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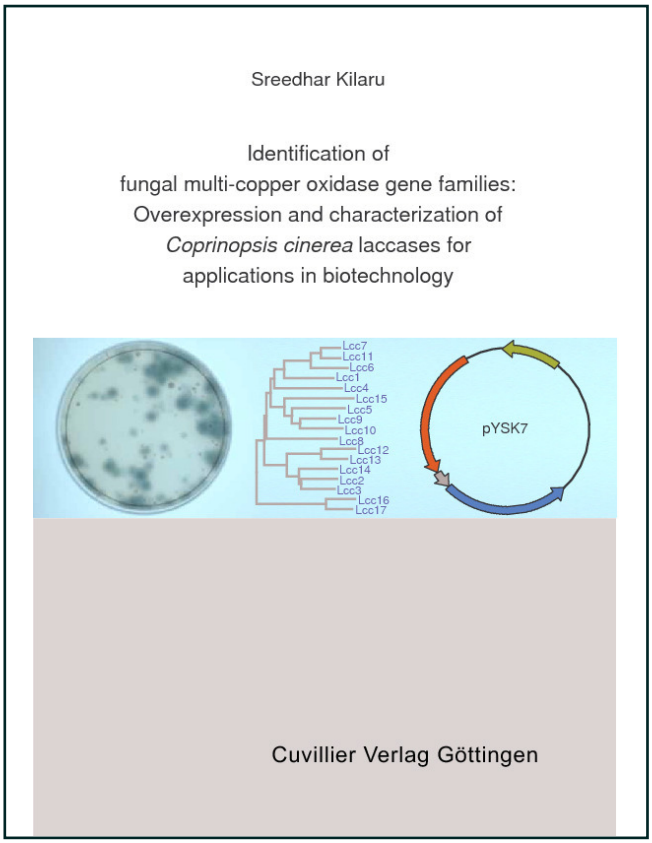
Identification of fungal multi-copper oxidase gene families: Overexpression and characterization of Coprinopsis cinerea laccases for applications in biotechnology



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Part I

I Introduction

I.1 Background and aims of this thesis

White-rot fungi are able to degrade lignocellulosic material such as straw and wood by secretion of various types of enzymes (Leonowicz et al. 2001, Martinez et al. 2005). One of the key enzymes in lignin degradation likely is laccase (EC 1.10.3.2) (Bourbonnais et al. 1995, Fukushima and Kirk 1995, Eggert et al. 1997). Laccases attack the phenolic groups of the lignin, thereby producing activated phenoxy radicals which will easily polymerize with each other (Bourbonnais et al. 1995). This principle is made use off in the production of enzyme-mediated wood composites (Hüttermann et al. 2001, Mai et al. 2004). In the paper industry, laccases are used in pulp bleaching to remove the lignin compounds which are responsible for the dark brown color of the pulp (Addleman and Archibald 1993, Reid 1998, Camarero et al. 2004). Laccases have a broad substrate spectrum. The enzymes for example convert xenobiotic compounds, chlorinated phenols, synthetic dyes, poly-cyclic aromatic hydrocarbons into unstable radicals (Claus et al. 2002, Mayer and Staples 2002, Baldrian 2005). Therefore, laccases are important enzymes also for other biotechnological applications of quite different nature such as in bioremediation, textile dye decolorization, in detection of phenolic compounds by biosensors, stabilization of beer and wine, and cosmetic preparations. All these different applications explaining the broad industrial interest in laccases are further described in the literature overview on biotechnological applications in chapter I.2 of the introduction of this thesis.

For application in industry, cheap sources of enzyme are required. Naturally, when enzyme production has been induced, basidiomycetes tend to produce between 1 to 10 U of laccases (defined as the amount of enzyme necessary to oxidize one µmol of the ABTS in one minute) per ml culture medium, in an exceptional case up to 400 units/ml has been described (Nagai et al. 2002, Xiao et al. 2003, Hou et al. 2004, Ullrich et al. 2005). However, for high scale production, fermentation conditions have to be established for individual fungal species or even individual fungal strains (Yoshiyama and Itoh 1994, Koroleva et al. 2000, Fenice et al. 2003, Ikehata et al. 2004). For high efficient production, inducers such as copper, 2,5-xylidine, vanillin and ABTS are used (Eggert et al. 1996, Baldrian and Gabriel 2002**,** de la Rubia et al. 2002, Hou et al. 2004, Minussi et al. 2005). Copper and 2,5-xylidine have the disadvantage that they are toxic and vanillin and in particular ABTS are very expensive. To become independent from such toxic and/or expensive substances and, in addition, to make

use of fungal organisms with well established fermentation technologies (Hensing et al. 1995, Gibbs et al. 2000, Liden 2002, Punt et al. 2002, Macauley-Patrick et al. 2005), many attempts have been made to overexpress basidiomycete laccases in ascomycete yeasts or in filamentous ascomycetes (see Table 2 in chapter I.3). Unfortunately, when basidiomycete laccases were overexpressed in ascomycetous hosts, recombinant enzymes in many instances were found to exhibit altered properties and the enzymatic yields were also not satisfactory (details are given in chapter I.3). Differences in protein glycosylation are made responsible for alteration of enzyme properties (Madzak et al. 2005, Nevalainen et al. 2005). Little is known about glycosylation in basidiomycetes (Giardina et al. 1999, Tams et al. 1999), but it is established from other eukaryotes that there are striking differences between different groups of organisms (Brooks 2004). One possibility to overcome problems with protein glycosylation is to alter the fungal glycosylation machinery by replacing the own glycosylation genes with foreign ones. Such approaches have been followed in construction of humanized yeasts for production of human proteins with natural glycosylation (Hamilton et al. 2003, Gerngross 2004, Wildt and Gerngross 2004, Macauley-Patrick et al. 2005). In the basidiomycete with yet not characterized glycosylation machinery, such approach appears currently too ambitious.

Developing an overexpression system for basidiomycetes is an alternative solution to the problem. This task was a main aim of this thesis. Parallel to this thesis, two other groups have initiated projects in this direction. In Japan, the group around Shishido used *Trametes versicolor* as host organism for enzyme overproduction from its laccase III gene under control of the species-own *gpd* promoter. 1.0 nmol/ml laccase activity (with guaiacol as test substrate) was achieved (Kajita et al. 2004). In the Netherlands, Wösten´s group expressed the *Pycnoporus cinnabarinus* gene *lacI* under control of the *Schizophyllum commune gpd* promoter in *P. cinnabarinus*. Enzyme activities of 145 nkat/ml culture (8.7 U/ml; measured with ABTS as substrate) were obtained in standard growth medium that by ethanol treatment surprisingly could be increased to values of 4660 nkat/ml (260 U/ml) (Alves et al. 2004). In this context, however, it should be noted that such values from different studies are difficult to compare when different enzymatic test systems with different substrates are used (see also the literature overview on enzyme properties and production in chapter I.3 of the introduction of this thesis).

In this study, *Coprinopsis cinerea* (former name *Coprinus cinereus*), a well known model fungus for studying growth and development in higher basidiomycetes (Kües 2000),

served as an expression host for laccases. An advantage of this species for recombinant protein production is the most efficient and reliable transformation system described for any basidiomycete. The fungus produces abundant uninuclear, haploid asexual spores (oidia; Polak et al. 1997, 2001), that easily can be protoplasted and regenerated upon treatment with DNA with several hundreds up to thousands of transformants per single experiment (Binninger et al. 1987, Granado et al. 1997). Obtaining transformants with *T. versciolor* and *P. cinnabarinus* is much more difficult and a transformation rate of about 10 transformants per µg of DNA in a positive experiment is a great success (Alves et al. 2004, Kajita et al. 2004). Compared to the other species, *C. cinerea* has also growth advantages. At the optimal temperature of 37°C, strains grow on agar plates several mm per day (Polak et al. 2001). In liquid medium in shaken culture, a few days are sufficient to complete biomass production (Ikehata et al. 2004). A high speed of biomass production of an organism is of financial benefit in biotechnological productions because of the short times needed for batches of fermentations and a high growth temperature will reduce energy costs for cooling in large scale fermentations (Punt et al. 2002).

Laccase genes used in this study for overexpression also came from *C. cinerea*. Six different genes were known from the literature (Bottoli et al. 1999, Yaver et al 1999, Kües 2000), of which one (*lcc1*) was shown to encode a functional protein (Schneider et al. 1999, Yaver et al. 1999). Two further genes were identified from PCR-directed cloning work by P. J. Hoegger. These eight genes were the starting point of the molecular genetic work of this thesis. Genes and proteins have been compared in structure (chapter II.1), an analysis that was extended to further laccase genes (chapter II.2) once the whole genome of *C. cinerea* strain Okayama 7 was released on 16 July 2003 to the public by the Broad Institute (http://www.broad.mit.edu/). To learn more from evolution of multi-copper oxidase genes about functions of their products, whole gene families from the fungal genomes and, in addition, all available fungal multi-copper oxidase genes from the NCBI database (http://www.ncbi.nlm.nih.gov/) were to be compared (see chapter II.3 and chapter II.4).

A third requirement for a successful establishment of a host-vector-system for overexpression of proteins is a suitable highly efficient promoter. Shishido´s group used in the past the *priA* promoter from *Lentinus edodes* for expression of the manganese (II) peroxidase gene *mnp* from *Pleurotus ostreatus* and of xylanase genes from *Aspergillus oryzae* and *Bacillus subtilis* in *C. cinerea* (Ogawa et al. 1998, Kikuchi et al. 1999, 2004). The *priA* promoter was kindly supplied by Prof. K. Shishido for this study. Successful expression of the

bacterial hygromycin resistance gene *hph* was first achieved in *C. cinerea* under control of the homologous *tub1* promoter (Cummings et al. 1999). Prior to publication (Burns et al. 2005), information was given to us about the successful use of the *Agaricus bisporus gpdII* promoter for expression of the green fluorescent protein gene *gfp* in *C. cinerea* and constructs with the promoter sequence were kindly provided by Dr. C. Burns and Prof. G.D. Foster for testing in laccase gene expression. The *Schizophyllum commune Sc3* promoter, known to efficiently drive expression in vegetative mycelium of *S. commune* (Schuren and Wessels 1990) as well as in the heterologous host *P. cinnabarinus* (Alves et al. 2004), was kindly supplied by Prof. H.A.B. Wösten. This promoter was not tested before for activity in *C. cinerea*. Whilst the functionality of four of the promoters in *C. cinerea* had been shown, nothing was known on the efficiencies with which they work in the species. Comparing and defining the best possible promoter for laccase expression was therefore another main task of this thesis. The known functional gene *lcc1* served thereby as a reporter gene (see chapters III.1 and III.3). Efficient replacement of promoter sequences in vectors for transformation was achieved by *in vivo* cloning through homologous recombination in the ascomycete yeast *Saccharomyces cerevisiae* (Raymond et al. 1999). The same quick strategy was used in replacing different *C. cinerea* laccase genes for overexpression (see Fig. 1 in chapters III.1 and Fig. 3 in chapter III.3).

More than 100 laccases have been purified from fungi and more or less characterized in their properties. A complete data set (MW, pI, pH optimum and Km for the different substrates ABTS (2,2′-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid]), DMP (2,6-dimethoxy phenol), GUA (guaiacol), SGZ (syringaldazine), temperature optimum) is however available only for a laccase from *Daedalea quercina* and for POXA1w, POXA2, and POXC from *Pleurotus ostreatus*. pH optimum and Km values for two or more substrates are available for additional 18 enzymes. In contrast, in 22 cases, the protein characterization is restricted to protein size determination and in 12 further cases, an additional value for the pI is given (see the compilation of enzyme data published at the end of 2005 by Baldrian, chapter I.3). Often, fungi produce several laccase isoenzymes from the same but also from different genes (Palmieri et al. 1997, Galhaup et al. 2002). When assessing the data on properties of purified laccase by Baldrian (2005), one has to keep in mind that in many of the studies protein purification might not have separated isoenzymes from each other (further explanation in chapter IV.2).

60 different laccase genes were known at the beginning of this thesis (Kumar et al. 2003). For the encoded protein products of most of these genes, there are no further data available (see Table 1 in chapter I.3 and Table 2 in chapter II.3). The 2760 references in the literature data base Web of Science appearing under the keyword laccase in January 2006 reflect the importance the enzyme is given in science and biotechnological application. The lack of full data sets on laccase genes and protein properties is therefore rather surprising. Once the system for overexpression of laccases in *C. cinerea* was established, the remaining time in this thesis was therefore allotted to production of enzymes for further characterization as a start to fill this hole of knowledge between genes and their proteins (see chapters IV.1 and IV.2). The overall data obtained in this thesis are discussed in chapter V.

I.1.1 References

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