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Development of Chimeric Laccases by Directed Evolution

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ABSTRACT: DNA recombination methods are useful tools to generate diversity in directed evolution protein engineering studies. We have designed an array of chimeric laccases with high-redox potential by in vitro and in vivo DNA recombination of two fungal laccases (from *Pycnoporus cinnabarinus* and PM1 basidiomycete), which were previously tailored by laboratory evolution for functional expression in *Saccharomyces cerevisiae*. The laccase fusion genes (including the evolved α-factor prepro-leaders for secretion in yeast) were subjected to a round of family shuffling to construct chimeric libraries and the best laccase hybrids were identified in dual high-throughput screening (HTS) assays. Using this approach, we identified chimeras with up to six crossover events in the whole sequence, and we obtained active hybrid laccases with combined characteristics in terms of pH activity and thermostability.

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KEYWORDS: chimeric laccases; DNA shuffling; α-factor prepro-leader; high-throughput screening; *S. cerevisiae*

Introduction

Laccases (EC 1.10.3.2) are blue-multicopper oxidases capable of acting on a wide range of aromatic compounds, especially phenolic compounds and aromatic amines. These enzymes were first discovered in the 19th century and they are widely distributed in nature, where they participate in synthesis and degradation processes (Claus, 2004; Thurston, 1994). White-rot basidiomycete fungi, the only organisms capable of complete degradation of lignin, are the most important producers of laccases. Laccases use molecular oxygen as the final electron acceptor and they release water as the only by-product. They contain four copper atoms arranged in three oxidation-reduction sites. The reducing substrate is oxidized at the T1 Cu site and the electrons from four monovalent oxidations are transferred to the trinuclear Cu cluster, formed by one T2 and two T3 Cu sites, where one molecule of oxygen is reduced to two molecules of water (Morozova et al., 2007). Depending on the redox potential at the T1 Cu site, laccases are sorted as low- (<470 mV), medium- (470–700 mV), or high- (>700 mV) redox potential enzymes (Shleev et al., 2005).

Due to their oxidative versatility and low catalytic requirements, fungal laccases, especially the high-redox potential laccases (HRPLs), are of particular interest as potential biocatalysts in different industrial sectors. However, for successful biotechnological application, the intrinsic properties of the enzyme usually need to be adjusted by protein engineering to comply with operational requirements. Unfortunately, white-rot fungi, the only known HRPL producers, are not amenable to genetic manipulation and it is difficult to heterologously express HRPLs (Rodgers et al., 2010). Recently, we described the in vitro evolution of HRPLs from PM1 basidiomycete (PM1L) and *Pycnoporus cinnabarinus* (PcL), which share 76% sequence identity, resulting in their successful functional expression in *S. cerevisiae* (Camarero et al., 2012; Maté et al., 2010). To accelerate directed evolution for improved secretion, native signal leaders were replaced by the prepro-leader of the α-mating factor from *S. cerevisiae* and the whole α-laccase constructs were subjected to random mutagenesis and recombination. Laccase secretion by yeast was significantly increased in the final evolved mutants (2–8 mg/L), which also showed improved kinetic values for phenolic and non-phenolic compounds. Moreover, the pH activity profile of evolved PcL shifted upwards and the evolved PM1L had greater thermal stability.

DNA shuffling has become the most commonly used method to create chimeric genes since it was described by Stemmer in 1994. In the present study, we sought to build complex chimeric libraries through DNA shuffling of HRPL genes to generate new rearranged proteins with modified/improved characteristics over the parent laccases. Using the

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combinatorial libraries enhanced by recombination in yeast (CLERY) strategy (Abécassis et al., 2000), the two evolved PM1L and PcL genes (Camarero et al., 2012; Maté et al., 2010) were recombined in vitro and in vivo, and a new collection of active chimeras was obtained after high-throughput screening (HTS) of the chimeric libraries. The selected laccase hybrids with combined properties constitute the first successful example of chimeragenesis of HRPLs from different fungi.

Materials and Methods

Reagents and Enzymes

DMP (2, 6-dimethoxyphenol) and the *S. cerevisiae* transformation kit were purchased from Sigma-Aldrich (Madrid, Spain). ABTS (2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), DNase I from bovine pancreas lyophilisate, and the high pure plasmid isolation kit were purchased from Roche (Madrid, Spain). Zymoprep yeast plasmid miniprep II kit was purchased from Zymo Research (Orange, CA), and the Plasmid midi kit and QIAquick gel extraction kit from QIAGEN (Valencia, CA). The restriction enzymes BamHI, NotI, and BsaXI were obtained from New England Biolabs (Hertfordshire, UK), and Pfu Ultra High-Fidelity DNA polymerase was purchased from Strategene (La Jolla, CA).

Strains and Culture Media

The protease deficient *S. cerevisiae* BJ5465 strain was purchased from LGCPromochem (Barcelona, Spain). The uracil-independent and ampicillin-resistance shuttle pJRoC30 vector carrying the 3PO (evolved PcL) and OB-1 (evolved PM1L) constructs (including their evolved α prepro-leaders) under the control of the GAL1 promoter were generated as described previously (Camarero et al., 2012; Maté et al., 2010). The BJ5465 yeast strain was grown in YPD medium. Specific uracil-deficient media used to grow yeast transformants and the laccase expression media supplemented with ethanol and copper have been described previously (Camarero et al., 2012). The DH5α *E. coli* strain was used to amplify the plasmids and it was grown in LB medium.

Construction of Chimeric Libraries using CLERY

General Aspects

All PCR products and/or digested fragments were purified by loading the reaction products into 0.8% medium EEO agarose gels and using the QIAquick gel extraction kit. The fragments were cloned into the pJRoC30 vector (previously linearized with NotI and BamHI to remove the native gene) by designing overhangs that specifically annealed with the ends of the linearized vector (annealing regions of 40 and 66 bp).

Optimization of In vitro DNA Shuffling

In vitro DNA shuffling was conducted as described previously (Joern, 2003; Zhao and Arnold, 1997) with some modifications. Parental DNA was obtained by digesting pJRα3PO and pJRαOB-1 with BsaXI, as the plasmid contains two restriction sites flanking the complete fusion gene. Thus, 2.8 kbp fragments were generated, purified, and concentrated. Then, a mixture of 1.25 μg of each parental gene was digested with 0.02 units of DNase I in a volume of 50 μL for 5 min at 15°C in the thermocycler (Eppendorf Mastercycler pro S). Digestion was stopped by adding 5 μL of 0.5 M EDTA on ice and the reaction products were separated in an agarose gel, producing a typical smear of DNA fragments ranging from 80 to 1,500 bp. The 80–350 bp and 350–700 bp fragments were purified and processed separately to create libraries L1 and L2, respectively. The PCR reassembly reaction mixture contained 25 μL of fragments (of either 80–350 or 350–700 bp), 5 μL of 10× PfuUltra HF buffer, 1 μL of 40 mM dNTP mix and 2.5 units of PfuUltra HF DNA polymerase in a final volume of 50 μL. PCR cycles were carried out as described previously (Abécassis et al., 2000) and fragments were amplified with primers designed to generate the aforementioned overhangs: RMLN sense (5′-CCTCTATACTTTAACGTCAAGG-3′) which binds to nucleotides 160–180 of the plasmid; and RMLC antisense (5′-GGGAGGGCGTGAATGTAAGC-3′), which binds to nucleotides 2031–2050 of the plasmid. The reaction mixture contained 1, 5, or 10 μL of the reassembly product, 5 μL of 10× PfuUltra HF buffer, 3% DMSO, 1 mM dNTP mix, 0.25 μM of each primer, and 2.5 units of PfuUltra HF DNA polymerase in a final volume of 50 μL. Reactions were performed as follows: 95°C for 2 min; 28 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min; and a final elongation step at 72°C for 10 min. Maximum amplification yield was obtained with 10 μL of the reassembly product derived from small fragments and 5 μL derived from large fragments. Bands of 1.9 kb (1.8 kb of α-laccase plus the overhangs generated with RMLN and RMLC) were purified and subjected to in vivo DNA shuffling.

In vivo DNA Shuffling

Of the purified PCR products generated by in vitro DNA-shuffling, 400 ng was mixed with 100 ng of the linearized vector (the molar insert:vector ratio was 27:1) and used to transform *S. cerevisiae*-competent cells. Transformed cells were then plated on synthetic complete (SC) drop-out plates and the colonies of cells containing the whole autonomously replicating vector were grown for 48 h at 30°C. Up to 3,000 transformants per μg of DNA were obtained for L2 and 1,400 transformants per μg of DNA for L1. Of these, 550 individual colonies were picked from each library and

screened, using transformed cells with plasmids harboring the parental genes for reference during the library screening.

High-Throughput Screening

Laccase Activity Screening

Individual clones were picked and cultured in 50 μL of minimal medium in sterile 96-well plates (Falcon BD, Meylan Cedex, France), in which columns 6 and 7 were inoculated with the parental types, and one uninoculated well (H1) served as a negative control. The plates were sealed and incubated at 30°C in 60% humidity with agitation (200 rpm). After 48 h, 160 μL of expression medium (Camarero et al., 2012) was added and the plates were incubated for a further 24 h at 20°C, after which they were centrifuged at 2200*g* for 15 min at 4°C (Eppendorf centrifuge 5430 R). A 20 μL volume of supernatant was transferred to two replica plates (Greiner Bio-One) using a Quadra 96–320 Liquid Handler (Tomtec, Hamden, CT). To each replica plate 180 μL of 3 mM ABTS or DMP was added in 100 mM acetate buffer (pH 5.0) and the absorption was measured at 418 nm ($\varepsilon_{ABTS\cdot+} = 36000\,M^{-1}\,cm^{-1}$) and 469 nm ($\varepsilon_{DMP} = 27500\,M^{-1}\,cm^{-1}$) using a SPECTRAMax Plus 384 plate reader (Molecular Devices, Sunnyvale, CA). Plates were incubated in the darkness at room temperature (RT) until the color fully developed and the endpoint absorptions were measured. Relative activities were calculated from the differences between the final and initial absorption and normalized against each parental type in the corresponding plate.

First Re-Screening

Aliquots (5 μL) from microcultures of the selected clones (21 clones from L1 and 47 from L2) were removed from the master plates and used to inoculate 50 μL of minimal medium in columns 2 and 7 of new sterile 96-well plates. Wells D7 and E7 were inoculated with the two parental types. Columns 1 and 12, and rows A and H, were not used. Plates were sealed and incubated for 24 h at 30°C with agitation at 200 rpm. Next, 5 μL of growth medium was transferred to the four adjacent wells and the plates were incubated for another 24 h. Finally, 160 μL of expression medium was added to each well and the plates were incubated for an additional 24 h at 20°C. Laccase activity was measured as described above.

Second Re-Screening

Aliquots (100 μL) from the selected clones (12 clones from L1 and 29 from L2) were used to inoculate 3 mL of YPD medium, which was then incubated for 24 h at 30°C. The plasmids isolated from these cultures were transformed into *E. coli*, and single colonies of these cells grown on LB-Amp plates were expanded overnight at 37°C in 5 ml of LB-Amp medium and the amplified plasmids were recovered. *S. cerevisiae* was transformed with the purified plasmids and the parental types, and five single colonies were picked and screened for each clone, as described above. Plasmids from selected mutants were sequenced with the RMLN, RMLC, LAC-F, and LAC-R primers, the latter of which were designed to anneal to stretches of identity between parent laccases, except for one mismatch (underlined). LAC-F (5′-AGGGCAAGCGCTA**C**CGCTTCCGC-3′) binds to nucleotides 1057–1079 of pJRα3PO, and LAC-R (5′-CGGAT-CCAGTAGTT**G**TCCAC-3′) binds to nucleotides 1242–1261 of pJRα3PO (the positions for pJRαOB-1 are three nucleotides less).

Flask Production of Chimeras

Single colonies of the *S. cerevisiae* clones transformed with plasmids containing the selected genes were picked from SC drop-out plates, used to inoculate 3 mL of minimal medium in duplicate and incubated for 48 h at 30°C with agitation (220 rpm). An aliquot of the cells was used to inoculate a final volume of 5 mL in 100 mL flasks, adjusting the OD_{600} to 0.25, which were then incubated for two complete growth phases (6–8 h). Thereafter, the cells were diluted to $OD_{600} = 0.1$ in a final volume of 10 mL of expression medium (supplemented with 4 mM $CuSO_4$) in 100 mL flasks. When maximum activity was reached, the cells were separated by centrifugation at 13,000 rpm at 4°C and the supernatants were concentrated in 10,000 MWCO Amicon-Ultra Centrifugal filters (Millipore Iberica S.A.U., Madrid, Spain).

Characterization of Chimeric Laccases

pH Activity Profiles

Appropriate dilutions of the concentrated crude extracts were prepared such that 10 μL aliquots produced a linear response in the kinetic mode in the plate reader. Plates containing 10 μL of laccase samples and 180 μL of 100 mM Britton and Robinson buffer were prepared at pH values of 2, 3, 4, 5, 6, 7, and 8. The assay commenced when 10 μL of 60 mM ABTS or DMP was added to each well to give a final substrate concentration of 3 mM. Activities were measured in triplicate in kinetic mode and the relative activity was calculated as a percentage of the maximum activity of each variant in the assay.

Thermostability

T_{50} values were assessed in the gradient thermocycler, defining the T_{50} as the temperature at which the enzyme retains 50% of its activity after a 10 min incubation. Appropriate dilutions were prepared such that 20 μL aliquots produced a linear response in the kinetic mode in the plate reader. Subsequently, 35 μL aliquots were used

in triplicate at each point of the following temperature scale (°C): 39.9, 40.4, 41.7, 43.5, 45.8, 48.4, 51.1, 53.8, 56.2, 58.1, 59.5, 61.4, 63.3, 65.6, 68.2, 70.9, 73.6, 76.0, 77.9, 79.3, and 79.9. After a 10 min incubation, samples were chilled on ice for 10 min and incubated at RT for another 5 min. Thereafter, 20 μL of each sample was added to 180 μL of 100 mM acetate buffer (pH 5) containing 3 mM ABTS and the activity was measured in the kinetic mode. The thermostability values were deduced from the ratio between the residual activities incubated at different temperature points and the maximum activity for each variant in the assay.

K_m values for ABTS and DMP

Oxidation of increasing substrate concentrations was analyzed by measuring absorption at 418 nm (ABTS) and 469 nm (DMP) in the plate reader. Reactions were conducted in triplicate by adding 20 μL of the crude extract dilutions to 100 mM acetate buffer (pH 5.0) to a final volume of 250 μL. To calculate the K_m value, the relative activity (relative to maximum activity in each assay) was plotted against the substrate concentration and fitted to a single rectangular hyperbola function in SigmaPlot software, where $b = K_m$.

Results

Constructing and Screening Chimeric Libraries

The evolved laccase variants finally obtained through the directed evolution of PM1L and PcL, the OB-1 and 3PO mutants (Camarero et al., 2012; Maté et al., 2010), were used as the parental lacasses for chimeragenesis. These mutants accumulate several different beneficial mutations in their evolved α-factor prepro-leaders that promote their secretion by yeast, along with other mutations in the mature protein that enhances their activity/stability. These two evolved fusion genes were shuffled by CLERY, which combines the PCR-dependent reassembly of the fragmented genes (in vitro DNA shuffling) with the recombination and cloning of the PCR products in *S. cerevisiae* (in vivo DNA shuffling, Abécassis et al., 2000). Gene fragments of different sizes (80–350 and 350–700 bp) were tested to create libraries with distinct crossover profiles, given that in vitro DNA shuffling of smaller fragments generates more crossover events and larger fragments are easier to reassemble (Abécassis et al., 2000; Moore et al., 2001). The higher probability of correct PCR-reassembly using large fragments was confirmed by the enrichment of the desired 1.9 kb band in the final amplification step (see Materials and Methods section for details). Two chimeric libraries were tailored by in vitro DNA shuffling, one of small fragments (L1) and the other of large fragments (L2), and they were separately transformed in the yeast to optimize the final laccase family shuffling process.

The laccase activity in the chimeric libraries was screened independently by the oxidation of ABTS and DMP (Camarero et al., 2012; Maté et al., 2010). We sought active laccase variants that performed better than the parental laccases in our experimental conditions (due to either improved secretion, enhanced catalytic activity, or altered optimum pH). Since the OB-1 parent exhibited higher activity for both substrates than the 3PO parent, the best clones were selected based on their activity with respect to OB-1. More specifically, the best hybrids were chosen based on the Total Activity Improvement (TAI) value, defined as the combined improvement in secretion and k_{cat} relative to the OB-1 parental type. In general, we observed a direct correlation of laccase activities with the two substrates (Fig. 1), which allowed us to select clones with the best TAI values for both compounds. The protocol used to build L2 seemed to be more efficient in generating active hybrids,

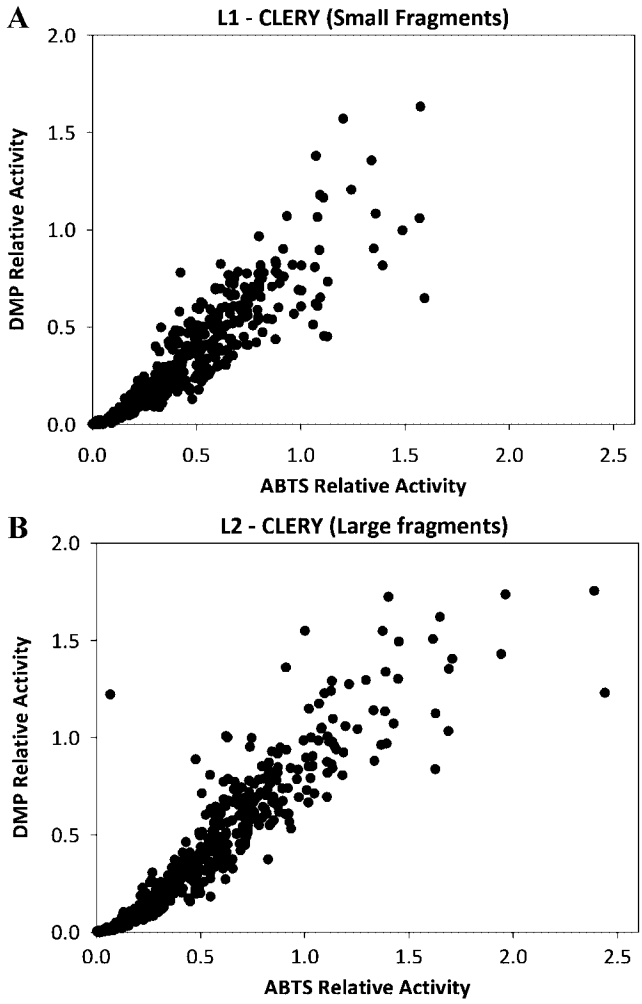

Figure 1. Landscapes obtained from the HTS of chimeric libraries L1 (**A**) and L2 (**B**), showing the laccase activities of clones for ABTS and DMP in comparison with that of the OB-1 parental type (1).

given that 5.2% of the clones screened in L2 exhibited higher laccase activity than the parental types, as opposed to 1.9% in L1. These data indicate that the higher recombination frequency among small fragments might limit the generation of active chimeras in L1. After two consecutive re-screenings, seven clones from L1 and 19 clones from L2 were selected and further characterized, having TAI values for both substrates approximately twofold those of the OB-1 parent.

DNA sequencing revealed that all selected clones were chimeric (Fig. 2), although some were overrepresented in the same library as commonly occurs in DNA shuffling (Chaparro-Riggers et al., 2007), and three clones acquired a single mutation, in concordance with the rate of inadvertent point mutations for in vitro DNA shuffling using *Pfu* polymerase (Chaparro-Riggers et al., 2007; Zhao and Arnold, 1997). Most genes selected combined beneficial mutations from the two evolved α-factor prepro-leaders. In general, the new hybrid products included three different types of chimera: Shuffled prepro-leaders fused to the unaltered OB-1 mature laccase, mature protein chimeras with parent prepro-leaders, and global chimeras (Fig. 2). DNA shuffling was produced at stretches of identity of 3–93 bp. Many of the crossover events in the mature protein sequence occurred at the four conserved signature laccase motifs that contain the preserved ligands of the T1, T2, and T3 Cu sites. Other crossover events occurred repeatedly in distinct regions of high homology between both parent sequences (Fig. S1). In both libraries, the number of crossover events in the whole chimeric sequence ranged from 1 to 6. The in vivo shuffling may have contributed to the similar number of crossover events observed in L2 and L1, as some recombined fragments found in L2 were very small and yeast favors crossovers within homologous stretches as small as 2–20 bp (Mezard et al., 1992). However, the higher recombination frequency following in vitro shuffling of small fragments (L1) was evident through the higher proportion of chimeras with six crossovers selected from L1 (29%) than from L2 (5%).

Chimera Characterization

Chimeras were grown in flasks to obtain sufficient secreted laccase for preliminary characterization studies. The pH activity profiles for oxidation of ABTS and DMP (Fig. 3A, B) and the thermostability (Table I) of the mature laccase hybrids were measured to identify possible changes in laccase properties due to sequence block exchange. The 2C4 variant (containing a higher proportion of OB1) exhibited the most significant differences in its pH activity profile to all the other laccase chimeras analyzed. Its pH profile shifted considerably toward more neutral values, with a sharp maximum at pH 5.0 for both substrates, and there was

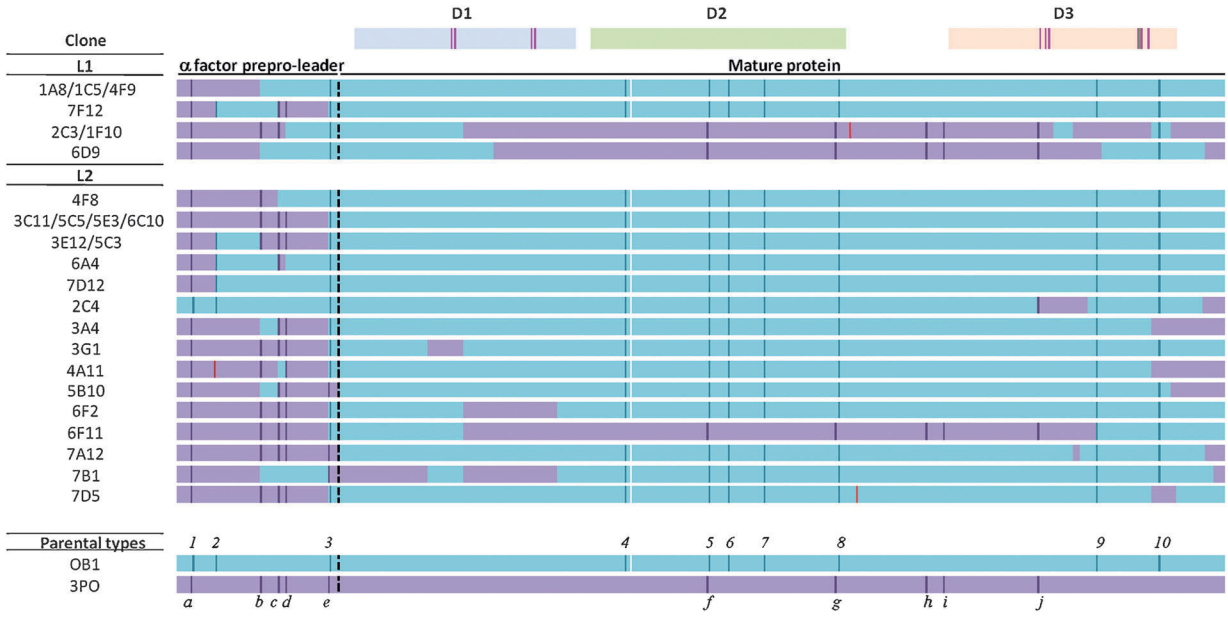

Figure 2. Schematic illustration of the sequence block exchanges produced in the selected hybrid sequences. Light blue and purple boxes represent the OB-1 and 3PO parental types, respectively. The distinct mutations (dark blue and purple lines) accumulated in both the mature laccase and the α-factor prepro-leader sequences during the parallel evolution pathways are indicated. For simplification, block exchange is represented in the first residue that differs between the two laccases. Mutations in OB-1 are: (1) Vα10D, (2) Nα23K, (3) Aα87T, (4) V162A, (5) H208Y, (6) S224G, (7) A239P, (8) D281E, (9) S426N, and (10) A461T. Mutations in 3PO are (a) Aα9D, (b) Fα48S, (c) Sα58G, (d) Gα62R, (e) Eα86G, (f) N208S, (g) R280H, (h) N331D, (i) D341N, and (j) P394H. The T1, T2, and T3 Cu ligands are depicted as magenta lines at the laccase domains (D1, D2, and D3). The white line represents a 1 residue gap introduced for sequence alignment, and the red lines represent inadvertent point mutations introduced during PCR amplification.

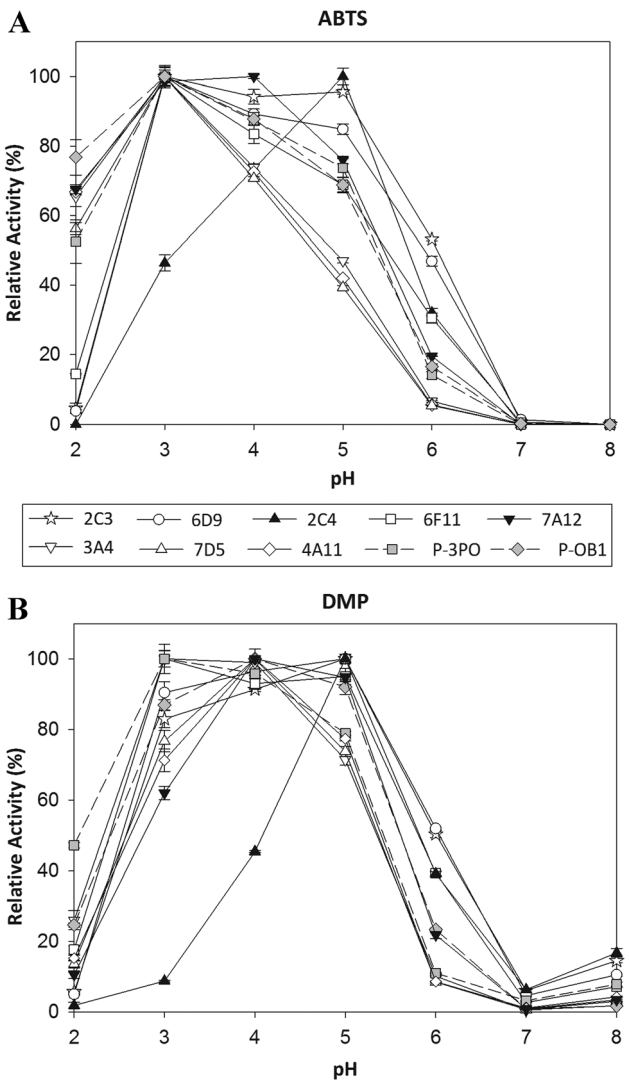

Figure 3. pH activity profiles of chimeric laccases for the oxidation of ABTS (**A**) and DMP (**B**). For simplification, only laccase hybrids with activity profiles distinct from the parental forms are depicted. Each point represents the average of three independent experiments ± standard deviation.

a significant decrease in activity at pH 3.0 and 4.0, values at which other laccase hybrids displayed maximal activity. The activity of the chimeras 2C3, 6D9, and 6F11, whose protein sequences shared greater identity with the 3PO parent (Fig. 2), was enhanced at more neutral pHs, retaining 40–50% activity at pH 6.0 for both substrates (as opposed to ~10–20% for the parental types). By contrast, 3A4, 7D5, and 4A11 chimeras (holding a higher proportion of OB1) exhibited narrower pH profiles, which unpredictably shifted towards more acidic values. Only 40% ABTS activity was retained at pH 5 compared to the 70–80% of the parental types (Fig. 3A).

Unexpectedly, five laccase hybrids (2C4, 3A4, 4A11, 7A12, and 7D5) exhibited significantly higher T_{50} values than both parental types, with increases of up to 3°C over that of OB-1 (Table I). The most unstable variants ($T_{50} \leq 62$°C) were 2C3 and 6F11, which shared the highest sequence content with the 3PO parent. However, variant 6D9 (also containing a 3PO scaffold) retained a T_{50} value similar to that of 3PO. The substrate affinities for most promising chimeras (with improved stability or modified pH activity profiles) were determined (Table II) and as expected, the K_m values of the 6D9 chimera were comparable to those of the 3PO parent. Similarly, the 3A4, 7D5, and 7A12 chimeras, in which the OB-1 sequence was largely retained, exhibited a similar K_m for ABTS as the OB-1 parent, and an even higher affinity for DMP.

Table I. T_{50} values for the chimeric and parental laccases.

Library	Clone	T_{50} (°C)
L2	6F11	59.1
L1	2C3	61.9
L2	7B1	65.0
L2	3G1	67.1
L2	6F2	68.1
L2	5B10	68.7
L1	6D9	69.5
Parent	3PO	70.9
Parent	OB-1	73.3
L2	7D5	74.9
L2	2C4	75.5
L2	3A4	76.1
L2	4A11	76.6
L2	7A12	76.6

Discussion

Family Shuffling and Sequence Analysis

DNA recombination by family shuffling is based on the use of distinct parental genes with sufficient sequence homology to allow the assembly of whole genetic products without jeopardizing enzyme folding and function. The present study describes the family shuffling of two fungal HRPLs that are functionally expressed in *S. cerevisiae* to generate

Table II. Substrate affinities for ABTS and DMP of selected hybrid and parental laccases.

	K_m (μM)	
Laccase	ABTS	DMP
6D9 chimera	46.5 ± 1.0	361.4 ± 12.0
3A4 chimera	9.8 ± 1.1	61.8 ± 4.8
7D5 chimera	7.6 ± 0.5	53.4 ± 2.5
7A12 chimera	8.1 ± 0.4	86.2 ± 2.9
2C4 chimera	8.0 ± 0.4	777.8 ± 22.2
OB-1 parent	9.1 ± 0.5	93.1 ± 3.3
3PO parent	42.9 ± 1.3	243.5 ± 6.7

chimeric laccases. We selected the CLERY strategy that combines in vitro and in vivo DNA shuffling (Abécassis et al., 2000) in order to create as much diversity as possible through the high recombination frequency between the parental types. The generation of chimeric laccases has been described previously using isoenzymes from the same fungal strain (Cusano et al., 2009). Here, we used laccase genes from different basidiomycete fungi, which in conjunction with the DNA recombination strategy employed, allows access to different regions of the sequence space and promotes the exchange of sequence blocks, thereby yielding chimeras with larger sequence divergence (Arnold and Volkov, 1999; Crameri et al., 1998) than those described previously (Cusano et al., 2009; Nakagawa et al., 2010).

Laccases are organized into three cupredoxin-like domains (D1, D2, and D3) and when the mature hybrid protein sequences generated by chimeragenesis were analyzed no crossover events were detected in the second laccase domain (D2). All crossover events were observed at D1 and D3, where the four highly conserved laccase signature motifs are located (Kumar et al., 2003), although many crossovers occurred outside of these conserved regions. In fact, the number of crossover events at D3 doubled that observed at D1, indicating a biased window of recombination in the 3′ region (C terminal), consistent with previous findings (Cusano et al., 2009). Crossovers preferably occur in regions of high homology and at positions that minimize disruptions of interactions (Joern et al., 2002). However, the random fragmentation of genes used in the present study reduced such bias in recombination when compared with other reported methods. The bias in the in vivo gap-repaired ligation method is introduced by linearizing the receptor fragment at the targeted restriction sites, where the eukaryotic machinery repairs the gap by recombination with the donor gene (Cusano et al., 2009). Likewise, by using the DNA swapping method, protein block exchange can only occur at the site at which the genes have been cut (Nakagawa et al., 2010).

DNA shuffling facilitates a markedly broader exploration of protein sequences by increasing the recombination frequency (Abécassis et al., 2000; Stemmer, 1994; Volkov and Arnold, 2000) and indeed, screening the chimeric libraries revealed active laccase hybrids with up to six crossover events. By contrast, only one protein block exchange and two crossovers were obtained by DNA swapping (Nakagawa et al., 2010) and in vivo gap-repaired ligation (Cusano et al., 2009), respectively. We do not dismiss that even higher recombination frequencies might have occurred generating less active chimeras that would have not been detected by the screening procedure, as only clones with enhanced activity with respect to parental types were chosen. The HTS protocol used also allowed us to get rid of the parental background usually produced during DNA shuffling (Abécassis et al., 2000; Joern et al., 2002; Stemmer, 1994).

Of the 12 mature chimeric sequences selected, nine retained a predominant fraction of the parental OB-1 laccase, in good agreement with previous studies of shuffled gene libraries (Joern et al., 2002). In principle, chimeras are inactivated by disrupting interactions that contribute to proper folding, stability, or activity. Hence, chimeric clones with a higher proportion of a single parent are less prone to inactivation as more interactions are conserved (Crameri et al., 1998). Most hybrid sequences exhibited shuffled signal peptides, including the Aα9D and Aα87T mutations from the 3PO and OB-1 evolved α-factor prepro-leaders, respectively. Interestingly, other mutations at similar locations were also evident in both variants (Vα10D in OB-1 and Eα86G in 3PO). Aα9 and Vα10 are non-polar amino acids in the hydrophobic core of the canonical pre-leader that is involved in the orientation and insertion of the nascent polypeptide during translocation to the endoplasmic reticulum (Nothwehr and Gordon, 1990). Their substitution by aspartic acid suggests that a reduction in hydrophobicity in this area may be beneficial for secretion. However, the Aα9D mutation, which led to the greatest improvement during the directed evolution of *P. cinnabarinus* laccase (Camarero et al., 2012), appears to be more beneficial than the Vα10D mutation (Aα9D was found in 18 of the 19 chimeras analyzed). The Aα87T mutation (and Eα86G) is located in the spacer dipeptide (Glu-Ala-Glu-Ala) that lies between the α-factor prepro-leader and the mature laccase, which is the processing site of the dipeptidyl aminopeptidase *STE13* in the Golgi compartment (Romanos et al., 1992). This mutation may be involved in the alternative processing that promotes the secretion of active laccases containing a 6 amino acid extension at the N-terminal, without compromising activity or stability (unpublished data). In general, the shuffled prepro-leaders fused to the unaltered OB-1 mature laccase increased their secretion by yeast. Along with the observed prevalence of certain beneficial mutations from the evolved prepro-leaders, these findings provide new evidence to support the hypothetical design of a ''universal'' prepro-leader for laccase expression in yeast.

Chimeric Laccases With Combined Features

The dual colorimetric assay used to screen the chimeric libraries allowed the selection of active hybrids that maintained a broad oxidation range for phenolic (DMP) and non-phenolic polar compounds (ABTS). The increase in laccase activity at neutral pHs may be of interest for certain biotechnological applications, such as enzyme-aided TCF bleaching of paper pulp or decolorization of textile effluents (Abadulla et al., 2000; Ibarra et al., 2006). A predictable increase in laccase activity at pH 5 was obtained for some laccase hybrids, although other unexpected features were also observed following chimeragenesis, such as increased thermostability and more acidic activity profiles. Laccase thermostability was significantly enhanced

in several chimeras (3A4, 4A11, 7D5, 2C4, and 7A12), and in some cases this increase in thermostability was accompanied by a sharper optimum pH. On the other hand, the recombination of OB-1 and 3PO genes gave rise to some laccase hybrids with a broader pH activity profile than parental types, and a significant increase in laccase activity at pH 6.0. These included chimeras 2C3, 6D9, and 6F11, which largely retained the 3PO scaffold while incorporating portions of the OB-1 protein, although 2C3 and 6F11 exhibited severely diminished thermostability. The activity of the 2C4 hybrid also improved at more neutral pH values but unlike the aforementioned chimeras, its pH profile narrowed notably and its activity was maximal at pH 5.0. 2C4 is mainly composed of the OB-1 parent type and two small segments of 3PO (Fig. 4). The first segment (residue 394–421 in 3PO) includes the third highly conserved laccase motif (with T1, T2, and T3 Cu ligands plus the P394H mutation from 3PO). The P394H mutation is contiguous with the His395 ligand of the T1 copper (Valderrama et al., 2003) and there is a noticeable mismatch between both parental laccases in this signature motif. The second segment introduced in 2C4 belongs to the last 10 C-terminal (C_t) residues of the 3PO parental enzyme. Interestingly, the 7A12 chimera shares the same scaffold and sequence block exchange at the Ct with 2C4, although it does not contain the 394–421 segment (Fig. 4). The 7A12 chimera was the most thermostable variant and, besides the substrate affinities for DMP or ABTS were not impaired by recombination. Comparing the biochemical features of the 2C4 and 7A12 chimeras revealed the contribution of the 394–421 segment to the regulation of substrate affinity and optimum pH.

It should be noted that other chimeric laccases with combined properties also incorporate several residues of the C_t from 3PO. Chimeras 3A4, 4A11, and 7D5 contain the C_t of 3PO in the OB-1 scaffold and displayed sharper acidic optimal pH, yet they were very thermostable with improved K_m values for phenolic compounds. These properties may be of interest for processes such as the enzymatic detoxification of thermo-chemically pretreated lignocellulosic biomass (Jurado et al., 2009). The C-terminal tail of ascomycete laccases plays a key role in enzyme stability and activity (containing a plug that regulates the traffic of oxygen to the trinuclear Cu cluster: (Andberg et al., 2009; Zumárraga et al., 2008a,b). Although the case of basidiomycete laccases is different (they only share ~33% of sequence identity with ascomycete laccases), as there are several mismatches between OB1 and 3PO in this region, the role of the C_t in the global folding and function of HRPLs cannot be underestimated and requires further investigation.

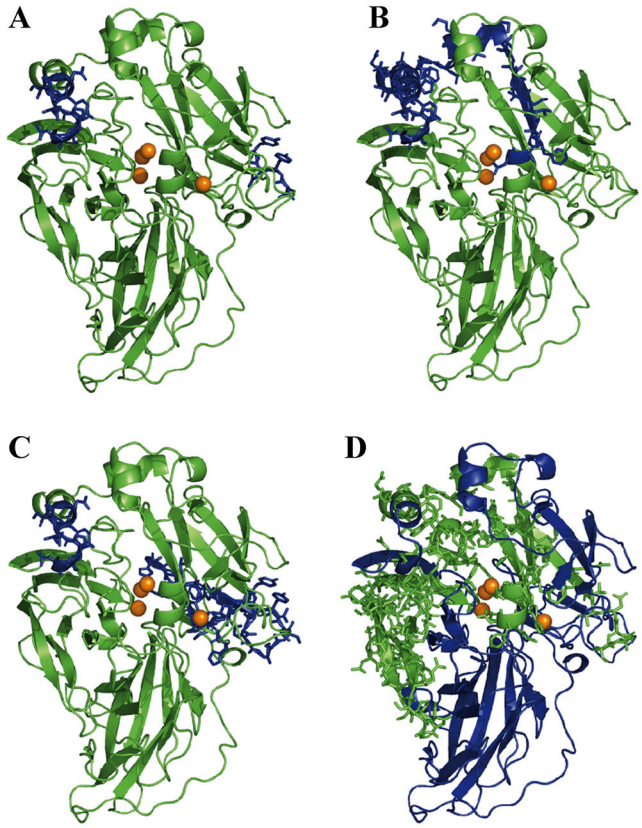

Figure 4. 3D models of selected laccase hybrids showing the exchange of protein fragments from the two parent laccases. The protein scaffold of the OB-1 parent (green) was retained in chimeras 7A12 (**A**), 3A4 and 4A11 (**B**), and 2C4 (**C**), and combined with different 3PO fragments (blue). 6D9 (**D**) retained the scaffold of the 3PO parent and contained two blocks from OB-1. Chimeras were modeled using the structures of *P. cinnabarinus* (pdb: 2XYB) and *T. troggi* (pdb: 2HRG) laccases as templates using PyMol software (DeLano, 2002).

Conclusions

In summary, we describe the engineering of chimeric laccases via family shuffling of two HRPLs from different fungi. The exchange of diverse protein blocks were obtained by in vitro plus in vivo recombination of the parental genes, and hybrid proteins active on phenolic and non-phenolic polar substrates were identified by dual high-throughput screening of the chimeric libraries. Selected chimeric laccases displayed modified pH activity profiles, enhanced thermostability, or improved substrate affinities. This collection of chimeras may provide a suitable starting point for tailoring HRPLs with novel properties.

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