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Characterization of the *Podospora anserina* (Rabenh.) Niessl peroxidase gene family

Roselyne FERRARI, Valérie GAUTIER, Narumon TANGTHIRASUNUN, Laetitia CHAN HO TONG & Philippe SILAR

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Tian QING
Center of Excellence in Fungal Research, Mae Fah Luang University 333 M. 1 T.Tasud Muang District, Chiang Rai 57100 (Thailand)

Sylvie RAPIOR
Laboratoire de Botanique, Phytochimie et Mycologie / UMR -CNRS 5175 CEFE, Faculté de Pharmacie, 15, avenue Charles-Flahault,
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Université de Montpellier II, CEFE/CNRS Campus du CNRS, 1919, route de Mende, 34293 Montpellier Cedex 5 (France)

Naritsada THONGKLANG
Center of Excellence in Fungal Research, Mae Fah Luang University, 333 M. 1 T.Tasud Muang District, Chiang Rai 57100 (Thailand)

Xiang-Hua WANG
CAS Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany,
Chinese Academy of Sciences, Lanhei Road 132, Kunming 650201, P. R. (China)

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Characterization of the *Podospora anserina* (Rabenh.) Niessl peroxidase gene family

Roselyne FERRARI
Valérie GAUTIER
Université Paris Diderot, Sorbonne Paris Cité, Laboratoire Interdisciplinaire des Énergies de Demain, 75205 Paris Cité Cedex 13 (France)

Narumon TANGTHIRASUNUN
Department of Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok, 10520 (Thailand)

Laetitia CHAN HO TONG
Philippe SILAR
Université Paris Diderot, Sorbonne Paris Cité, Laboratoire Interdisciplinaire des Énergies de Demain, 75205 Paris Cité Cedex 13 (France)
philippe.silar@univ-paris-diderot.fr (corresponding author)

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ABSTRACT
Major role of peroxidases in plant biomass degradation is well-established in the white rot basidiomycetes. On the contrary, peroxidases are not used for this purpose by brown rot basidiomycetes, which use instead a non-enzymatic mechanism. In the case of the ascomycetes, not much is known although these fungi have peroxidase genes. Here, we identify and characterize the peroxidase genes of *Podospora anserina* (Rabenh.) Niessl, an ascomycete used to study development and lignocellulose degradation. We show that this fungus has one class II peroxidase, one hybrid B peroxidase, one haloperoxidase, four functional aromatic peroxygenases, one glutathione peroxidase, one cytochrome C peroxidase and one alkyl peroxidase, but lacks a dye peroxidase. We show that potentially secreted peroxidases (i.e., the class II, hybrid B, haloperoxidase and aromatic peroxygenase peroxidases) present a patchy phylogenetic distribution compatible with an accessory role in finely adapting the different fungal species to their ecological niche, rather than being involved in fundamental roles in fungal biology. Accordingly, targeted gene deletions of the different *P. anserina* peroxidase genes identified only one phenotype, seemingly an alteration of the timing of ascospore maturation at intermediate concentration of vanillic acid. However, direct measure of peroxidase activity did not show drastic loss of activity in the tested mutants, suggesting compensation between the enzymes. Hence, in *P. anserina*, peroxidases appear to have a minor role in biomass degradation, unlike what has been described in white rot fungi, and in this regard *P. anserina* appears to be similar to brown rot fungi.

KEY WORDS
Peroxidase,
lignin,
ascomycetes.

RÉSUMÉ
Caractérisation de la famille des gènes codant les peroxydases chez Podospora anserina *(Rabenh.) Niessl.*
Le rôle majeur des peroxydases dans la dégradation de la biomasse végétale a été bien caractérisé chez les basidiomycètes provoquant des caries blanches. Au contraire, les peroxydases ne sont pas utilisées dans ce but chez les basidiomycètes provoquant des caries brunes qui à la place utilisent un mécanisme non-enzymatique. Concernant les ascomycètes, peu de choses sont connues sur le rôle des peroxydases, bien que ces champignons possèdent des gènes codant pour ces enzymes. Ici, nous identifions et caractérisons les gènes codant des peroxydases chez *Podospora anserina* (Rabenh.) Niessl, un ascomycète utilisé comme modèle pour étudier le développement et la dégradation de la biomasse végétale. Cette espèce possède une peroxydase de classe II, une peroxydase « hybrid B », une haloperoxydase, quatre péroxygenases d'aromatiques fonctionnelles, une peroxydase du glutathion, une peroxydase du cytochrome C et une peroxydase d'alkyl, mais ne possède pas de peroxydase de la famille « dye ». Nous montrons que les gènes de peroxydases potentiellement secrétées (i.e., les peroxydases de classe II, « hybrid B », les haloperoxydases et les péroxygenases d'aromatiques) présentent une répartition non homogène chez les champignons, suggérant un rôle dans l'adaptation fine des champignons à leur niche écologique plutôt qu'un rôle fondamental dans la biologie fongique. De fait, les mutants de délétion des gènes de peroxydase ne présentent apparemment qu'un phénotype de modification de la cinétique de maturation des ascospores sur des concentrations intermédiaires d'acide vanillique. Néanmoins, la mesure directe de l'activité peroxydase dans les différents mutants ne montre pas de modification importante de celle-ci, suggérant des compensations entre les différents enzymes. L'ensemble des données suggère un rôle mineur des peroxydases dans la dégradation de la biomasse chez *P. anserina*, ce qui le rapproche plutôt des caries brunes.

MOTS CLÉS
Peroxydase,
lignine,
ascomycètes.

INTRODUCTION

Plant biomass degradation is an essential phenomenon that enables the recycling of carbon trapped inside the cellulosic and lignin components of the plant cell wall. Indeed, in terrestrial ecosystems, it is estimated that photosynthesis transfers yearly into organic matter about 120 gigatons of carbon (GtC) from the 800 GtC stock, present in the atmosphere as carbon dioxide. This carbon ends up mainly in the plant cell wall as cellulose and lignin. Both polymers are difficult to degrade because of their structure and/or insolubility properties, especially lignin which account for about 20% of plant biomass. Therefore, there is a need for a recycling of this lignin-trapped carbon and in nature fungi are the main recyclers because they acquired during evolution a dedicated and efficient enzymatic machinery to do so.

The enzymes involved in lignin breakdown have been well studied in basidiomycetes and much less in ascomycetes, although these fungi are likely important actors owing to their domination of the fungal biomass in soils (Egidi *et al.* 2019; Ferrari *et al.* 2021). It is likely that similar mechanisms are used by both kinds of fungi (Ferrari *et al.* 2021). Degradation proceeds in two steps. The first one involves the breakdown of the lignin polymer by redox enzymes and the second one the mineralization of the highly-recalcitrant aromatic compounds freed from lignin (Pollegioni *et al.* 2015). Both stages may involve peroxidases (EC1.11.1.1 to EC1.11.1.19). Indeed, firstly, it is well-established that "lignin", "manganese" and "versatile" peroxidases are key actors of lignin depolymerization by many basidiomycetes, the white rot ones (Ruiz-Dueñas & Martínez 2009; Janusz *et al.* 2017). Secondly, peroxidases are known to transform toxic compounds into less toxic ones (Bansal & Kanwar 2013). Peroxidases may thus display important roles in biomass degradation by both participating directly in lignin depolymerization, but also in conferring resistance to toxic compounds present in woody materials such as "extractibles" (Valette *et al.* 2017) and/or to toxic by-products generated during lignin breakdown (Schweigert *et al.* 2001). Of particular importance for this process are 1) class II peroxidases to which "lignin", "manganese" and "versatile" peroxidases belong; 2) Dye-decolorizing peroxidases; 3) haloperoxidases (a.k.a. vanadium haloperoxidase); and 4) some heme-thiolate peroxidases such as aromatic peroxygenases and hybrid B peroxidases, since these enzymes are able to transform aromatic substrates like those present in lignin and extractibles. Accordingly, transcription of these enzymes is abundant in leaf litter and corresponding enzymatic activities can be detected in most investigated samples (Kellner *et al.* 2014).

Surprisingly, few studies dealing with determination of the role of these kinds of peroxidases potentially involved in biomass degradation through the targeted deletions of their genes have been published and none assessed the phenotypes of the mutants with respect to biomass degradation and resistance to toxic chemicals apart from H_2O_2 that is used as co-substrate by these enzymes. Deletion of genes encoding these peroxidases in *Fusarium graminearum* Schwabe showed no effect on vegetative growth, sexual and asexual development, as well as in trichothecene production, virulence and resistance/sensitivity to oxidative stress, except for a role of the cytochrome C peroxidase gene *fpx*1 in resistance to H_2O_2, as expected for the crucial role of this enzyme in electron transfer in the mitochondria (Lee *et al.* 2018). On the contrary, small role

in virulence and sensitivity to H_2O_2, but not on vegetative growth, conidiation, conidial germination and appressorium formation was evidenced in *Magnaporthe oryzae* (T.T. Hebert) M.E.Barr for all investigated genes (Mir *et al.* 2015). Similarly, roles of peroxidase genes on resistance to H_2O_2 and in conidiation, but not on vegetative growth, was evidenced in *Valsa mali* Miyabe & G.Yamada, and in this species only one of the investigated genes was shown to control virulence (Feng *et al.* 2018). In *Verticillium nonalfalfae* Inderb., H.W.Platt, R.M.Bostock, R.M.Davis & Subbarao, a peroxidase gene was shown to be involved in virulence but not in vegetative growth and conidiation (Flajsman *et al.* 2016). Finally, in *Botryosphaeria kuwatsukai* (Hara) G.Y.Sun & E.Tanaka, the class II peroxidase BkliP1 was shown to be involved in vegetative growth and virulence, while BkliP2 was not (Xiao *et al.* 2022).

Here, we present an analysis through targeted gene deletions of the roles of seven peroxidase genes in *Podospora anserina* (Rabenh.) Niessl, a saprobic ascomycete used as a model to study biomass breakdown by ascomycetes (Couturier *et al.* 2016). All these peroxidases may use aromatic compounds as electron donor substrate to reduce H_2O_2 into H_2O. This species is able to breakdown lignin (Dicko *et al.* 2020; van Erven *et al.* 2020) and is easily amenable to reverse genetics. Data show a minor role for peroxidases, including for a class II, in lignocellulose breakdown.

MATERIAL AND METHODS

Strain and culture conditions

The *P. anserina* strains used in this study were derived from the S strain ensuring a homogenous genetic background (Rizet 1952; Boucher *et al.* 2017). Standard culture conditions, media compositions and genetics methods for this fungus have been extensively described (Silar 2013, 2020). Primary and refined genome sequences and annotations for strain S are available in GenBank (Espagne *et al.* 2008; Grognet *et al.* 2014; Silar *et al.* 2019).

For sensitivity tests, chemical compounds were added to M2 medium at the following concentrations: ferulic acid 0.1-2 g/L, phytic acid 0.1-2 g/L, p-coumaric acid 0.1-2 g/L, quercetin 01.-2 g/L, caffeic acid 0.1-2 g/L, vanillic acid 0.1-2 g/L, tannic acid 0.1-2 g/L, humic acid 0.1-2 g/L, syringyl alcohol 0.1-2 g/L, veratryl alcohol 0.1-2 g/L, methylene blue 0.001-0.01 g/L, wood extractive 0.1-2 g/L, hydrogen peroxide 0.0004-0.01%, menadione 10^{-6}-5.10^{-5} M and ter-butyl hydroperoxide (10^{-5}-10^{-4} M). The highest concentrations were the sublethal doses.

Peroxide and superoxide secretion were assayed as described in Malagnac *et al.* (2004) and Hyphal Interference as in Silar (2005). Peroxide accumulation during wounding was assayed as described in Ferrari *et al.* (2018).

Gene deletions and quadruple mutant construction

The seven peroxidase genes were deleted using the split marker method (Silar 2013, 2020) with the primers reported in Appendix 1. Potential independent candidates obtained after transformation with the deletion cassettes were crossed to the wild type and in the progeny, strains carrying the proper resistance marker (see Table 1 for the markers used) were checked by Southern blot analyses (Appendix 2). One Southern blot-validated candidate for each deletion was then selected for further analyses.

To construct the strain deleted for its four aromatic peroxygenase genes, single mutants, each inactivated by a different resistance cassette (Table 1), were crossed and, in the progeny, double mutants were identified thanks to their resistance to the relevant drugs. Then, two double mutants inactivated for different genes were crossed and, in the progeny, quadruple mutants were identified thanks to their resistance to the four drugs used for deletion selection.

Azure B discoloration time course assays

Petri dishes (Ø = 50 mm) were prepared with the following medium: Sorbose 3 g/L; Yeast Extract 2 g/L and Agar 10 g/L. The Azure B dye was added to the cooling medium at 25 mg/L just before pouring the plates. Heterokaryotic mat+/mat- mycelia were then inoculated at the center of the plates. To this end, two plugs of the same size, one from a fresh mat+ mycelium and the other from a fresh mat- one, were cut with a punch and ground in 500 µL of sterile distilled water with a Fast-Prep apparatus. The Petri dishes were inoculated with 10 µL of the mix. All the inoculations were carried out in triplicate and the Petri dishes were incubated at 27°C in the dark. After a few days, the fungi grew as a compact colony around which a halo of discoloration appeared. To quantify the capability of the investigated strains to bleach Azure B, photographs of the Petri dishes were taken at regular intervals. The images were then analyzed with Adobe Photoshop 6 software, which allowed quantification of the pixels corresponding to the uniform blue coloration of the starting medium versus those corresponding to the discoloration halo. The discolored surface in pixels (+ Standard Deviation) was then plotted as a function of the incubation time.

UV-Vis time course and fluorescence time course assays

For UV-Vis time course assays, 10 µL of mat+/mat- fragmented heterokaryons (see above section) were inoculated in 7 mL of liquid M2 (containing 4.5 g/L dextrin as carbon source) or M4 medium (containing 4.5 g/L crystalline cellulose as carbon source) placed in 50 mm Ø Petri dishes. Lignin was added at the final concentration of 0.2 g/L. All the inoculations were carried out in triplicates and the Petri dishes were incubated at 27°C in presence or in absence of light. Degradation of lignin was monitored daily with spectroscopy by following the absorbance of the lignin on an aliquot of 100 µL taken from each of the Petri dishes and diluted to ⅒ in 900 µL sterilized water. UV-Vis absorption spectra were acquired using a Perkin-Elmer LambdaTM 25 UV-Vis spectrometer (Perkin Elmer, United States). Spectra for each sample were collected between 200 to 500 nm spectral range at 1 nm spectral resolution with reference to M2 medium diluted to ⅒ in

TABLE 1. — Peroxidase genes in *P. anserina* (Rabenh.) Niessl genome. Symbols: **ND**, not determined; **?**, ambiguous answers from the prediction programs; expression level ranged from weak (**+/-**) to average (**++**) and strong (**++++**) when compared to other genes.

Gene number	Predicted catalase/ peroxidase domain	In second. metab. cluster ?	Secretion signal with SignalP	Secretion signal with predisi	Expression level	Other domain	Mutant name/ Inactivation marker
Pa_1_5970	Class II peroxidase	no	no	no	+/-	–	5970Δ/HygR
Pa_2_9370	Hybrid B peroxidase	no	yes	yes	++++	4 WSC	9370Δ/GenR
Pa_1_5120	Haloperoxidase	no	yes	yes	++	–	5120Δ/GenR
Pa_5_12205	Aromatic peroxygenase	no	no	no	+/-	–	12205Δ/NourR
Pa_0_370	Aromatic peroxygenase	no	yes	yes	+++++	–	370Δ/HygR
Pa_2_7390	Aromatic peroxygenase	yes	yes	yes	++	–	7390Δ/PhleoR
Pa_2_6870	Aromatic peroxygenase	yes	no?	no?	++	–	6870Δ/GenR
Pa_2_10	Aromatic peroxygenase pseudogene	–	no	no	–	–	Naturally inactivated by a transposon in strain S
Pa_2_8460	Glutathione peroxidase	–	no	no	ND	–	Not inactivated in the present study
Pa_1_6960	Mitochondrial cytochrome C peroxidase	–	no	no	ND	Mitochondrial signal	
Pa_2_390	Alkylhydroperoxidase D-like	–	no	no	ND	–	

900 µL sterilized water. The first point corresponded to the zero time and gave the value of the absorbance of the lignin at the beginning of the experiment. The UV-Vis absorption spectra were plotted in the absorbance (Absorbance Units) wavelength (nm) coordinates. A zero of the spectrometer was made with the M2 medium diluted to ⅒ in 900 µL sterilized water without the fungus and the baseline was recorded. All the samples were then analyzed in the same order and in a room without direct light with the M2 medium diluted to ⅒ in 900 µL sterilized water.

Determination of the peroxidase activity on secretome

Peroxidase activity was performed using a method adapted from Archibald (1992) on an aliquot of the liquid M2 medium in which the strains were grown for three weeks in Petri dishes. These were inoculated with 10 µL of mat+/mat- fragmented heterokaryons. The analysis was carried out in a Perkin-Elmer LambdaTM 25 UV-Vis spectrometer (Perkin Elmer, United States), at 651 nm in a final volume of 1 mL consisting of 500 µL of the secretion volume and 480 µL of tartrate buffer and 10 µL of a 32 µM solution of Azure B. The Azure B decolorization reaction was initiated by the addition of 10 µL of 0.1 mM H_2O_2 and the reaction kinetics was read for five minutes. The absorbances were recorded as a function of time to calculate the slope of the reaction for one minute. The amount of Azure B decolorized in one minute was related to the amount of total protein present in the sample of culture analyzed. The protein assay was performed by the Bradford method in a 96-well microplate. All the experiments were carried out in triplicate.

Phylogenetic analysis

P. anserina peroxidase genes were searched by BLAST using the last version of the genome annotation (Silar *et al.* 2019) using the default parameters with the known peroxidase enzyme sequences as query. Hits with an e-value lower than 10^{-5} were selected. Alignments were made with MAFFT (Katoh *et al.* 2005) and manually refined with Jalview (Waterhouse *et al.* 2009). The alignments were used to construct phylogenetic trees using the maximum likelihood method (PhyML software using the default parameters) (Guindon & Gascuel 2003) and transferred to the iTOL server for visualization (Letunic & Bork 2007). Bootstrap values are expressed as percentages of 100 replicates.

RESULTS

Identification of peroxidase-encoding genes in the genome of *P. anserina*

The last version of the annotation of the *P. anserina* genome (Silar *et al.* 2019) was searched by Blast for putative peroxidase encoding genes and potential candidates were validated by searching for conserved peroxidase domains with the CD-search package from NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Fig. 1; Table 1). Firstly, we could not detect any gene encoding a protein belonging to the dye peroxidase family indicating that *P. anserina* does not possess an enzyme from this family, unlike the related fungus *Sordaria macrospora* Auersw., which has one gene encoding such peroxidase (Adamo *et al.* 2022). Secondly, in addition to

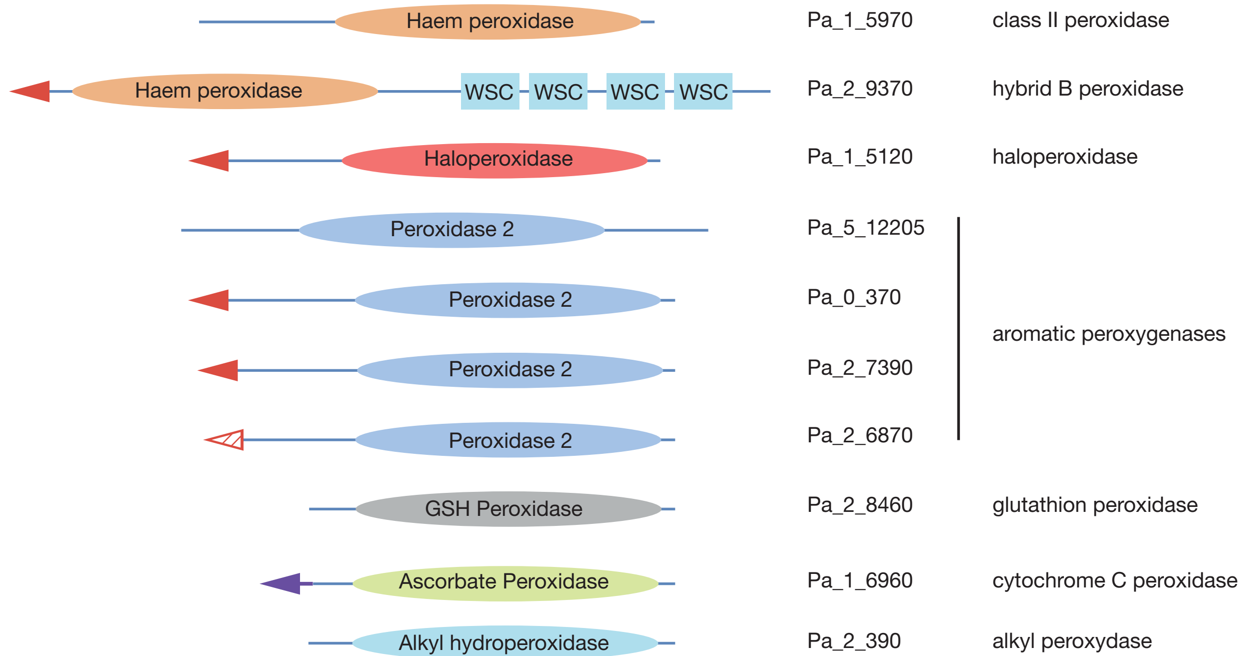


FIG. 1. — Peroxidases encoded in the *P. anserina* (Rabenh.) Niessl genome. Symbols: **WSC**, carbohydrate binding domain; **red arrows**, secretion signal; **red arrow with slanted lines**, possible secretion signal having a low probability; **violet arrow**, mitochondrial targeting signal peptide.

the previously identified catalase/peroxidase genes (Bourdais *et al.* 2012), the *P. anserina* genome encodes for one class II peroxidase and one "hydrid B haem" peroxidase (also known as Hybrid Ascorbate-Cytochrome C peroxidase), a class of peroxidase that contains carbohydrate binding domains (Zámocký *et al.* 2017), the *P. anserina* protein contains four such "WSC" domains (Fig. 1). The genome also encodes one haloperoxidase (also known as "Vanadium chloroperoxidase") and four aromatic peroxygenases (also known as haem haloperoxidases) – note that the fifth gene (a.k.a. *Pa_2_10*) included in databases such as fPoxDB (Choi *et al.* 2014) corresponds to a pseudogene inactivated by a transposon insertion in strain S (Silar *et al.* 2019). Finally searches uncovered one glutathione peroxidase; one cytochrome C peroxidase and one alkyl peroxidase. Because the last three peroxidases are intracellular enzymes that are involved in controlling H_2O_2 and/or alkyl hydroperoxide levels, they were not investigated further. On the contrary, enzymes from the five first families are known to use small aromatic molecules and H_2O_2 to potentially produce reduced aromatics and thereby detoxify them. Moreover, some of these reduced aromatics could be mediators interacting with lignin and promote its breakage.

Complex evolution of peroxidase genes in fungi

Phylogenetic analyses were carried out to understand the evolution of the peroxidase genes found in the *P. anserina* genome (Figs 2-5). All genes have undergone during evolution of fungi complex histories of gain and loss since many species, including closely related ones, may or may not have any particular enzyme and in many instances the peroxidases of a particular class may be encoded by small multigenic families resulting from recent amplifications (see for example the class II peroxidases in *Schizothecium vesticola* (Berk. & Broome) N.Lundq., Fig. 2) and/or horizontal transfers (see below the horizontal transfer of *Pa_2_7390*). Loss is evident for example in the case of the pseudogenization of the "*Pa_2_10*" gene in *P. anserina*. Overall, peroxidase genes, especially those coding for aromatic peroxygenases, tend to be abundant in the genomes of Agaricomycotina Doweld and Pezizomycotina O.E.Erikss. & Winka, and scarcer in early-diverging lineages of Eumycota D.J.S. Barr, Ascomycota Caval.-Sm. and Basidiomycota R.T.Moore (Fig. 4). However, even in Agaricomycotina and Pezizomycotina, peroxidase genes may be lacking, as seen by the fact that the five families of peroxidases may be missing in at least one species in these two clades. Note that the dye oxygenase genes, which are not present in *P. anserina*, have the same kind of evolution (Adamo *et al.* 2022), which is reminiscent of other genes encoding enzymes such as arylamine N-acetyltransferases (Martins *et al.* 2010) and lipoxygenases/cyclooxygenases (Ferrari *et al.* 2018) involved in the fine tuning of the different fungal species to their particular biotope/lifestyle. This kind of complex evolution is also encountered for secondary metabolic clusters (Rokas *et al.* 2018). Not unexpectedly, two *P. anserina* aromatic peroxygenase genes (*Pa_2_6870* and *Pa_2_7390*) are likely part of secondary metabolic gene clusters. *Pa_2_7390* is a homologue of *stc*C of *Aspergillus nidulans* G.Winter, which is present in the aflatoxin biosynthesis cluster, which was horizontally transferred into *P. anserina* from an *Aspergillus* P.Micheli ex Link species (Slot & Rokas 2011). *Pa_2_6870* is located close to a polyketide synthase gene (about 6 kb away) and two P450 cytochrome genes (14 kb and 25 kb away), which are typically involved in secondary metabolites production. The

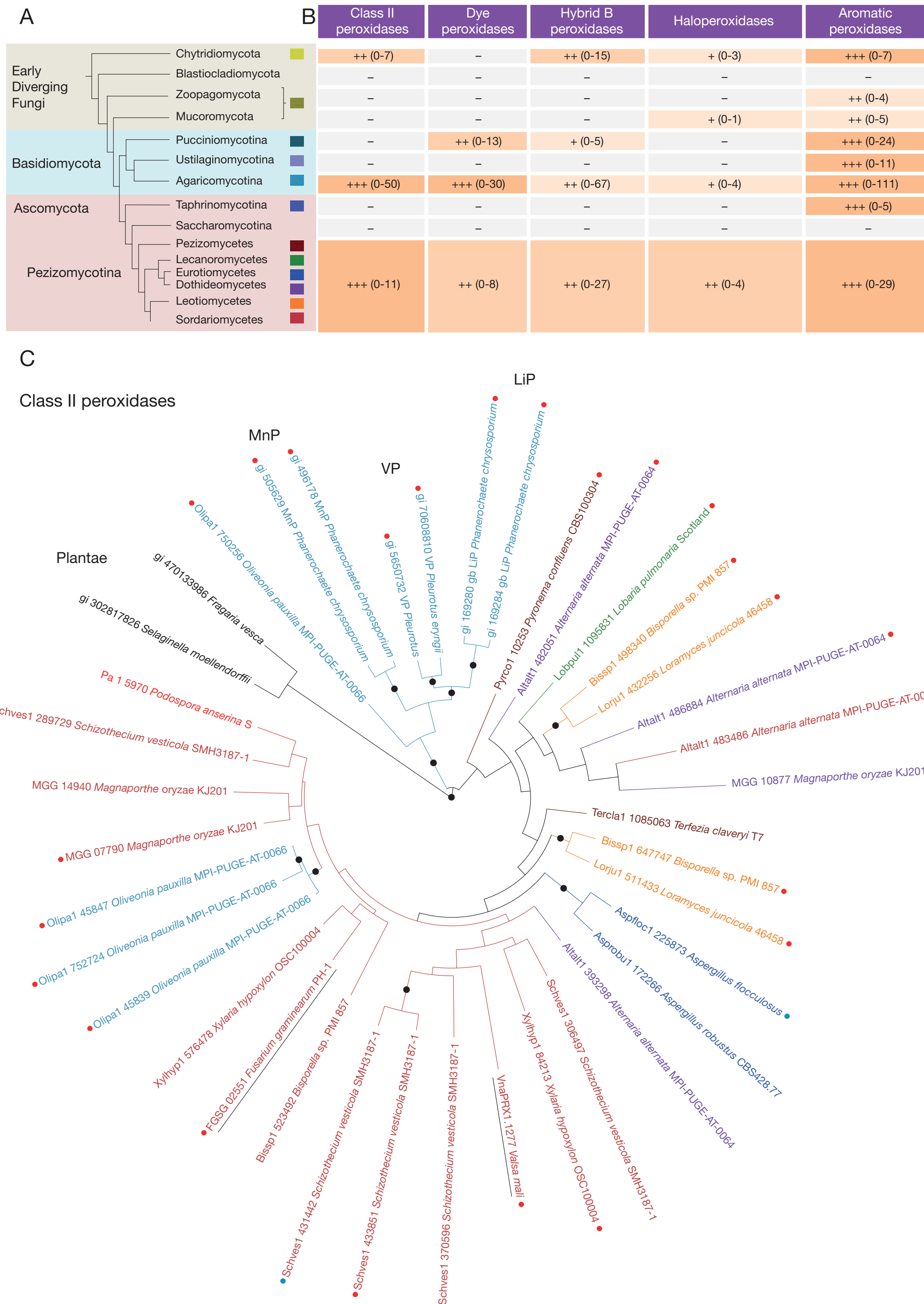
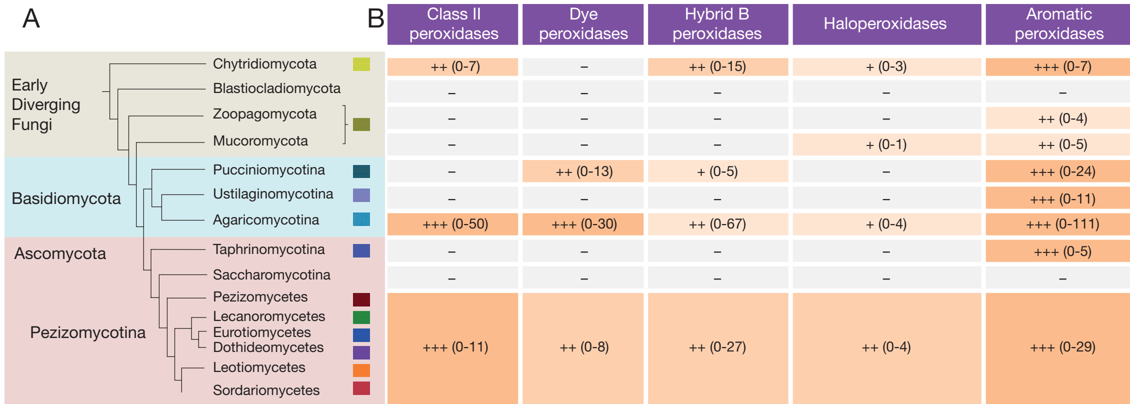


Fig. 2. — Evolution of peroxidases genes in fungi. **A**, known phylogeny of the major taxa of fungi; squares show the colors used in the phylogenetic tree; **B**, presence of the different families of peroxidases in the major fungal taxa: **-**, absent; **+**, few taxa (< 10%); **++**, many taxa (10-60%) having relevant peroxidase genes; **+++**, most taxa (> 60%) having relevant peroxidase genes. **Numbers in parenthesis** are the minimum and maximum numbers of genes detected in genomes of relevant taxa. **C**, phylogenetic tree of class II peroxidases. Tree was rooted with proteins from plants. **Dots** at the end of the protein names indicate the presence of a secretion signal predicted by SignalP (in **blue**), Predisi (in **green**) or both (in **red**). Proteins for which the encoding genes have been inactivated in other studies are **underlined in black**; the *P. anserina* (Rabenh.) Niessl protein is in **red**. Abbreviations: **LiP**, lignin peroxidase; **MnP**, manganese peroxidase; **VP**, versatile peroxidase.



A	Class II peroxidases	Dye peroxidases	Hybrid B peroxidases	Haloperoxidases	Aromatic peroxidases
Early Diverging Fungi – Chytridiomycota	++ (0-7)	–	++ (0-15)	+ (0-3)	+++ (0-7)
Early Diverging Fungi – Blastocladiomycota	–	–	–	–	–
Early Diverging Fungi – Zoopagomycota	–	–	–	–	++ (0-4)
Early Diverging Fungi – Mucoromycota	–	–	–	+ (0-1)	++ (0-5)
Basidiomycota – Pucciniomycotina	–	++ (0-13)	+ (0-5)	–	+++ (0-24)
Basidiomycota – Ustilaginomycotina	–	–	–	–	+++ (0-11)
Basidiomycota – Agaricomycotina	+++ (0-50)	+++ (0-30)	++ (0-67)	+ (0-4)	+++ (0-111)
Ascomycota – Taphrinomycotina	–	–	–	–	+++ (0-5)
Ascomycota – Saccharomycotina	–	–	–	–	–
Ascomycota – Pezizomycotina (Pezizomycetes, Lecanoromycetes, Eurotiomycetes, Dothideomycetes, Leotiomycetes, Sordariomycetes)	+++ (0-11)	++ (0-8)	++ (0-27)	++ (0-4)	+++ (0-29)

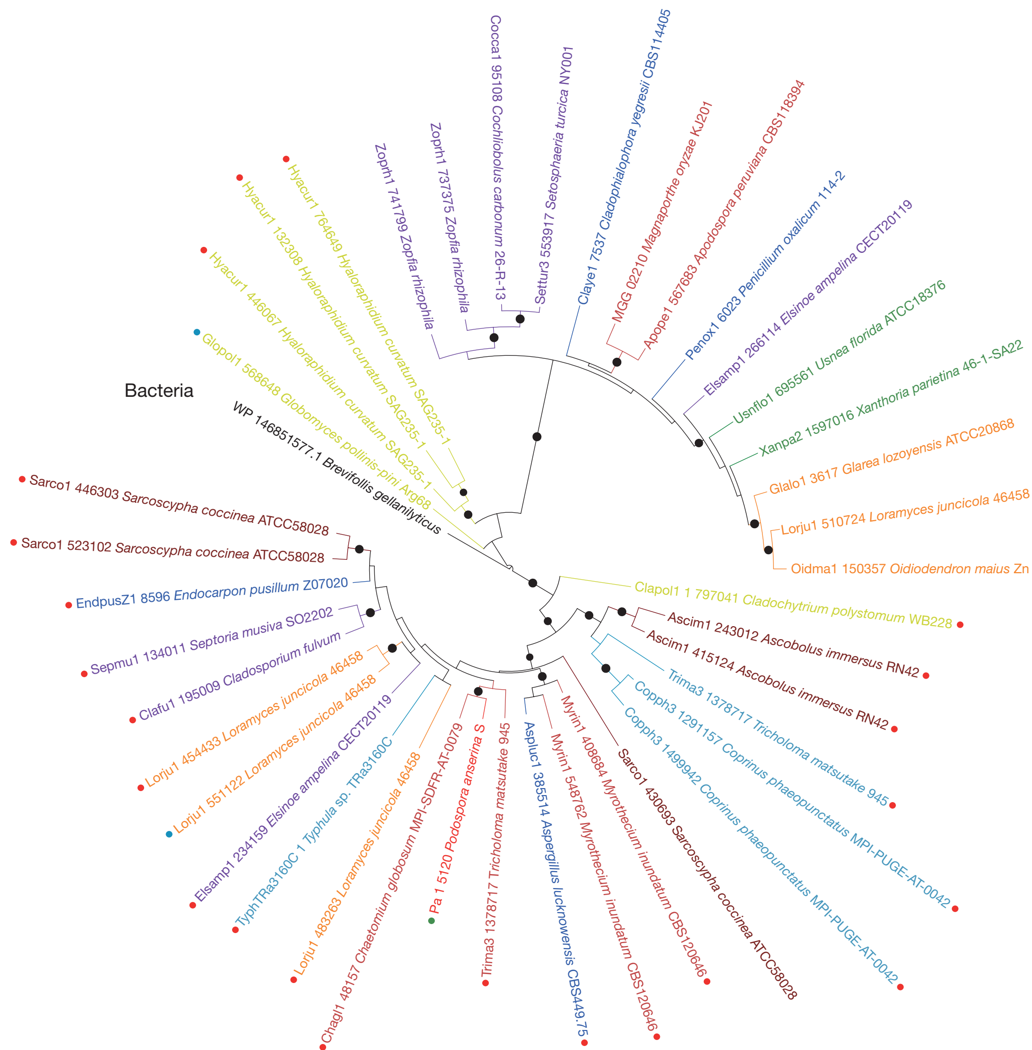


FIG. 3. — Evolution of peroxidases genes in fungi. **A**, known phylogeny of the major taxa of fungi; squares show the colors used in the phylogenetic tree; **B**, presence of the different families of peroxidases in the major fungal taxa: **-**, absent; **+**, few taxa (<10%); **++**, many taxa (10-60%) having relevant peroxidase genes; **+++**, most taxa (>60%) having relevant peroxidase genes. **Numbers in parenthesis** are the minimum and maximum numbers of genes detected in genomes of relevant taxa. **C**, phylogenetic tree of haloperoxidases. Tree was rooted with proteins from bacteria. **Dots** at the end of the protein names indicate the presence of a secretion signal predicted by SignalP (in **blue**), Predisi (in **green**) or both (in **red**). The *P. anserina* (Rabenh.) Niessl protein is in **red**.

Fig. 4. — Evolution of peroxidases genes in fungi. **A**, known phylogeny of the major taxa of fungi; squares show the colors used in the phylogenetic tree; **B**, presence of the different families of peroxidases in the major fungal taxa: **-**, absent; **+**, few taxa (<10%); **++**, many taxa (10-60%) having relevant peroxidase genes; **+++**, most taxa (>60%) having relevant peroxidase genes. **Numbers in parenthesis** are the minimum and maximum numbers of genes detected in genomes of relevant taxa. **C**, phylogenetic tree of aromatic peroxidases. Tree was rooted with proteins from oomycetes. **Dots** at the end of the protein names indicate the presence of a secretion signal predicted by SignalP (in **blue**), Predisi (in **green**) or both (in **red**). Proteins for which the encoding genes have been inactivated in other studies are **underlined in black**; the *P. anserina* (Rabenh.) Niessl proteins are in **red**.

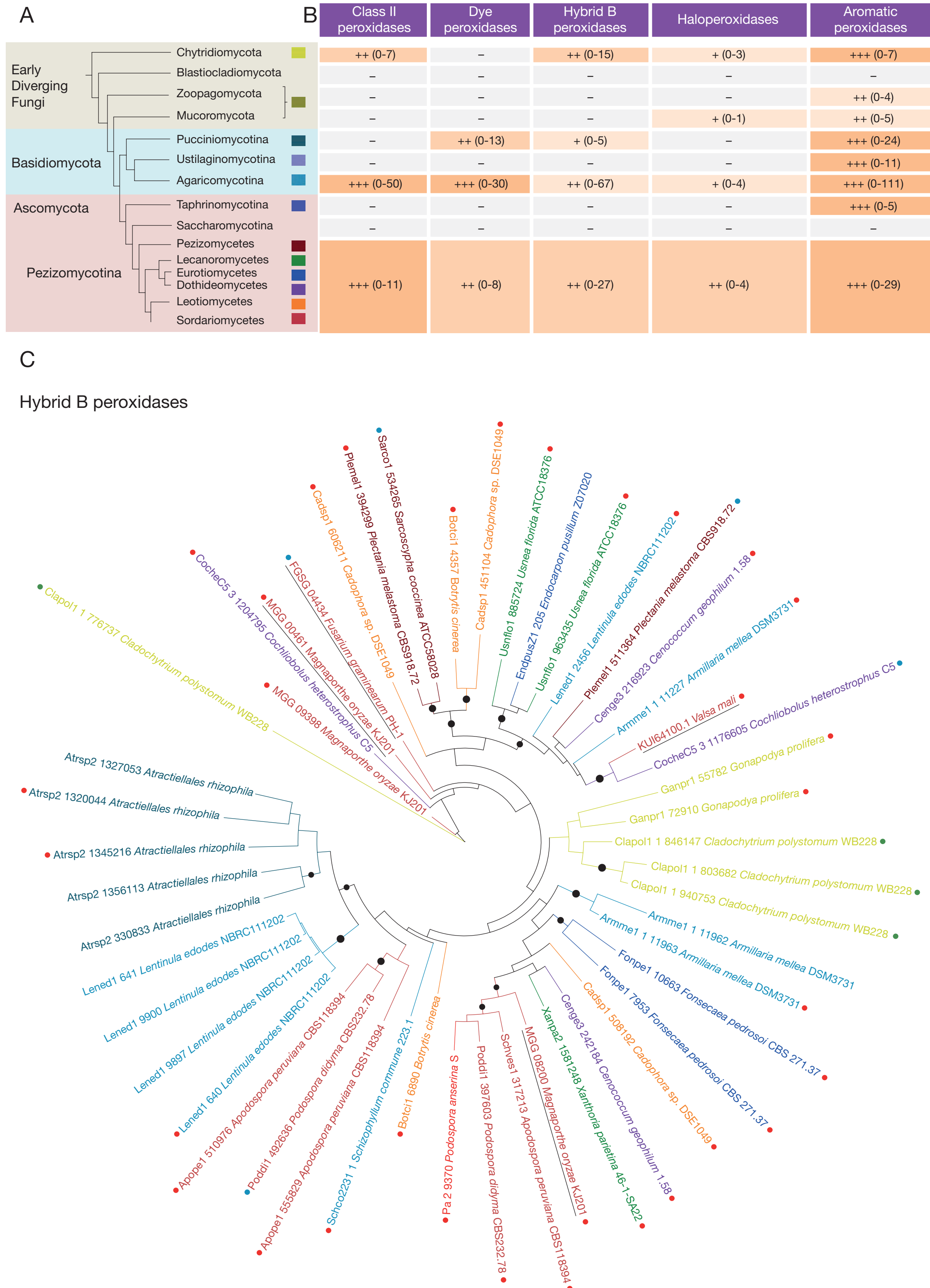


Fig. 5. — Evolution of peroxidases genes in fungi. **A**, known phylogeny of the major taxa of fungi; squares show the colors used in the phylogenetic trees; **B**, presence of the different families of peroxidases in the major fungal taxa: **-**, absent; **+**, few taxa (<10%); **++**, many taxa (10-60%) having relevant peroxidase genes; **+++**, most taxa (>60%) having relevant peroxidase genes. **Numbers in parenthesis** are the minimum and maximum numbers of genes detected in genomes of relevant taxa. **C**, phylogenetic tree of hybrid B peroxidases. Tree was rooted with a randomly chosen sequence from the early-diverging chytrid *Cladochytrium polystomum* Zopf, since these peroxidases are present only in fungi. **Dots** at the end of the protein names indicate the presence of a secretion signal predicted by SignalP (in **blue**), Predisi (in **green**) or both (in **red**). Proteins for which the encoding genes have been inactivated in other studies are **underlined in black**; the *P. anserina* (Rabenh.) Niessl protein is in **red**.

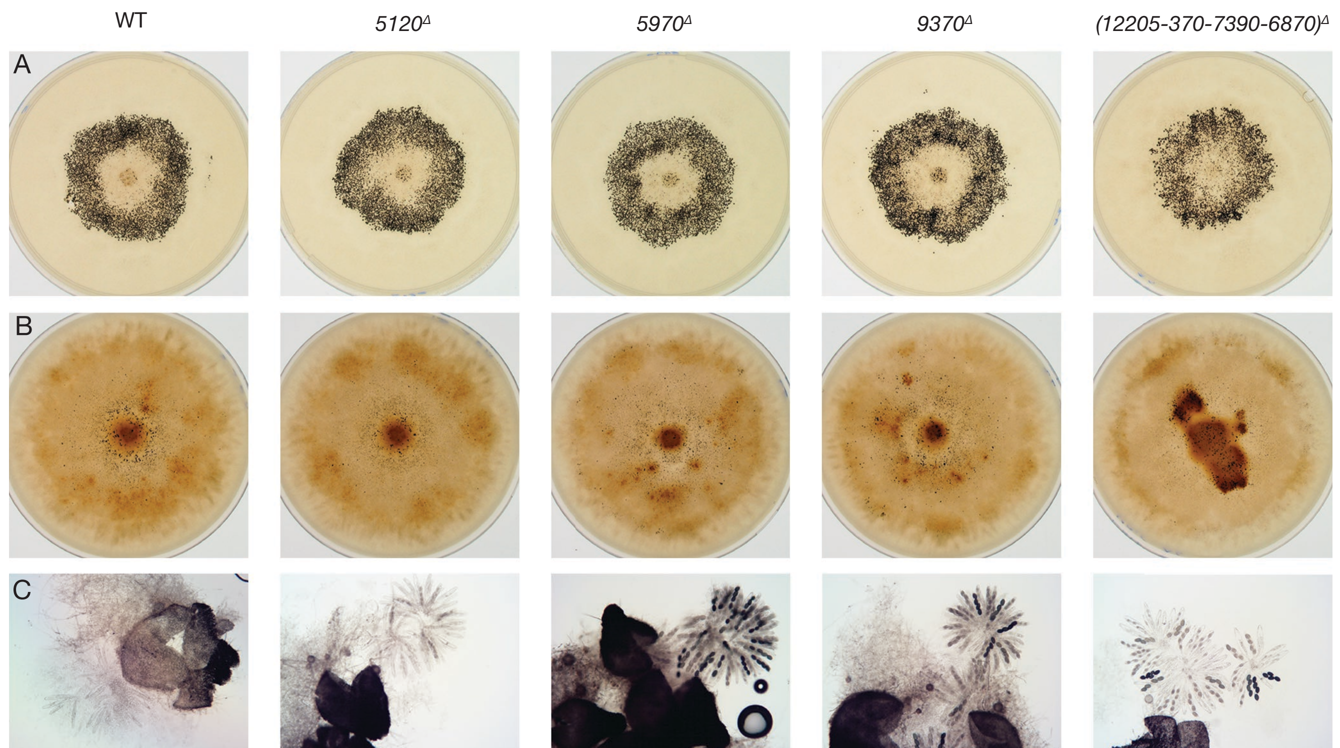


Fig. 6. — Phenotype of peroxidase mutants. **A**, Petri plates were inoculated on M2 medium with mat+/mat- heterokaryons with the indicated genotypes and incubated for one week at 27°C under constant illumination. The plates were then photographed. The mutant showed rings of perithecia (seen here as small black dots) similar to those of the wild type. Note that the irregular ring presented by *(12205-370-7390-6870)Δ* was not observed in all experiments; **B**, the same strains were inoculated on M2 + 0.5 g/L of vanillic acid and incubated for 10 days. Presence of vanillic acid severely inhibited the production of fruiting bodies in all strains. However, perithecia in mutants *5970Δ*, *9370Δ* and *(12205-370-7390-6870)Δ* matured slightly faster as seen by the presence of mature asci in the **C** pictures of rosettes, while these are not present in the wild type. On the contrary, presence of mature asci in rosette of *5120Δ* was delayed by one day compared to the wild type. These differences in the timing of maturation were seen in three independent experiments.

natural product(s) potentially synthesized by this cluster is unknown. Complex evolution of some peroxidase gene families and their likely involvement in finely tuning fungi to their biotope/lifestyle was previously noted (Zámocký *et al.* 2015, 2017; Mathé *et al.* 2019).

Potential secretion of the *P. anserina* peroxidases was investigated by searching for consensus secretion signals with SignalP (http://www.cbs.dtu.dk/services/SignalP/) and Predisi (http://www.predisi.de/) (Fig. 1; Table 1). Clear consensuses were found for the hybrid B peroxidase Pa_2_9370, the haloperoxidase Pa_1_5120 and the aromatic peroxygenases Pa_2_7390 and Pa_0_370. Additionally, a weak consensus was found for the aromatic peroxygenase Pa_2_6870. Surprisingly, no secretion signal could be detected by both programs for the class II peroxidase Pa_1_5970 and the aromatic peroxygenase Pa_5_12205. However, examination of previously published secretomes data (e.g. supplementary tables in Mäkelä *et al.* 2017; van Erven *et al.* 2020) showed that, despite lacking a signal, the class II peroxidase Pa_1_5970 was secreted; additionally, actual secretion of the hybrid B peroxidase Pa_2_9370, the haloperoxidase Pa_1_5120 and the aromatic peroxygenases Pa_2_7390, Pa_2_6870 and Pa_0_370, but not that of Pa_5_12205 could also be evidenced by analyzing these secretome data. Phylogenetic analyses of the presence of secretion signals (Figs 2-5) showed that they presented a patchy phylogenetic distribution for class II, aromatic and hybrid B peroxidases, i.e., genes from closely related species may or may not contain them, while phylogeny identified for haloperoxidases two paralogous families, one with a secretion signal, to which the *P. anserina* haloperoxidase Pa_1_5120 belongs, and one without a secretion signal. As seen by the *P. anserina* class II peroxidase Pa_1_5970, absence of a signal does not preclude secretion.

Expression of peroxidase genes in *P. anserina* mycelium

We evaluated the expression of peroxidase genes during two days and four days of growth on M2 medium (Table 1), by analyzing microarrays and RNAseq data generated in previous studies (Bidard *et al.* 2012; Silar *et al.* 2019) (Table 1). We could evidence expression of all peroxidase genes (except for the "*Pa_2_10*" pseudogene). The hybrid B peroxidase *Pa_2_9370* and the aromatic peroxygenase *Pa_0_370* genes were highly transcribed; on the contrary, the class II peroxidase *Pa_1_5970* and the aromatic peroxygenase *Pa_5_12205* gene were poorly expressed, a feature compatible with the lack of the aromatic peroxygenase *Pa_5_1220* in secretomes data. Note that analysis of RNAseq data from perithecia showed that the class II peroxidase *Pa_1_5970* gene was much more transcribed in these sexual fruiting bodies than in the mycelium, especially in four-day-old perithecia.

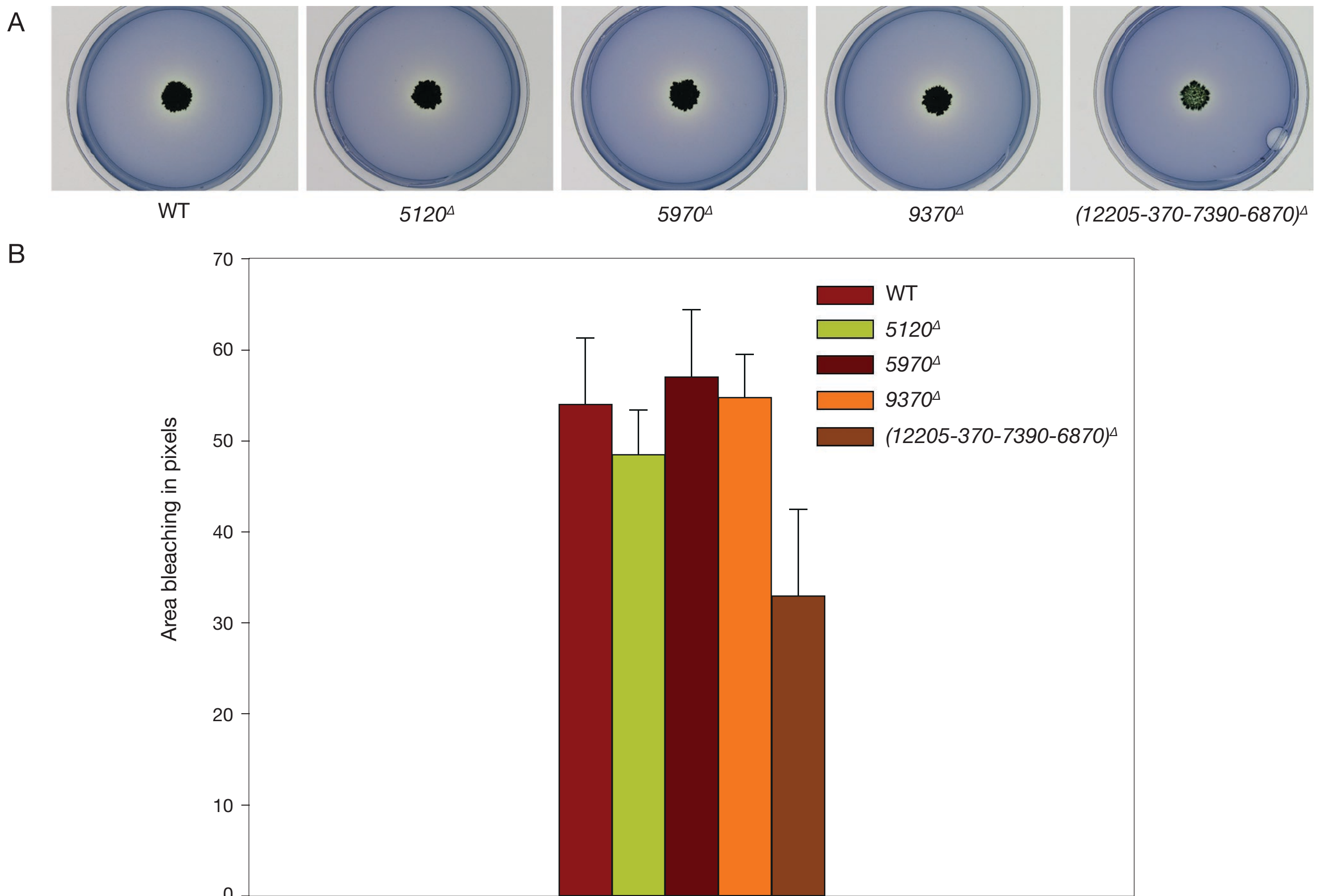


Fig. 7. — Decolorization of Azure B after two weeks of incubation: **A**, plates of Azure B for the wild type (**WT**) and four tested mutants after two weeks of incubation; **B**, quantification of Azure B bleaching. Measures were average of five experiments made in triplicates.

Targeted gene deletions did not uncover any obvious developmental phenotype of peroxidase mutants

To address the role of peroxidases in *P. anserina* development and ability to scavenge nutrients, we deleted each gene by replacing their coding sequence with a resistance marker (see Table 1 for the used markers). Because aromatic peroxygenases could have redundant functions, we constructed a strain deleted for all four genes by genetic crosses (see Material and methods). It was called *(12205-370-7390-6870)*$^{\Delta}$.

Vegetative growth was not affected in the *P. anserina* peroxidase mutants, because growth speed and mycelium morphology were identical to that of the wild type when grown on the standard M2 minimal medium (Fig. 6). Fertility on solid media with various carbon sources – e.g. dextrin (M2 medium, Fig. 6), glucose (M3 medium), paper (M0 + 3 × 3 cm Whatman paper pads), miscanthus (M0 + 0.5 g/plate of fragmented *Miscanthus giganteus*), *Guibourtia demeusii* (M0 + 0.5 g/plate of *Guibourtia demeusii* wood shavings) – was also not affected, indicating no major problem in retrieving nutrients even from complex carbon sources to fuel sexual development. We could not detect differences in the fertility of the mutants when compared to the wild type in liquid media supplemented or not with 0.01, 0.04 and 0.1 g/L of lignin. All had an increased fertility in the presence of lignin as previously described (Dicko *et al.* 2020) showing no detectable alteration of development signaling by lignin. Production of *Cladorrhinum*-like anamorph (Boucher *et al.* 2017) at 18°C was also not affected in the mutants, as well as that of the appressorium-like structures that enable the fungus to breach cellophane (Demoor *et al.* 2019). Ascospore germination rate and speed were also not modified in the peroxidase mutants. Finally, longevity as well as the Crippled Growth cell degeneration were identical in the wild-type and the peroxidase mutant.

We also assayed peroxide and superoxide secretion, as well as Hyphal Interference, a defense mechanism involving an oxidative burst presented by *P. anserina* when encountering a fungus of a different species (Silar 2005), and could not evidence any difference in the mutant when compared to the wild type. We also assayed peroxide accumulation during wounding and did not detect any difference with the wild type. Moreover, the mutants had no modification of sensitivity to hydrogen peroxide, ter-butyl hydroperoxide and menadione (an intracellular generator of superoxide).

We also tested growth and fertility of the mutant on ferulic acid, phytic acid, p-coumaric acid, quercetin, caffeic acid, vanillic acid, tannic acid, humic acid, syringyl alcohol, veratryl alcohol, methylene blue and oak wood extractives. We did not detect any

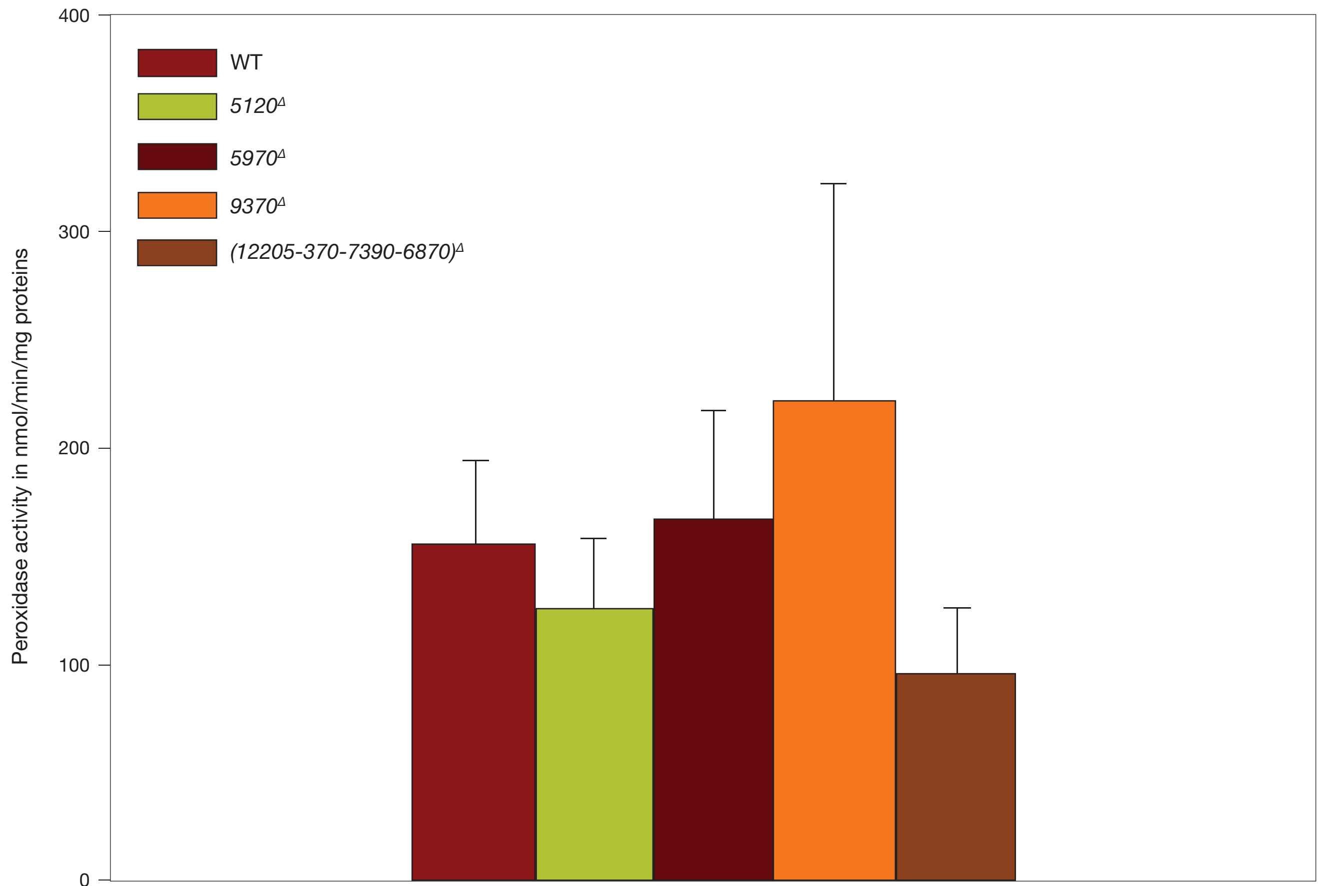


FIG. 8. — Peroxidase activity of *P. anserina* (Rabenh.) Niessl wild type and mutants. The activity was measured as described by Archibald (1992) and expressed in nmoles Azure B decolorized per minute and per mg proteins in the assay for the indicated strains. Measures were average of seven experiments made in triplicates.

modification of growth of the mutants compared to the wild type. Similarly, perithecium production was identical to that of the wild type on all media except on 0.5 g/L of vanillic acid, on which the mutants deleted for the class II peroxidase (e.g. mutant *5970Δ*), the hybrid B peroxidase (e.g. mutant *9370Δ*) and for the four aromatic peroxygenases (e.g. mutant *(12205-370-7390-6870)Δ*) had a slightly improved fertility, while the mutant deleted for the haloperoxidase (e.g. mutant *5120Δ*) appeared to have a slightly decreased fertility (Fig. 6). This was not seen in the amount of perithecia and ascospore produced but by the timing of ascospore production. However, the fertility phenotypes were not observed at the other tested concentration of vanillic acid (e.g. 0.1 g/L, 1 g/L and 2 g/L) and were very weak, with at most one day of differences between the maturation of the ascospores (Fig. 6). The phenotypes co-segregated with the deletions indicating that they were linked to them. Although we cannot completely rule out that linked mutations may have been responsible for the observed phenotype, the fact that mutants in independent peroxidase genes presented the same phenotype strongly argued that it was caused by loss of peroxidase genes.

EFFECT OF THE MUTATIONS ON AZURE B AND LIGNIN DECOLORIZATION BY *P. ANSERINA*

We assayed Azure B decolorization on Petri plates as described in Dicko *et al.* (2020) as a measure of total secreted peroxidase activity. As seen in Figure 7, we observed a diminution of secreted peroxidase activity only for the *(12205-370-7390-6870)Δ* mutant; however, owing to the large variability of the assay, this diminution was not statistically significant. Nevertheless, it was observed in most experiments, suggesting that for this strain a small decrease in secreted peroxidase activity was indeed present. This was confirmed by direct measure of peroxidase activity in supernatants of three-week old cultures (Fig. 8) for which a small decrease of activity, albeit statistically-non-significant, was also measured for the *(12205-370-7390-6870)Δ* mutant. We also observed a statistically-non-significant increase of activity in the *9370Δ* mutant (lacking hybrid-B peroxidase).

Lignin degradation in the supernatant of four-day-old culture was assayed by spectrophotometry as described in Dicko *et al.* (2020). We did not find statistically-significant differences between the strains. Nevertheless, the *(12205-370-7390-6870)Δ* mutant appeared slightly less efficient in degrading lignin and mutant *9370Δ* was slightly more efficient, results which are in line with their efficiency in degrading Azure B.

DISCUSSION

In previous studies, crucial role of catalases in biomass degradation were evidenced in *P. anserina* (Bourdais *et al.* 2012),

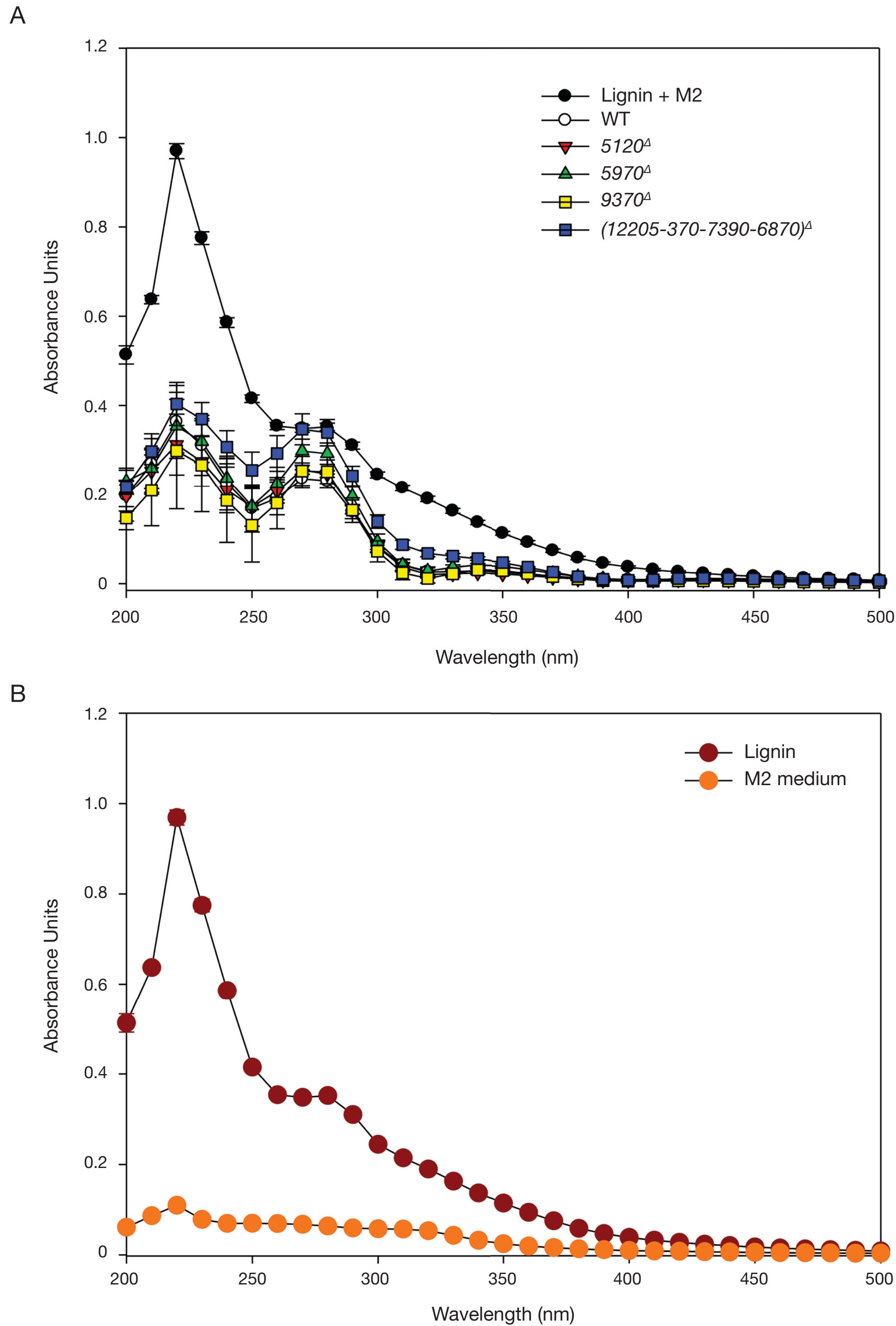


Fig. 9. — Lignin degradation in *P. anserina* (Rabenh.) Niessl wild type and mutants: **A**, UV-visible absorption spectra of lignin (20 µM) diluted in M2 medium after four days of incubation in the absence (**black circle**) or in the presence of *P. anserina* wild type and mutants; **B**, UV-visible absorption spectra of the lignin alone (20 µM) diluted in M2 medium (**brown circle**) and M2 medium alone (**orange circles**) after four days of incubation showing that lignin is stable in M2 medium and that the M2 medium does not contribute to changes in absorption. Measures were average of three experiments assayed in triplicates.

arguing for an important role of hydrogen peroxide in lignocellulose breakdown in this fungus. Here, we evidence minor roles for peroxidases for this process, as we were only able to evidence a small role in resistance/sensitivity to vanillic acid, a toxic degradation product of lignin. Note that the hybrid B peroxidase and the aromatic peroxygenases peroxidases appear to transform vanillic acid into more toxic product(s), while the haloperoxidase appear to transform it into less toxic product(s). Seemingly, *P. anserina* relies rather on laccases and other multicopper oxidases for degradation of plant biomass

(Xie *et al.* 2014, 2015). However, we did assay as multiple mutant only the one lacking all four aromatic peroxidases. In this mutant, we observed a small, albeit non-statistically-significant decrease in Azure B and lignin breakdown. Possibly, redundancy between the different peroxidase genes, even from different families, enable to compensate for the deletion of a single gene, as seen by the lack of drastic effect of gene deletions on peroxidase enzymatic activity. Necessity to inactivate several genes to observed drastic phenotypes was also demonstrated for catalases and laccases (Bourdais *et al.* 2012; Xie *et al.* 2014); however for catalases and laccases simple inactivation of some genes had detectable phenotypes, especially some single mutants produced less perithecia on complex biomasses, a feature that we did not observed here.

We also did not detect in the mutant any modification in vegetative growth, in sexual reproduction, in the formation of the *Cladorrhinum*-like anamorph and appressorium-like structures, in its interaction with other fungi (e.g. Hyphal Interference; Silar 2005) as well as during senescence, which is related to mitochondrial disfunction (Hamann & Osiewacz 2022), and in the Crippled Growth cell degeneration that is in part controlled by NADPH oxidase and a possible redox signaling cascade (Malagnac *et al.* 2004; Lalucque *et al.* 2017). All developmental features seem thus unaffected in the peroxidase mutants. This is in line to what is observed in other fungi in which deletion of peroxidases gene does not lead to growth impairment, except in *B. kuwatsukai*, and in conidiation except in *V. mali* and *B. kuwatsukai* (Mir *et al.* 2015; Flajsman *et al.* 2016; Feng *et al.* 2018; Lee *et al.* 2018; Xiao *et al.* 2022). All the other fungi investigated by gene deletion of peroxidase genes were phytopathogens and, in many instances (but not all), diminished virulence was observed in the mutants. Possibly, small fitness decreases, even if not detectable in laboratory conditions on Petri plates, may result in severe loss of fitness in more complex situations such as the one encountered during interaction with host plants. This suggests that even small advantages conferred by the presence of peroxidase may greatly improve fitness in the wild. It is thus possible that peroxidases promote small phenotypic differences, such as the one we observed in the presence of vanillic acid, which help the fungus to cope in nature with the presence of toxic product(s) made by plants and other microorganisms or generated during lignin degradation. This is also in line with the patchy phylogenetic distribution of the peroxidase genes, indicating that they are not essential but rather involved in fine adaptation of fungi to their biotope.

Authors' contributions

RF, VG and NT equally contributed to this work.

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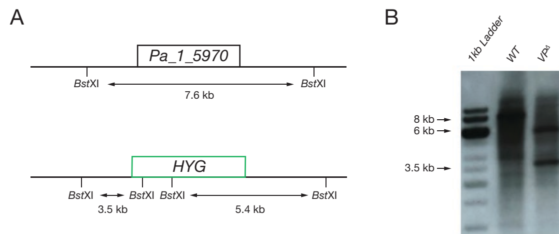
APPENDICES

APPENDIX 1. — Sequences of primers used for gene deletions.

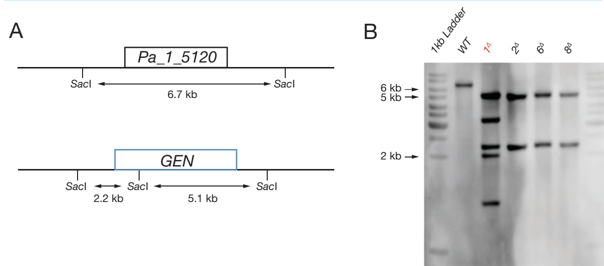
Gene	Primer names	Sequences
Pa_0_370	5’-Pa_0_370A	agctctttgcaagcgagagagttgtatcgt
	5’-Pa_0_370B	ctatttaacgaccctgccctgaaccgatagcctccttcgcccaggtatgtaatgt
	mk_0_370E	acattacatacctgggcgaaggaggctatcggttcagggcagggtcgttaaatag
	mk_0_370F	tacaggtgttgcagcctggttctttagctacatcgaactggatctcaacagcggtaag
	3’-Pa_0_370C	cttaccgctgttgagatccagttcgatgtagctaaagaaccaggctgcaacacctgta
	3’-Pa_0_370D	tatgactgtgctgtatcggaagcgctagat
Pa_1_5120	5’-Pa_1_5120A	ataaattgttgggagatgcgttcctgctac
	5’-Pa_1_5120B	ctatttaacgaccctgccctgaaccgggtggattgatgaacacagatgaaggatg
	mk_1_5120E	catccttcatctgtgttcatcaatccacccggttcagggcagggtcgttaaatag
	mk_1_5120F	atccatttacttatcacatcgctcacgtcccatcgaactggatctcaacagcggtaag
	3’-Pa_1_5120C	cttaccgctgttgagatccagttcgatgggacgtgagcgatgtgataagtaaatggat
	3’-Pa_1_5120D	ctacatcctcggagattcagagactggtgt
Pa_1_5970	5’-pa_1_5970a	cttccagaataggcatcaataaacagat
	5’-pa_1_5970b	ctatttaacgaccctgccctgaaccgttcagttgagatctaagtccgaaactct
	mk_5970_e	agagtttcggacttagatctcaactgaacggttcagggcagggtcgttaaatag
	mk_5970_f	tgctccccgtatttatttacttattttgcatcgaactggatctcaacagcggtaag
	3’-pa_1_5970c	cttaccgctgttgagatccagttcgatgcaaaataagtaaataaatacggggagca
	3’-pa_1_5970d	ccctaccgacgactatatcatcaaag
Pa_2_6870	5’-pa_2_6870a	atatcttgcgctgcaggagtgttgatacat
	5’-pa_2_6870b	ctatttaacgaccctgccctgaaccgagcaaaaggatcaaaacaggtccgagact
	mk_2_6870e	agtctcggacctgttttgatccttttgctcggttcagggcagggtcgttaaatag
	mk_2_6870f	gtgcaagtccaaaaggtgatgacataacgacatcgaactggatctcaacagcggtaag
	3’-pa_2_6870c	cttaccgctgttgagatccagttcgatgtcgttatgtcatcaccttttggacttgcac
	3’-pa_2_6870d	agataaagaagtgtagggggcggttggactc
Pa_2_7390	5’-pa_2_7390a	taacaacactcatcggtcagcgaaaaattc
	5’-pa_2_7390b	ctatttaacgaccctgccctgaaccgtaaaaacctgaagctgttggtgatggtgat
	mk_2_7390e	atcaccatcaccaacagcttcaggtttttacggttcagggcagggtcgttaaatag
	mk_2_7390f	tggtggtggatagttttattgctggtgttccatcgaactggatctcaacagcggtaag
	3’-pa_2_7390c	cttaccgctgttgagatccagttcgatggaacaccagcaataaaactatccaccacca
	3’-pa_2_7390d	aaaatcaaggctgatgcaatgagaggaga
Pa_5_12205	5’-Pa_5_12205A	gctgcccacgataacatagtcaaactcaga
	5’-Pa_5_12205B	ctatttaacgaccctgccctgaaccgaaataatcactccgctgaagcctgaaagc
	mk_12205E	gctttcaggcttcagcggagtgattatttcggttcagggcagggtcgttaaatag
	mk_12205F	gatgtaaggagggtggttgatgccaataacatcgaactggatctcaacagcggtaag
	3’-Pa_5_12205C	cttaccgctgttgagatccagttcgatgttattggcatcaaccaccctccttacatc
	3’-Pa_5_12205D	gtacggggtaccaaacctttcgttttcaa
Pa_2_9370	5’-Pa_2_9370A	caaaccccaagcgttctagg
	5’-Pa_2_9370B	ctatttaacgaccctgccctgaaccgagcgagtgagcgagtgaatg
	mk_E-9370	cattcactcgctcactcgctcggttcagggcagggtcgttaaatag
	mk_F- 9370	cgtctgccaatgtctggatccatcgaactggatctcaacagcggtaag
	3’-Pa_2_9370C	cttaccgctgttgagatccagttcgatggatccagacattggcagacg
	3’-Pa_2_9370D	gcaggcaggagaagtcgttc

APPENDIX 2. — Southern blot analyses of gene deletions. For each deletion: **A**, schematic representation of wild-type and deleted loci with relevant restriction sites for the enzymes used to cut DNA; probes were made for each gene with the amplified regions bordering the replaced regions and used in the deletion experiments. Expected sizes for the bands revealed by the probes are indicated below the **double-headed arrows**; **B**, autoradiograms for the tested candidates with expected size for the wild-type and deleted loci. For haloperoxidase *Pa_1_5120*, the candidate in **red** did not have the expected pattern and was eliminated.

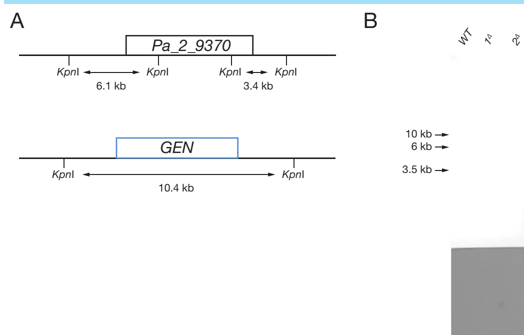
Class II peroxidase *Pa_1_5970*



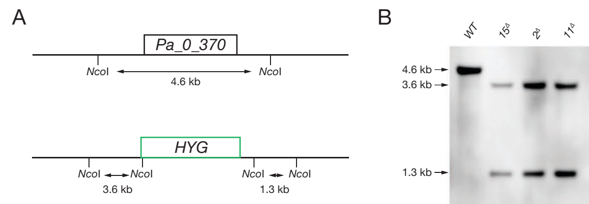
Haloperoxidase *Pa_1_5120*



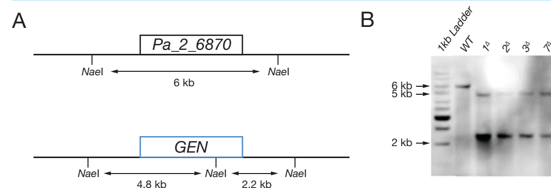
Hybrid B peroxidase *Pa_2_9370*



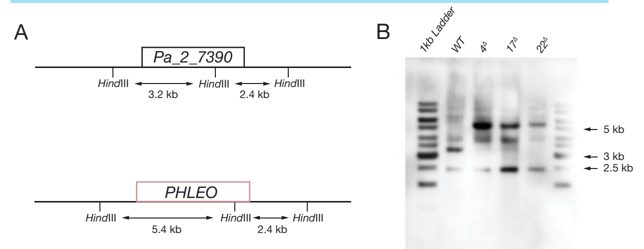
Aromatic peroxygenase *Pa_0_370*



Aromatic peroxygenase *Pa_2_6870*



Aromatic peroxygenase *Pa_2_7390*



Aromatic peroxygenase *Pa_5_12205*