate these enzymatic activities, or it is a gene that has a role in the transport system or a kind of effector in the exportation machinery of the protein to the culture medium.

Selection of *Pleurotus ostreatus* Strains in a Genetic Breeding Program

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The basidiomycete *Pleurotus ostreatus*, commonly known as oyster mushroom, is the second largest edible mushroom crop behind the white button mushroom, *Agaricus* bisporus. It accounts for nearly one-quarter of the total worldwide mushroom production. Furthermore, *P. ostreatus* has a high industrial interest because it is a good source of enzymes and other products with biotechnological, industrial and medical applications, it is easy to cultivate and because of its good organoleptic characteristics.

Since of 2003, our group research has carried out genetic breeding programs based on the determination of QTLs controlling production and quality in industrial cultures of this fungus. In this breeding program the first test consisted in putting under fructification conditions 130 strains obtained from the crossing of protoclon PC21 (*P. ostreatus* var. ostreatus wild strain) by a collection of monokarions derived from N001 (*P. ostreatus* var. florida commercial strain). For this purpose, 2 kg (3 repetitions per strain) bags of industrial sustrate were inoculated and cultivated at 21ºC. Mature fruiting bodies were collected and weighted daily during the fructification period.

The second test was made using the six strains that performed the better in Test1, but were cultivated at 18ºC and with 15 repetitions per strain were performed. From this test, three strains were selected and used in Test3. In this test, other three strains obtained from the crossing between monokarions descending of N001 and selectioned for their high growth rate were introduced. In this test the weight of the bags was increased to 5 kg and the cultures were cultivated at 18ºC.

The strains obtained from PC21 have good characterictics for mushroom size, with similar behaviour for yield and precocity.

The strains obtained from the crosses between N001 descendants have better mushroom size and similar yield and precocity than N001, then breeding was obtained.

The candidate strains for next tests are PC21xMA046 and PC21xMA027 for their high yield and the mushroom good features.

Genetics and Cellular Biology of Basidiomycetes-VI. A.G. Pisabarro and L. Ramírez (eds.)
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Scientific Program

GENETICS AND CELLULAR BIOLOGY OF BASIDIOMYCETES VI
Pamplona, June, 3rd to 6th, 2005

Friday June 3rd, 2005

Morning Session:

09:00-13:00 Registration
12:30-13:00 Opening.

Evening Session

1. GENETICS AND BREEDING
Chairs: **Lucy Ramírez, Rick Kerrigan**

15:00-16:00 Full lecture: Molecular approach to the breedeing of basidiomycetes. The *Pleurotus ostreatus case* Antonio G. Pisabarro, Pamplona, Spain.

16:00-16:40 Oral presentation 1.1: Natural and Artificial Hybridization of *Agaricus subrufescens* Peck (= *A. Blazei* Murrill Sensu Heinemann): Lessons from the Quasi-Alleles of the rDNA ITS1+2 Region. R. Kerrigan
Oral presentation 1.2: Expression of mating type genes in heterologous basidiomycetes. U. Kües

16:40-17:10 Coffee break

17:10-18:30 Oral presentation 1.3: Development of a sporeless strain of oyster mushroom (*Pleurotus ostreatus)*. Johan J. Baars
Oral presentation 1.4: Molecular characterisation and expression analysis of developmentally regulated genes in *Agaricus bisporus*. S. Sreenivasaprasad
Oral presentation 1.5: Mushroom Breeding Program In Iran Hamid R. Gordan

18:30-19:00 General discussion of genetics and breeding

Saturday June 4th, 2005

Morning Session

2. GENOME ANALYSIS
Chair: **Allen Gathman**

09:00-10:30 Oral presentation 2.1 QTL Mapping of Pathogenicity in *Heterobasidion annosum sensu lato*. Å. Olson
Oral presentation 2.2: Gene Expression During Symbiosis in the Ectomycorrhizal Fungus *Tricholoma vaccinum*. E. Kothe
Oral presentation 2.3: Soybean Rust, a Rising Star in Phytopathology. M.L. Posada-Buitrago
Oral presentation 2.4: Evidence for a 200 Gene Ribosome and rRNA Biosynthesis (rrb) Regulon in Fungi. M. A. McAlear
10:30-11:00 Coffee break
11:00-13:00 General round table about genome analysis and genome-sequencing projects
13:00-15:00 Lunch time

Evening Session

3 CELLULAR AND MOLECULAR BIOLOGY
Chairs: **Regina Kahmann, Erika Kothe**

15:00-16:00 Full lecture: Gero Steinberg, Marburg, Germany
16:00-16:40 Oral presentation 3.1: Ectopic expression of constitutively activated small GTPase cdc42 alters the morphology of haploid and dikaryotic hyphae in the filamentous homobasidiomycete *Schizophyllum commune*. M. Raudaskoski.
Oral presentation 3.2: Biochemistry of volatile compounds synthesis in *Agaricus bisporus*. E. Combet.
16:40-17:10 Coffee break
17:10-18:30 Oral presentation 3.3: *In situ* RNA-RNA hybridization: useful method for analyzing the distribution of transcripts of various genes in *Lentinula edodes* fruiting bodies. K. Shishido
Oral presentation 3.4: Molecular characterisation and expression analysis of developmentally regulated genes in *Agaricus bisporus*. S Sreenivasaprasad
Oral presentation 3.5: Sequence analysis and expression of a RecQ gene homologue from *Lentinula edodes.* S. Katsukawa
Oral presentation 3.6: Multiple hydrophobin genes in mushrooms. S. Peddireddi
18:30-19:00 General discussion of cellular and molecular biology

Sunday June 5th, 2005
Morning Session

4. INDUSTRIAL APPLICATIONS
Chairs: **Giovanni Sannia, Kerry Burton**

09:00-10:00 Full lecture: Leif.Jonsson. Karlstad, Sweden.
10:00-10:40 Oral presentation 4.1: Expression profiling of natural antisense transcripts in *Agaricus bisporus*. K Burton
Oral presentation 4.2: Anticancer activity of polysaccharides produced by *Pleurotus ostreatus* in submerged culture. H. El-Enshasy
10:40-11:10 Coffee break
11:10-12:30 Oral presentation 4.3: Rat Cytochrome P_{450}-mediated Transformation of Dichlorodibenzo-p-dioxins by Recombinant White-rot Basidiomycete *Coriolus hirsutus*. K. Shishido
Oral presentation 4.4: Molecular Toolkit Development for Gene Expression and Gene Silencing Technologies in the Homobasidiomycete Fungi *Agaricus bisporus* and *Coprinus cinereus*. G. Foster.
Oral presentation 4.5 Overexpression of Laccases in *Coprinopsis cinerea* S Kilaru
Oral presentation 4.6. Atypical laccase from the white-rot fungus Pleurotus ostreatus and its application for the treatment of industrial coloured effluents. G. Festa
12:30-13:00 General discussion of industrial applications
13:00-15:00 Lunch time

Evening Session

5. PLANT AND ANIMAL PATHOGENS
Chair: **José Pérez**

15:00-16:00 Full lecture: Christina Hull, Madison, USA
16:00-16:40 Oral presentation 5.1 Investigations into the fungal-fungal interaction between *Verticillium fungicola* and *Agaricus bisporus*. G Foster
Oral presentation 5.2: Characterisation of *Agaricus bisporus* response genes to *Verticillium fungicola* infection. M. Challen
16:30-17:00 Coffee break
17:00-18:30 Oral presentation 5.3: Pathogenicity in *Heterobasidion annosum* S.L. J Stenlid
Oral presentation 5.4: Virus related symptoms in crops of the button mushroom *Agaricus bisporus*. A. Sonnenberg
Oral presentation 5.5. The roles of SC3 and SC15 of *Schizophyllum commune* during growth on wood. J. de Jong
Oral presentation 5.6 Double stranded RNAs in the mushroom Virus X complex of *Agaricus bisporus*. Pr. Mills
18:30-19:00 General discussion of plant and animal pathogens
21:00-23:00 Congress Dinner

Monday June 6th, 2005

Morning Session

6. BIODIVERSITY

Chair: **Philippe Callac**

09:00-10:00	Full lecture: Fungal diversity adds value to biotechnology and agriculture. George Zervakis, Greece.
10:00-10:40	Oral presentation 6.1: Wild strains of *Agaricus bisporus*: a source of tolerance to dry bubble disease M.L. Llargeteau Oral presentation 6.2 *Agaricus devoniensis* complex comprises a group of heterothallic isolates constituting a basis for breeding. P. Callac
10:40-11:10	Coffee break
11:10-12:30	Oral presentation 6.3 Monstrosities under the Inkcap Mushrooms M Navarro González Oral presentation 6.4. Genetic variability of *Flammulina velutipes* collections from Armenia Suzanna M. Badalyan
12:30-13:00	General discussion of biodiversity
13:00-13:30	Closing and check out

Author Index