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par:

Andrew ANAK NGADIN

Caractérisation biochimique et fonctionnelle de glutathion-S-transferases (GSTs) chez *Phanerochaete chrysosporium*

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Membres du jury:

Rapporteurs

Pr. Michel PENNINCKX	Professeur, UPEM, Université de Libre, Brussel
Dr. Anissa L. HADJ-SAHRAOUI	Maître de conférences, UCEIV, Université du Littoral Côte d'Opale, Calais

Examinateurs

Pr. Daniel WIPF	Professeur, UMR PME, Université de Bourgogne, Dijon
Dr. Patrick BILLARD	Maître de conférences, LIMOS, Nancy-Université

Directeurs de thèse

Pr. Eric GELHAYE	Professeur, UMR IAM, Nancy-Université
Dr. Mélanie MOREL	Maître de conférences, UMR IAM, Nancy-Université

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DEDICACE

To my parents
for letting me pursue my dream
for so long
so far away from home
&
To my close friends
for giving me
new dreams to pursue

ABREVIATIONS

α	alpha
4-HNE	4-hydroxynonenal
4NPG	S-(4-nitrophenacyl) glutathione
5-HPETE	acide 5-hydroxyeicosatetraeonique
ABC	ATP binding cassette
ADN/DNA	deoxyribonucleic acid
ATP	adenosine-5'-triphosphate
Cd	cadmium
CDNB	1-chloro-2, 4-dinitrobenzene
CLIC	chloride ion channels
CO_2	carbon dioxide
CuOOH	cumene hydroperoxide
D_a	dalton
DCM	dichloromethane
DHA	dehydroascorbate
DHAR	dehydroascorbate reductase
DMA	dimethylarsenate
DTT	dithiothreitol
EOR/ROS	reactive oxygen species
FLAP	5-lipoxigenase activating protein
GR	glutathione reductase
GSH	reduced glutathione
G-site	glutathione-binding site
GSSG	glutathione disulfide redox state
GST	glutathione S-transferase
H_2O_2	hydrogen peroxide
HAP/PAH	polycyclic aromatic hydrocarbon
HED	β- hydroxyethyl disulphide
HPLC	high-performance liquid chromatography
H-site	substrate binding site of glutathione transferase
IPTG	isopropyl β-D-1-thiogalactopyranoside
J_2(15d-PGJ_2)	15-deoxy-Δ12,14-proostaglandin J_2
KDa	kilo dalton
Km $_{(GSH)}$	affinity constant Michaelis-Menten
Lig	lignin
Lips	lignin peroxidase
LTC4S	leukotriene C4 synthase
MAPEG	membrane associated proteins involved in eicosanoid and glutathione metabolism
MAPK	mitogen activated protein kinase
MDR	multi-drug resistance
MMA	monomethylarsenate
MMDB	molecular modeling database
Mn	manganese
MnP	manganase peroxidase
MnSOD	manganase superoxyde dismutase

NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National centre for biotechnology information
NCR	nitrogen catabolite repression
PDB	protein data bank
PGD_2	prostaglandine D_2
PGES1	prostaglandine E synthase
PGF_{2a}	prostaglandine $F_{2\alpha}$ synthase
PMSF	phenylmethylsulfonyl fluoride
Rpm	rotations per minute
RT PCR	reverse transcription polymerase chain reaction
RT	reverse transcription
SAM	S-adenosyl methionine
SOD	superoxide dismutase
t-BOOH	tert-butyl hydroperoxyde
TCHQ	tetrachlorohydroquinone
VA	veratryl alcool
YPD	yeast peptone dextrose

INTRODUCTION

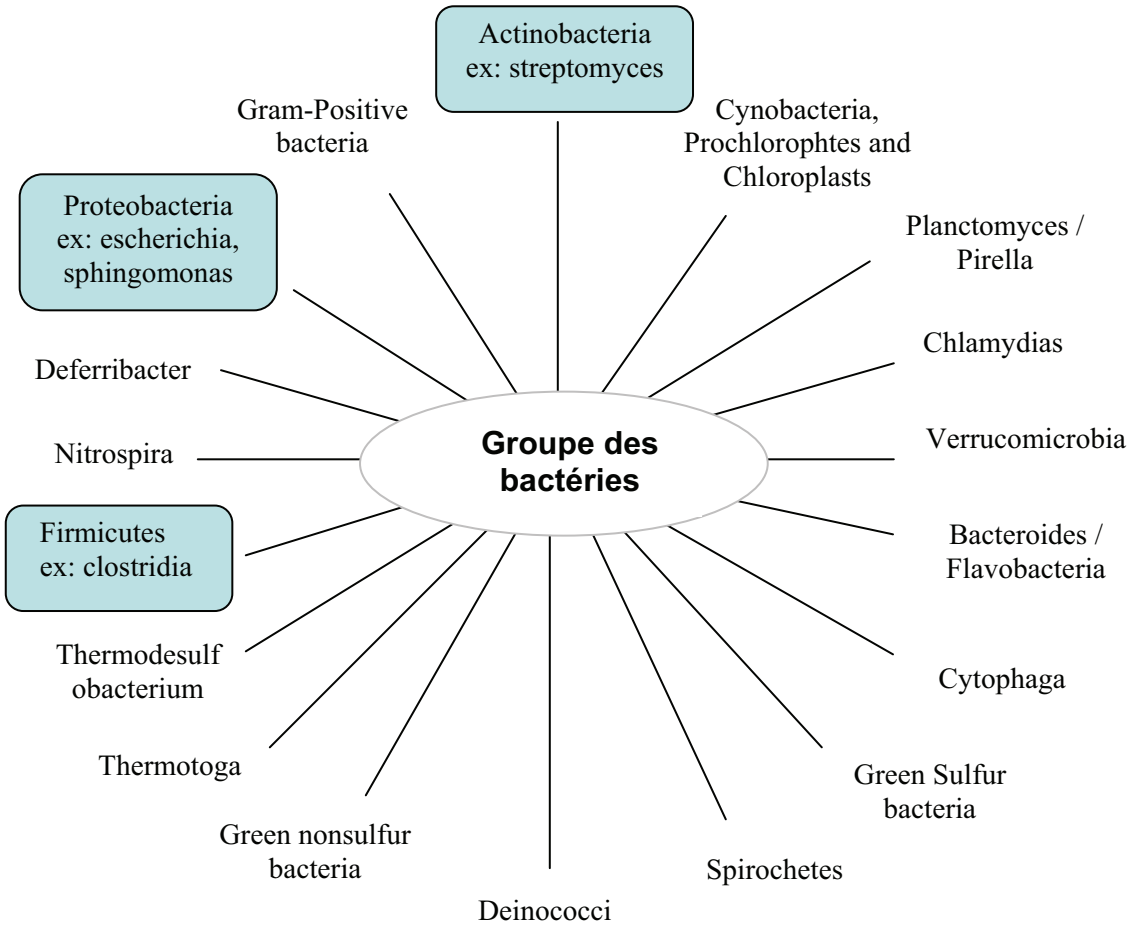


Figure 1 : Diversité du groupe des bactéries. Les proteobactéries, les actinobactéries et les firmicutes sont particulièrement imliquées dans la dégradation de la matière organique.

1. Diversité des microorganismes saprophytes

Les microorganismes saprophytes sont des bactéries et des champignons hétérotrophes capables de mobiliser le carbone et l'azote à partir de composés organiques complexes. Ces microorganismes décomposeurs sont cruciaux dans de nombreuses fonctions environnementales, tel que le cycle du carbone et de l'azote mais aussi la dégradation de polluants. La composition, la biomasse et l'activité chimique des communautés saprophytes dans le sol dépend d'importants facteurs physiques et chimiques tels que l'oxygénation, la température, le pH, l'humidité et la richesse nutritionnelle des sols (Garbeva et al., 2004; Killham, 1994; Lavelle et Spain, 2001). L'intérêt pour l'environnement est double:

1- la dégradation de la matière organique représente un atout trophique pour les microorganismes dont la nutrition carbonée et azotée n'est alors pas limitée par la biodisponibilité des éléments dans les sols.

2- les propriétés de dégradation de molécules complexes qu'ont ces organismes en font de bons candidats pour les projets de bioremèdiation des sols pollués.

1.1 Les bactéries

Parmi les microorganismes saprophytes, les bactéries représentent le groupe le plus abondant et le plus diversifié. Dix-huit phylums ont été identifiés en fonction de leur rôle dans l'écosystème (Figure 1). Une séquence d'apparition des espèces dans les processus de dégradation a pu être établie. Les bactéries du genre Escherichia interviennent au début des processus de dégradation des animaux morts en particulier, en décomposant physiquement les muscles en filaments protéiques. Ensuite, les bactéries des ordres Eubacteriales, Actinomycetales, (ex Streptomyces sp) et les bactéries cellulolytiques (ex : Clostridia) continuent le processus de dégradation des tissus morts. Ces bactéries participent activement à la mobilisation de la matière organique du sol grâce à leur forte représentabilité à savoir 2 milliards de bactéries par gramme de sol. Enfin, les bactéries dites lignivores sont capables de dégrader les composés récalcitrants (ex : Sphingomonas).

Sans l'action de ces bactéries, la matière organique issue d'animaux morts ou de résidus végétaux s'accumulerait très rapidement, empêchant le développement des nouvelles générations.

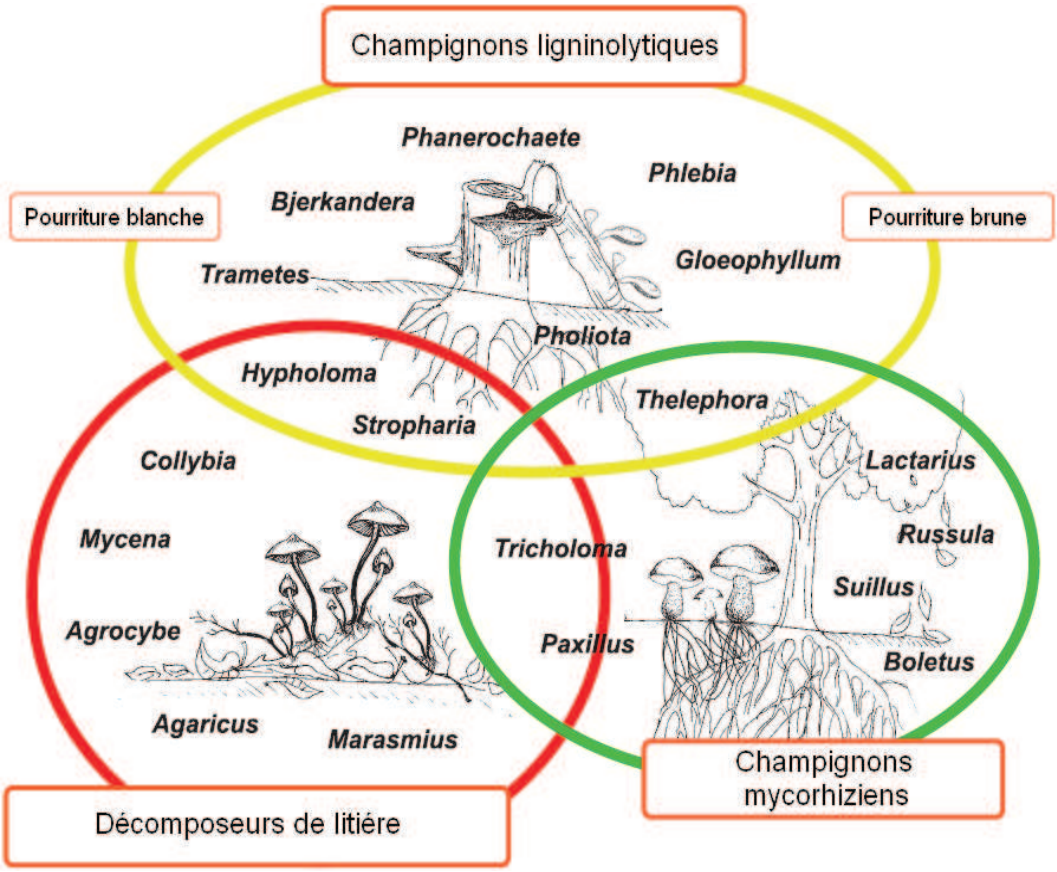


Figure 2 : Les différents groupes de champignons saprophytes (Steffen, 2003).

1.2 Les champignons

Les champignons saprophytes peuvent se subdiviser en 3 groupes principaux: les champignons mycorhiziens, les champignons décomposeurs de litière et les champignons ligninolytiques (Figure 2). Ces champignons sont essentiellement des décomposeurs de matière organique morte et leur mycélium, composé d'hyphes souterraines est important pour la prospection et la translocation des nutriments au sein du champignon (Hunt et Wall 2002; Setala et McLean 2004). Une partie des champignons saprophytes se développent sur du bois et possèdent donc un système enzymatique spécifique leur permettant de dégrader ses différents constituants que sont la cellulose, les hémicelluloses et la lignine. Parmi ces champignons ligninolytiques, on distingue les champignons de pourriture blanche, les champignons de pourriture brune et les champignons de pourriture molle.

1.2.1 Les champignons de pourriture brune

Le syndrome de pourriture brune est causé par des champignons qui dégradent la cellulose et les hémicelluloses. En revanche, ils sont incapables de digérer complètement la lignine, ils ne peuvent que la modifier chimiquement. Le bois dégradé par ces champignons a un aspect craquelé (Figure 3A). Les champignons impliqués sont des basidiomycètes qui appartiennent majoritairement à la famille des polyporaceae et seulement 6% des champignons ligninolytiques font partie de ces champignons dits de pourriture brune (Schwarze *et al.*, 2000). *Postia placenta*, *Serpula lacrymans*, *Coniophora puteana*, *Meruliporia incrassate*, and *Gloeophyllum trabeum* en sont quelques exemples (Blanchette, 1995). Ces champignons utilisent des mécanismes spécifiques basés sur la réaction de Fenton et la production de radicaux hydroxyles pour dégrader la cellulose et les hémicelluloses. Des réactions de démethylation, d'oxydation et de dépolymérisation sont responsables de la modification de la lignine (Figure 4).

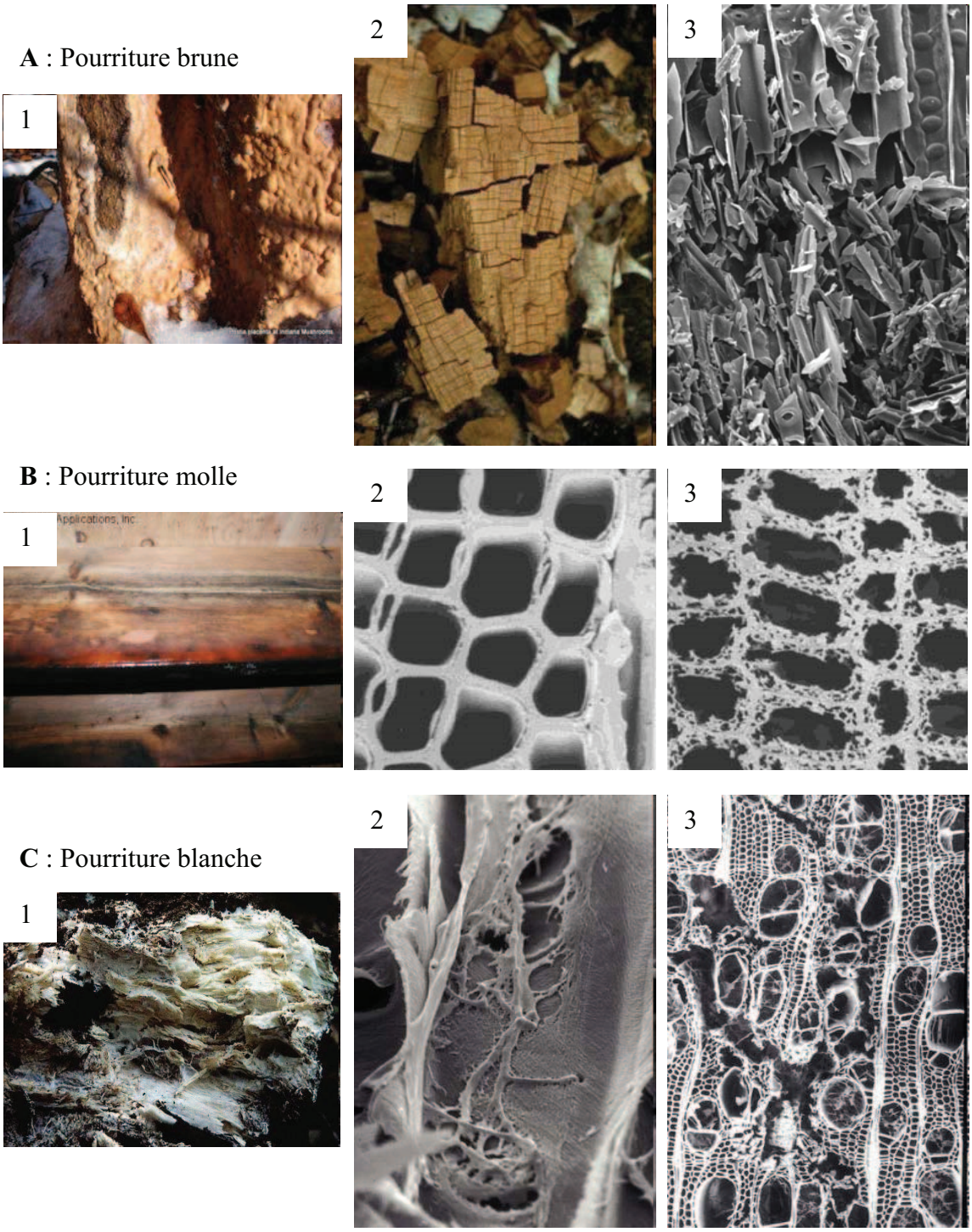


Figure 3 : Photographies des différents types de champignons ligninolytiques. A : *Postia placenta* en milieu naturel (1), bois dégradé par *P. placenta* (2), bois dégradé par *P. placenta* vu en microscopie électronique à balayage (3). B : *Chaetomium sp* se développant sur du bois (1), structure du bois non dégradé (2) et du bois dégradé par un champignon de pourriture molle (3). C : *Phanerochaete chrysosporium* en milieu naturel (1), bois dégradé par *P. chrysosporium* vu en microscopie électronique à balayage (2 et 3).

1.2.2 Les champignons de pourriture molle

Ce groupe comporte des espèces capables de dégrader la cellulose, les hémicelluloses et seulement partiellement la lignine. La pourriture molle concerne essentiellement des bois humides contenant beaucoup d'azote mais peu de lignine. Les hyphes du champignon s'insèrent dans la paroi secondaire en direction des microfibrilles de cellulose créant des cavités selon un axe longitudinal qui apparaissent alors en coupe comme de petits trous circulaires dans la paroi secondaire (Figure 3B) (Greaves, 1975). Le champignon crée ces cavités dans la paroi des cellules grâce à des systèmes enzymatiques et non-enzymatiques, mais ceux-ci sont encore mal connus car peu étudiés. Les espèces concernées sont des ascomycètes et des deutéromycètes et plus de 300 espèces font partie de ce groupe comme par exemple *Cephalosporium*, *Acremonium*, et *Chaetomium.*

1.2.3 Les champignons de pourriture blanche

Ces champignons sont représentés dans tous les principaux groupes de Basidiomycètes et quelques Ascomycètes (Sutherland et Crawford, 1981). Ils sont capables de dégrader complètement les composants majeurs de la lignine à savoir la cellulose, les hémicelluloses et la lignine. La désignation de pourriture blanche a été utilisée pour décrire le type de dégradation dans lequel le bois prend une apparence décolorée et où la lignine, la cellulose et les hémicelluloses sont fragmentés (Figure 3C).

Deux formes de pourriture blanche ont été décrites (Blanchette, 1984), la délignification sélective et la pourriture simultanée, qui sont fonction des caractéristiques chimiques et morphologiques du bois. Un même champignon peut causer ces deux formes de pourriture blanche. La délignification sélective conduit à la pourriture blanche de poche, qui se traduit par des taches claires au niveau de la lignine. La pourriture simultanée laisse des taches de cellulose pure. Parmi ces champignons de pourriture blanche, *Phanerochaete chrysosporium* est le modèle le plus étudié.

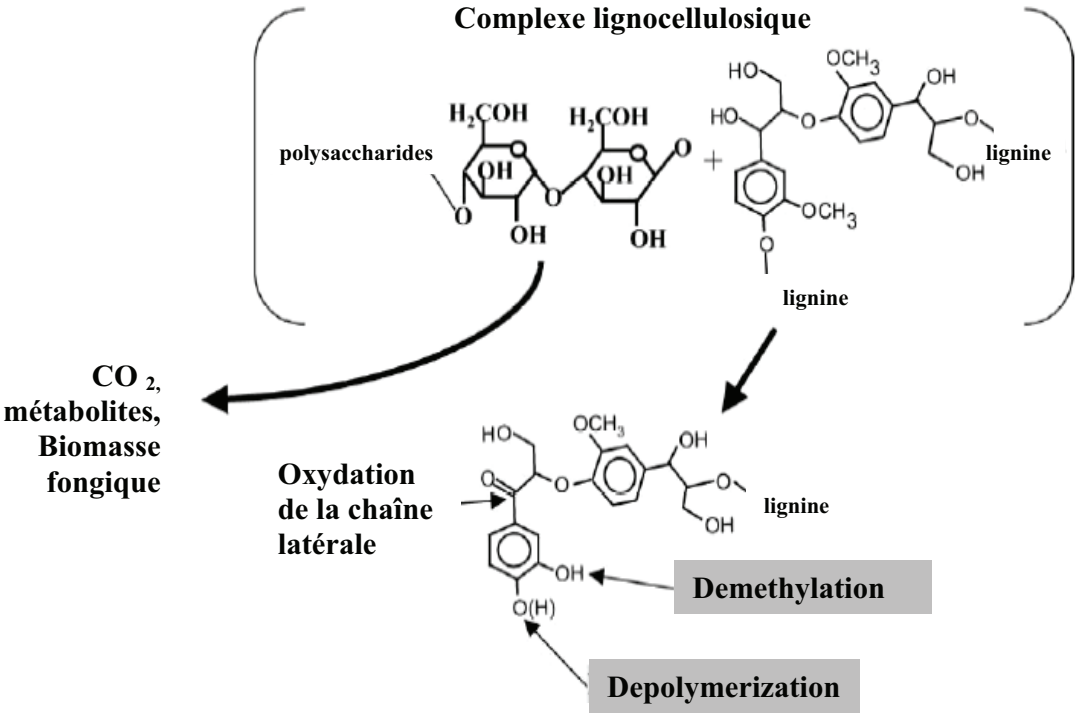


Figure 4 : Mécanisme de dégradation du bois par les champignons de pourriture brune. Les celluloses sont complètement digérées et le carbone ainsi relargué permet d'alimenter la biomasse fongique. La lignine, elle, est modifiée par des réactions d'oxydation, de dépolymérisation et de déméthylation.

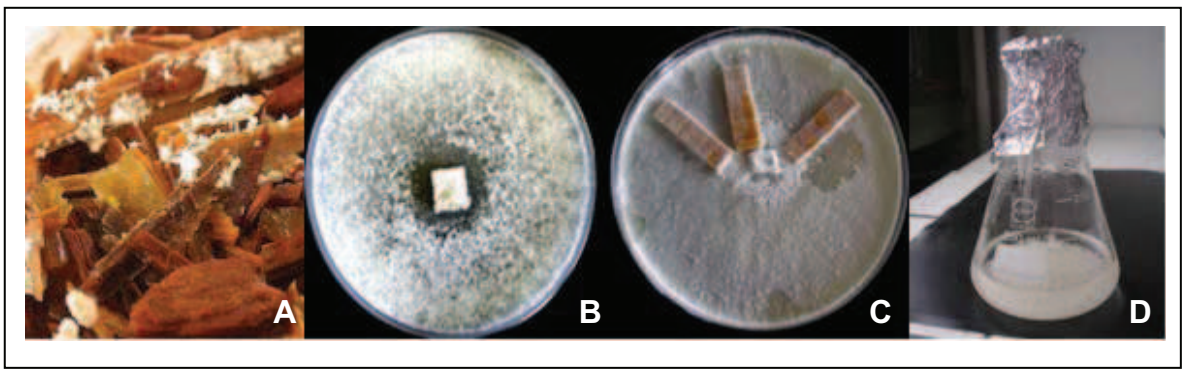


Figure 5 : Systèmes de culture de *Phanerochaete chrysosporium*. A: en conditions naturelles B: *in vitro* sur milieu riche gélosé; C: sur des éprouvettes de bois; D: en forme de pelotons en milieu liquide.

2. *Phanerochaete chrysosporium*

2.1 Description

Le genre Phanerochaete est un assemblage hétérogène de plus de 90 espèces (Parmasto, 1997 Cortbase ver.1.4 2002) présentant des caractéristiques phénotypiques variées notamment en ce qui concerne les fructifications plus ou moins diffuses ou tuberculeuses, la morphologie des hyphes, des basides et des basidiospores (Eriksson et al., 1978; Wu, 1995; 1998). *P. chrysosporium* est un champignon homobasidiomycète de pourriture blanche qui présente un optimum de température de 40°C, lui permettant de croître dans des niches de décomposition où la température est en général élevée de par l'activité de certaines bactéries. Dans son habitat naturel, *P. chrysosporium* se développe directement sur du bois (Figure 5A). *In vitro*, différents systèmes de culture sont utilisés : le champignon peut être cultivé sur des boites de milieu riche gélosé où il est capable de sporuler (Figure 5B), sur des éprouvettes de bois (Figure 5C) ou en milieu liquide dans lequel les spores germent en formant des pelotons (Figure 5D). Le génome de *P. chrysosporium* est le premier génome basidiomycète à avoir été séquencé (Martinez, 2004). Son assemblage a révélé 10 048 modèles de gènes et sa taille est de 35,1 mégabases.

2.2 Dégradation de la matière organique

2.2.1 Généralités

Dans les sols, la dégradation de la matière organique passe par plusieurs étapes dépendantes à la fois de facteurs biotiques et abiotiques:

- Le lessivage est la perte des éléments solubles de la matière organique.
- La fragmentation des résidus est un processus physique et chimique, biotique et abiotique qui permet d'augmenter l'accessibilité des nutriments aux microorganismes.
- La minéralisation fait intervenir des microorganismes (bactéries et champignons) capables d'excréter tout un panel d'enzymes pour la dégradation des protéines, des sucres, des lipides, des acides nucléiques et des composés plus complexes comme la cellulose ou la lignine.

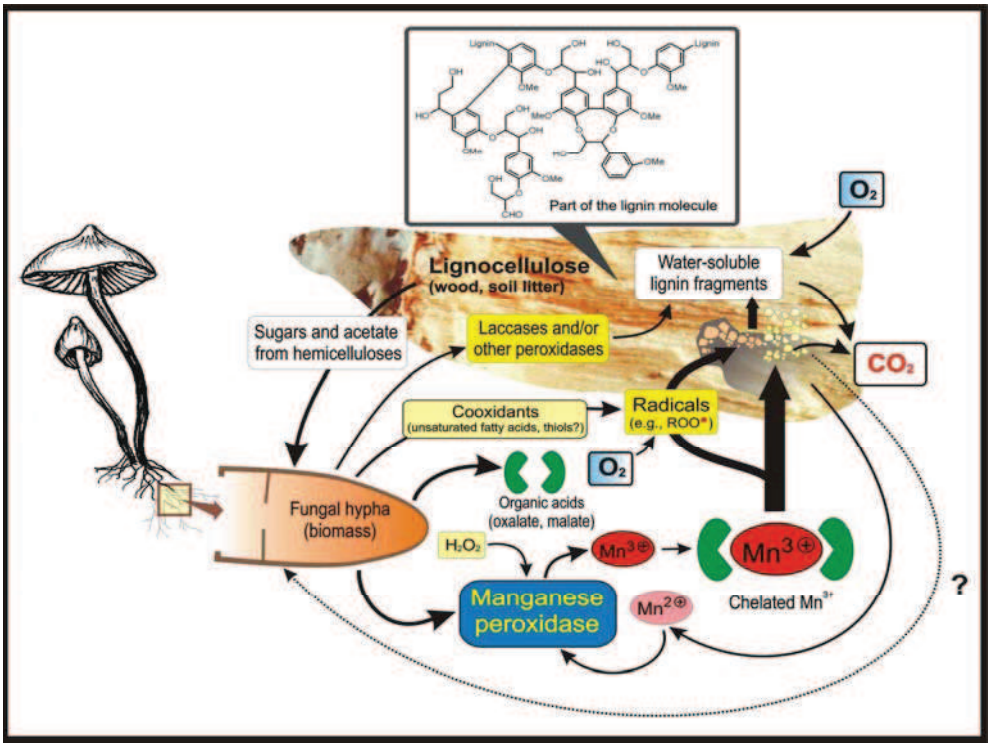


Figure 6 : Système de dégradation du bois par les champignons ligninolytiques (Hofrichter, 2001). Pour dégrader le bois, les champignons excrètent:
1- des oxydants qui vont générer des radicaux très réactifs
2- des manganèse peroxydases qui vont générer du Mn^{3+} qui va se complexer avec des acides organiques excrétés par le champignon. Ce complexe très réactif pourra agir sur la 3- d'autres enzymes telles que des laccases ou des peroxydases (ex: lignine peroxydases).

Tableau 1 : Propriétés physico-chimiques des LiPs (Schoemaker et Leisola, 1990; Farrell et al., 1989; Tien et Kirk, 1983; Teunissen and Field, 1998; Teunissen et al., 1998b).

E.C. (Enzyme Commission code)	LiP 1.11.1.14
Groupe prosthétique	Diarylpropane, O_2, H_2O_2 Oxidoreductases, Hème
Poids moléculaire (kDa)	38-47
Glycosylation	N -
Isoformes	monomères
Point isoélectrique	3.2 – 4.7
pH	2.0 - 5.0
E^0 (mV)	1450
Clivage des liaisons C-C	oui
Regulation par H_2O_2	oui
Stabilité	+
Médiateur	Alcool vératryl , 2-chloro-1,4-dimethoxybenzene
Specificité	large, composés aromatiques et non phénoliques

Une des particularités de *P. chrysosporium*, qui lui a d'ailleurs valu d'être choisi comme modèle de nombreuses études, est l'excrétion d'enzymes impliquées dans la dégradation de molécules complexes et en particulier la lignine. Ces enzymes sont essentiellement des lignine peroxydases et des manganese peroxidases (Figure 6).

2.2.2 Les lignine peroxydases (LiPs)

Ce sont des glycoprotéines à hème. Elles catalysent l'oxydation de structures phénoliques générant des radicaux (Kirk et Farell, 1987; Eriksson et al., 1990; Higuchi 1990; Hatakka 2001; Martinez, 2002). Les réactions catalysées par ces enzymes sont le clivage des liaisons carbone-carbone (Cα-Cβ), l'oxydation des carbones en position α, le clivage des liaisons alkyl-aryl et des cycles aromatiques. Ces enzymes ont également des activités de déméthoxylation, d'hydroxylation et de polymérisation (Tien, 1987; Higuchi, 1990). *P. chrysosporium* possède 10 isoformes de LiPs, leur masse moléculaire variant entre 35 et 48 kDa et leurs points isoélectriques entre 3 et 4 (Tableau 1). Le sécrétome de *P. chrysosporium* a récemment été analysé et a révélé que 790 protéines sur un total de plus de 10 000 gènes modèles, pourraient être excrétées dans le milieu et parmi elles 1% sont des lignine peroxydases (Bouws et al., 2008). De nombreux efforts se sont concentrés sur les conditions requises pour l'excrétion de ces enzymes. Il a été suggéré que les espèces oxygénées réactives induisaient l'expression des LiPs (Belinky et al., 2003). En effet, l'activité des LiP dans le milieu est induite lorsque de l'oxygène pur est appliqué par bullage à des cultures liquides de *P. chrysosporium* (Rothschild et al., 1995;1999). Cette induction a également été observée en carençant le milieu de culture en manganèse. Dans ces conditions, le superoxyde dismutase mitochondriale dépendante du manganèse (MnSOD) n'est pas active, ce qui résulte en une accumulation d'ions superoxydes intracellulaires (Rothschild et al., 1999). Cependant, l'induction des LiPs n'est pas seulement due au stress oxydant généré par l'absence d'activité SOD, puisque chez des mutants MnSOD obtenus par interférence ARN, aucune activité LiP n'a pu être mise en évidence (Matityahu et al., 2008).

Tableau 2 : Propriétés physico-chimiques des MnPs (Willmann et Fakoussa, 1997; Lobos et al., 1994; Urzúa et al., 1995; Schneegaβ et al., 1997; Heinzkill et al., 1998; Maltseva et al., 1991; Sarkar et al., 1997).

E.C.	MnP 1.11.1.13
Groupe prosthétique	Mn (II): H_2O_2 Oxidoreductases, Hème
Poids moléculaire (kDa)	32- 62.5
Glycosylation	N -
Isoformes	monomères
Point isoélectrique	2.8 - 7.2
pH	2.6 - 4.5
E^0 (mV)	1510
Clivage des liaisons C-C	oui
Régulation par H_2O_2	oui
Stabilité	+ + +
Médiateur primaire	Mn^{2+}; Mn^{3+}
Specificité	Mn^{2+}
Médiateurs secondaires	Thiols, acides gras unsaturés

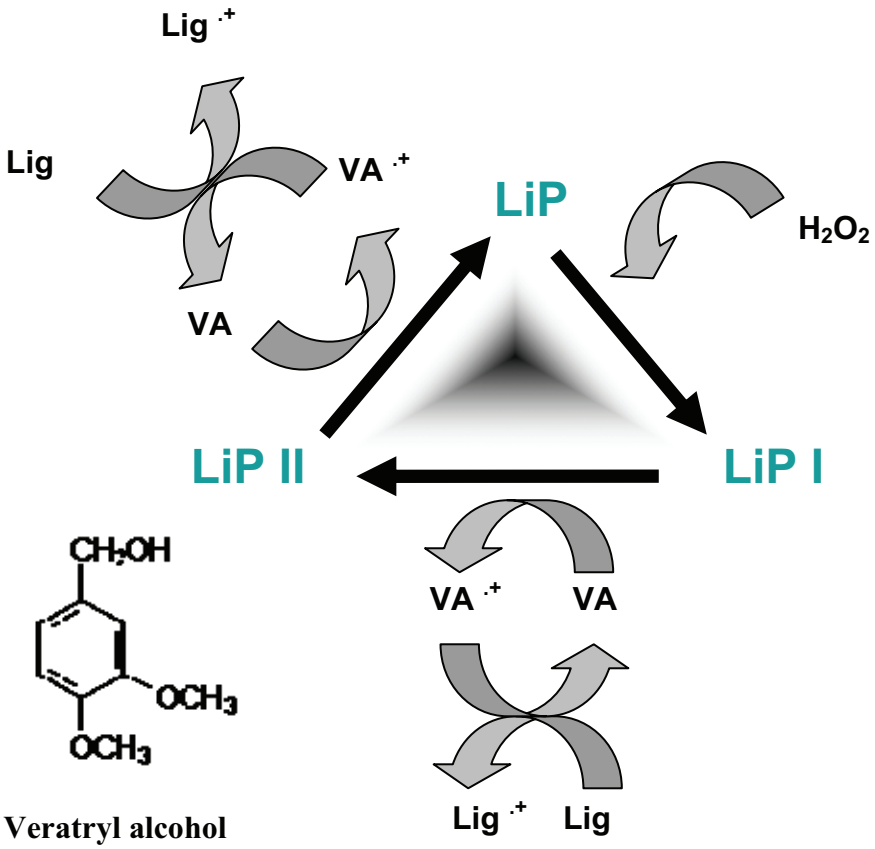


Figure 7 : Mécanisme d'action des lignine peroxydases sur la lignine utilisant l'alcool veratryl comme inducteur. Lig: lignine, LiP: Lignine peroxydase, VA: alcool vératryl.

D'autres études ont montré que l'azote régulait le niveau de transcrits des LiPs, une carence azotée induisant leur expression (Li et al., 1994). Cependant dans une condition riche en azote, l'induction des LiPs peut être régie par une carence en carbone (Singh and Chen, 2008, Dosoretz et al., 1993). Les conditions de production de ces enzymes sont assez complexes puisque d'autres facteurs tels que la biomasse, la taille des pelotons formés en culture liquide ou la température peuvent moduler la production de ces enzymes (Hormiga et al., 2008). Un modèle mathématique a été réalisé mettant en relation la croissance du champignon, sa consommation en substrat et sa dynamique ligninolytique. Les auteurs ont montré que le système atteignait un état stable avec une production de biomasse et une activité de dégradation maximales lorsqu'il y a addition simultanée de glucose et de diphénol en présence d'alcool vératryl (Hormiga et al., 2008). L'alcool vératryl, dont la structure est similaire aux unités constituant la lignine, est un inducteur de l'activité LiP grâce à la formation de radicaux (Figure 7).

2.2.3 Les manganèse peroxydases (MnPs)

Les manganèse peroxidases (MnPs) jouent un rôle important dans la dépolymérisation de la lignine (Wariishi *et al.* 1989; Hofrichter *et al.* 1999; 2002; Kapich *et al.* 1999). Ce sont les principales enzymes impliquées dans ce processus chez les champignons de pourriture blanche et d'autres champignons décomposeurs de litière (Hofrichter, 2002). Des études ont d'ailleurs montré qu'elles étaient majoritaires par rapport aux lignine peroxidases (Hatakka 1994; 2001; Vares et Hatakka 1997; Steffen *et al.,* 2000; Hofrichter 2002; Martínez, 2002). Elles sont capables d'oxyder et de dépolymériser les lignines naturelles et synthétiques, tout comme les lignocelluloses dans des systèmes acellulaires (Hofrichter, 2002). Cependant, elles ne peuvent pas oxyder d'autres structures non phénoliques comme les acides gras insaturés et leurs dérivés, sans co-oxydants (Kapich *et al.* 1999; Hofrichter, 2002). Les MnPs sont des enzymes à hème, glycosylées avec un poids moléculaire de 38 à 62.5 kDa selon les isoformes et leur p*I* est acide (pH3-4) (Tableau 2) (Hofrichter, 2002). L'oxydation de composés phénoliques et non phénoliques par les MnPs est à la fois dépendante du Mn libre et du peroxyde d'hydrogène (H_2O_2) (Glenn *et al.* 1986; Hofrichter, 2002). Les MnPs oxydent le Mn(II) en Mn(III), qui va ensuite oxyder des substrats organiques ou des cycles phénoliques en radicaux phénoxy jusqu'à la décomposition totale de la molécule. Le Mn(III), très réactif est stabilisé par la formation de complexes avec des acides organiques qui sont produits par le champignon, comme le lactate, le malonate ou l'oxalate. Ce complexe ainsi formé sert alors

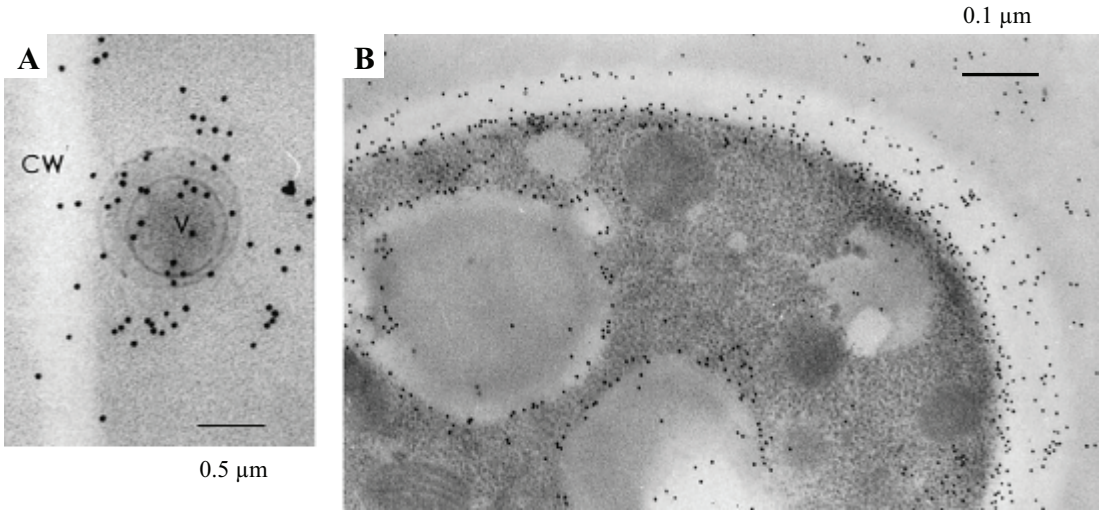


Figure 8 : Localisation des MnPs dans les chlamydospores de *P. chrysosporium*. A: distribution des particules d'or à la périphérie des chlamydiospores à 4 jours de culture. P : Paroi, V : vésicule. B: Distribution des particules d'or à 6 jours de culture (Jiménez-Tobon *et al*., 2003).

de médiateur redox de faible poids moléculaire qui attaque les structures phénoliques de la lignine et génère des radicaux libres qui ont tendance à se désintégrer spontanément (Hofrichter, 2002). *P. chrysosporium* possède 5 isoformes de MnPs. La production de ces enzymes a été visualisée par immunogold en microscopie électronique à transmission grâce à des anticorps anti-MnP (Jiménez-Tobon *et al*., 2003). Les signaux correspondant aux MnPs ont été détectés principalement dans les chlamydospores, en particulier dans les vésicules golgiennes à la périphérie du cytoplasme pour des cultures de 3-4 jours et à proportions égales dans les vésicules golgiennes et la paroi après 6 jours de culture (Figure 8).

2.2.4 Les autres enzymes extracellulaires

A côté des LiPs et MnPs, le système enzymatique extracellulaire comprend de nombreuses enzymes hydrolytiques telles que des cellulases, xylanases, proteases et pectinases. Les cellulases désignent un système enzymatique complexe comprenant des endo-1,4-β-D-glucanase glucanase (EC-3.2.1.4), des exo-1,4-β-glucanases (exocellobiohydrolase, EC-3.2.1.91) et des β-D-glucosidase (β-D-glucoside glucanhydrolase, EC-3.2.1.21). Ces enzymes, tout comme les hémicellulases et les pectinases sont les enzymes clé de la biotransformation des composés lignocellulosiques pour la production de biocarburant et de matières premières pour l'alimentation des animaux (Wong et Sanddler, 1993). Les xylanases (endo-1,4-β-D-xylanase, EC-3.2.1.8) hydrolysent les xylanes et trouvent une application dans la fabrication de la toile de jute (Shoham et al., 1993; Suurnakki at al., 1997). Ces enzymes, tout comme les cellulases sont utilisées en industrie textile pour le traitement des fibres.

2.3 Dégradation de polluants

Cet aspect a fait l'objet de nombreuses études dans les dernières décennies. Ces polluants sont majoritairement des pesticides et des herbicides issus des pratiques agricoles, des métaux lourds et d'autres composés aromatiques complexes tels que les hydrocarbures aromatiques polycycliques (HAP) issus des activités anthropiques. Récemment, les études se sont focalisées sur la capacité des champignons saprophytes et en particulier *P. chrysosporium* à dégrader des colorants. Ces composés sont en général des molécules toxiques pour les cellules vivantes et ils sont particulièrement nocifs en milieu aquatique

Tableau 3 : Capacité de dégradation des colorants *in vitro* par *Phanerochaete chrysosporium*, *Pleurotus ostreatus* et *Trametes hispida.* Les champignons ont été cultivés sur un milieu solide (agar), la dégradation mesurée a alors été qualifiée de métabolique. La dégradation « enzymatique » a également été estimée en testant le milieu de culture des champignons en système liquide. NM: non mesuré, BASF et COLORFAN sont deux producteurs de colorants (Rodriguez et al., 1999).

	Dégradation métabolique		Dégradation enzymatique	
	P. chrysosporium (ATCC-24725)	*P. ostreatus* (IE8)	*P. ostreatus* (IE8)	*T. hispida* (8260)
BASF				
Acid black 194	+	+	+	+
Acid blue 185	-	+	+	+
Direct black 22	NM	NM	-	+
Disperse blue 56	+	+	-	+
Disperse blue 79	NM	NM	-	+
Disperse orange 30	-	-	-	-
Disperse yellow 54	-	+	-	-
Disperse red 161	-	-	-	-
Reactive blue 19	-	+	-	+
Reactive blue 158	-	+	+	+
Reactive red 141	-	-	-	-
Reactive red 180	-	-	-	-
Reactive yellow 84	-	-	-	-
Sulfur black 1	+	+	-	+
Vat blue 6	+	+	-	-
Vat red 10	-	+	-	-
Vat yellow 46	-	+	-	-
COLORFAN				
Orisol black 2V	NM	NM	-	+
Orisol blue BH	+	+	+	+
Orisol orange S	-	-	-	-
Orisol scarlet 4BS	-	-	-	-
Orisol turquoise JL 2	-	+	+	+
Orisol yellow 4JLZ	-	-	-	-

puisqu'ils peuvent affecter l'activité photosynthètique en réduisant la pénétration de la lumière (Daneshvar et al., 2007). La capacité de dégradation des colorants a été étudiée pour plusieurs champignons. Le tableau 3 présente les résultats obtenus pour 3 espèces ; *P. chrysosporium*, *Pleurotus ostreatus, Trametes hispida***.** *P. ostreatus* et *T. hispida* sont capables de dégrader la majorité des polluants testés. *P. chrysosporium* est capable d'en métaboliser 5. Ces colorants peuvent être dégradés par le système enzymatique extracellulaire des champignons de pourriture blanche (ex : les laccases, les lignine peroxydases, les manganèse peroxydases) (Rodriguez et al., 1999).

L'intérêt de ces microorganismes parait alors évident dans les processus biotechnologiques de bioremèdiation développés pour optimiser la décontamination des sols pollués. La dégradation extracellulaire des polluants est possible grâce aux enzymes excrétées décrites ci-dessus, en particulier grâce à leur capacité à métaboliser des molécules complexes et aromatiques. Au niveau intracellulaire, les processus de dégradation décrits chez les organismes saprophytes regroupent à la fois la transformation et la compartimentation des composés toxiques et se subdivisent en trois phases (Figure 9).

2.3.1 Phase I

La première (phase I) est une phase de bioactivation des molécules toxiques. Les enzymes de phase I sont principalement des cytochromes P450 qui transforment les xénobiotiques en molécules intermédiaires, toujours toxiques pour la cellule. Cette bioactivation nécessite du NADH comme cofacteur et génère la formation d'espèces oxygénées réactives. Les cytochromes p450 sont très largement représentés chez *P. chrysosporium*, les séquences codant pour ces enzymes représentant 1% du génome soit environ 150 gènes (Doddapaneni et Yadav 2005; Martinez et al. 2004). Les P450 sont impliqués dans la biosynthèse de composés endogènes, la détoxication oxydative et l'élimination de nombreux polluants hydrophobes comme les drogues ou les pesticides (Jefcoate 1986; Omura 1999; Wislocki et al. 1980). Chez *P. chrysosporium*, ces enzymes se répartissent en 12 familles et 23 sous-familles, et sont exprimées à la fois dans des conditions ligninolytiques et non-ligninolytiques (Doddapaneni et Yadav, 2005). Les gènes codant certains P450 (CYP63A1, CYP63A3 and CYP63A3) sont induits par divers composés aromatiques chez *P. chrysosporium*, cependant les activités de ces enzymes sont encore mal connues (Doddapaneni et Yadav 2004). Quelques études ont montré un rôle de ces P450 dans

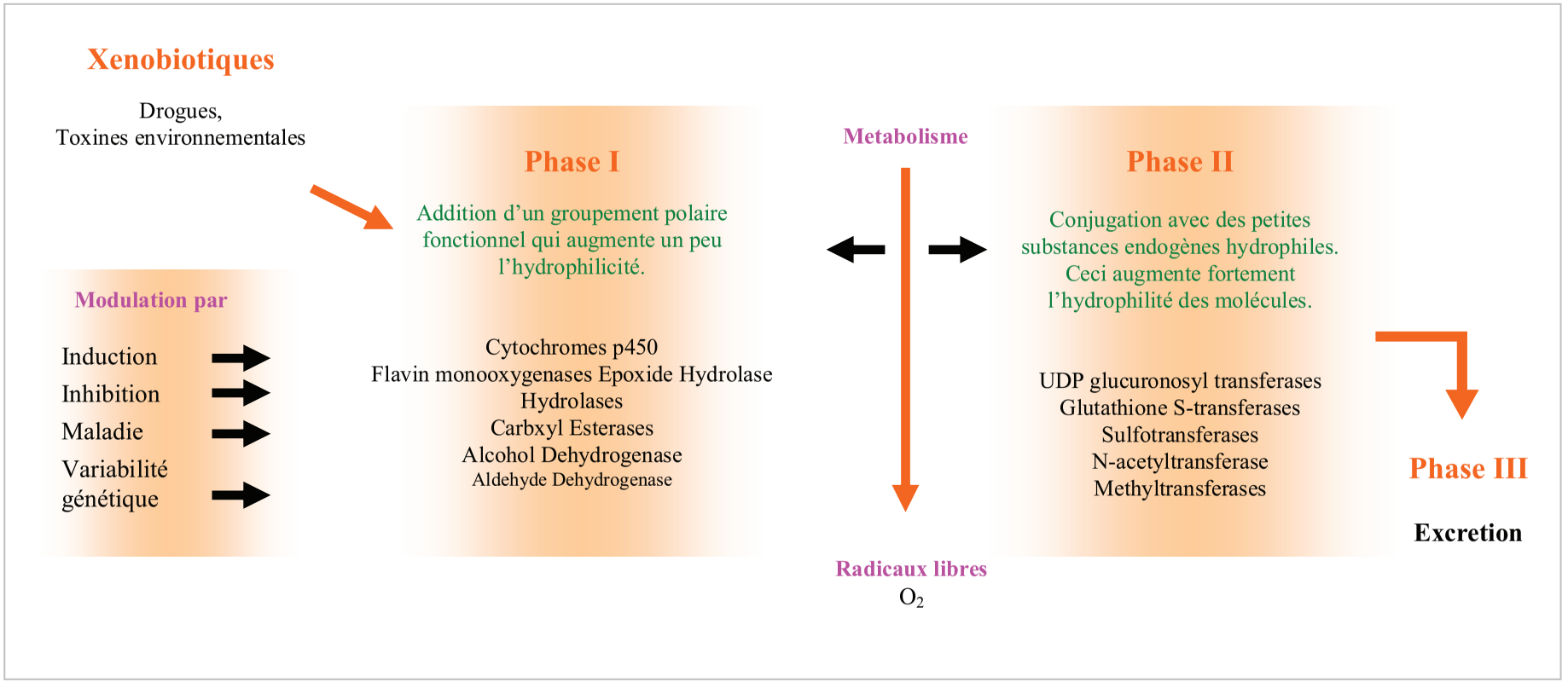


Figure 9 : Processus de dégradation des polluants en 3 phases par les champignons saprophytes. (Copyright© 2004 Oxford Biomedical Research Inc. All rights reserved. http://www.oxfordbiomed.com/commerce/ccc2433-xenobiotic-metabolism.htm)

la dégradation de lindane, d'endosulfane, d'atrazine, d'éther diphényl, de sulfide dibenzyl, de nitrotoluène et de nitrophénol (Mougin et al. 1996; Kullman et Matsumura 1996; Mougin et al. 1997; Hiratsuka et al. 2005; Van Hamme et al. 2003).

2.3.2 Phase II

La phase II permet de convertir les molécules réactives toxiques en molécules plus hydrosolubles et donc moins toxiques et plus facilement éliminables par la cellule. Il s'agit de réactions de glucuronidation, de sulfatation, de conjugaison du glutathion (GSH) ou d'acides aminés, de méthylation, et d'acétylation. Ce système est consommateur de soufre, de glutathion et d'acides aminés puisque ces composés sont fixés sur la molécule toxique et donc éliminés en même temps qu'elle. Cette phase nécessite également une grande quantité d'énergie sous forme d'adénosine triphosphate (ATP). Les enzymes impliquées dans ce processus sont des UDP glucuronosyl transferases (King et al., 2000), des glutathion S transférases (Sheehan et al., 2001) des sulfotransferases, des N-acetyltransferase (Al-Buheissi et al., 2006), et des méthyltransferases.

2.3.3 Phase III

La dernière étape de ce processus de détoxication est la phase III. Elle consiste en la compartimentation des conjugués formés en phase II (Coleman et al., 1997). Cette phase fait intervenir des transporteurs membranaires en général de type MDR (Multi-Drug Resistance) qui ont des activités d'antiport permettant ainsi de diminuer la concentration intracellulaire des composés toxiques (Benet, 1997 pers. Com.; Homolya et al., 2003; Chin et al., 1993). Le transport de composés conjugués au GSH est de type primaire et implique les glycoprotéines P, les transporteurs ABCB1 de la famille ABC (ATP binding cassette), MDR1 de la famille MDR et les MRPs (Multidrug Resistance associated Proteins).

2.4 Production d'espèces oxygénées réactives (EOR) chez *P. chrysosporium*

Les systèmes de dégradation mis en place par le champignon sont accompagnés de processus oxydatifs. Un certain nombre d'oxydases générant du peroxyde d'hydrogène sont excrétées dans le milieu extracellulaire créant un environnement très oxydant pour le champignon.

2.4.1 Les EOR : molécules à deux facettes

Les EOR ont deux facettes. L'une découle de leur réactivité importante et les rend nocives pour les cellules. L'autre a été mise en place par les cellules, qui vont les utiliser comme molécules de signalisation, tant que leur concentration peut être contrôlée par les systèmes antioxydants.

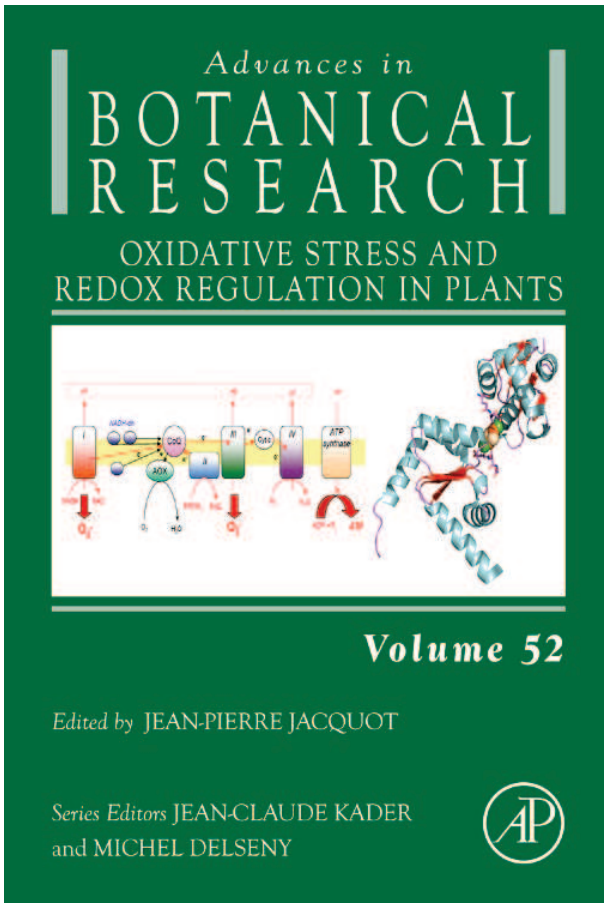
En particulier, chez *P. chrysosporium*, ces EOR sont essentielles au fonctionnement des peroxydases extracellulaires (lignine peroxydases et manganèse peroxydases) responsables de la dégradation de la lignine. Il a également été montré à plusieurs reprises que ces EOR étaient impliquées dans la régulation de la reproduction sexuée, dans les interrelations champignon pathogène/plante, dans le contrôle de la germination et de la prolifération des spores fongiques (Gessler et al., 2007). Les signaux générés par les EOR empruntent les cascades classiques des voies de transduction comme les systèmes de phosphorylation, les protéines G ou encore les MAPK (mitogen activated protein kinase) (Bahn et al., 2007; Banno et al., 2007; Hagiwara et al., 2007). L'efficacité et la rapidité de cette signalisation cellulaire va conditionner l'adaptation des champignons à des changements environnementaux et leur survie en conditions de stress important.

Cependant ces EOR peuvent être nocives pour les cellules vivantes. Elles sont susceptibles d'endommager les macromolécules constituant les cellules vivantes. Ainsi, l'oxydation des bases de l'ADN, et notamment celle de la thymine, peut conduire à des mutations qui sont susceptibles de perturber le fonctionnement cellulaire (Bao et al., 1997). Les membranes cellulaires sont aussi sensibles à l'oxydation à travers des peroxydations en chaîne au sein des couches lipidiques qui peuvent aboutir à une perte de l'intégrité membranaire ou à la rigidification des membranes (Stark, 2005). Enfin les protéines cellulaires peuvent subir différents types d'altération suite à leur réaction avec des ROS. Des dommages peuvent être causés par l'oxydation de différents acides aminés, et la perturbation

des structures tertiaire et quaternaire par une oxydation excessive et non contrôlée, peut aussi causer une perte de l'activité de la protéine et sa dégradation par la cellule (Davies, 2005).

2.4.2 Les systèmes antioxydants

Le champignon a développé des systèmes de résistance pour pouvoir évoluer dans cet environnement très oxydant. Dans la publication ci-dessous nous avons fait le bilan et mis en relation les systèmes antioxydants extracellulaires à l'origine des capacités saprophytes de *P. chrysosporium* et les systèmes intracellulaires dont le champignon dispose pour se protéger des EOR ainsi générées.



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Reactive Oxygen Species in *Phanerochaete chrysosporium*: Relationship Between Extracellular Oxidative and Intracellular Antioxidant Systems

MÉLANIE MOREL,[1] ANDREW A. NGADIN, JEAN-PIERRE JACQUOT AND ERIC GELHAYE

IFR 110 Ecosystèmes Forestiers, Agroressources, Bioprocédés et Alimentation, Unité Mixte de Recherches INRA UHP 1136 Interaction Arbres Microorganismes, Université Nancy I BP 239, 54506 Vandoeuvre-lès-Nancy Cedex, France

[1]Corresponding author: Email: mmorel@scbiol.uhp-nancy.fr

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ABSTRACT

The basidiomycete *Phanerochaete chrysosporium* is a model of ligninolytic fungus which has been studied for a long time. The lignin degradation mediated by this fungus occurs through oxidative processes involving a large set of extracellular enzymes including lignin oxidases and lignin-degrading auxiliary enzymes. In this context, the production of reactive oxygen species (ROS) by this fungus occurs in physiological conditions, that is, during the wood degradation. Ligninolytic basidiomycetes have thus had to develop strategies to protect themselves against oxidative damages induced during lignin oxidation. The excretion of extracellular ligninolytic enzymes is indeed linked at least partially to the fungal intracellular redox state, suggesting a relationship between the intracellular antioxidant system and the production of extracellular ROS by this fungus. This review describes the extracellular systems involved in ROS production, the intracellular systems protecting against ROS, as well as the relationship between them.

I. EXTRACELLULAR REACTIVE OXYGEN SPECIES (ROS) FORMATION

Lignin plays a key role in the carbon cycle as the most abundant aromatic compound in nature, providing the protective matrix surrounding the cellulose microfibrils of plant cell walls. This amorphous and insoluble polymer lacks stereoregularity and, in contrast to cellulose and hemicellulose, it is not susceptible to hydrolytic attack. Lignin is made up of phenylpropanoid units which are linked by a variety of carbon–carbon and carbon–oxygen bonds, making it very difficult to degrade. However, its degradation by certain fungi was recognized and described nearly 125 years ago. Collectively referred to as white-rot fungi (since they degrade brown lignin and leave behind white cellulose), these are the only microbes capable of efficient depolymerization and mineralization of lignin. All are basidiomycetes, a fungal group that includes both edible mushrooms and plant pathogens such as smuts and rust. They secrete an array of peroxidases and oxidases that act non-specifically via the generation of lignin-free radicals, which then undergo spontaneous cleavage reactions. The non-specific nature and exceptional oxidation potential of the enzymes has attracted considerable interest for application in bioprocesses such as organopollutant degradation and fibre bleaching.

The enzymes which are part of the ligninolytic system have been first discovered in *Phanerochaete chrysosporium* (Tien and Kirk, 1983). This system is composed of lignin peroxidases (LiPs), manganese peroxidases (MnPs) and glyoxal oxidase (Glenn *et al.*, 1983; Kersten and Kirk, 1987; Tien and Kirk, 1983). LiPs as well as MnPs represent each 1% of the *Phanerochaete* secretome, and total oxidases represent 11% (Bouws *et al.*, 2008). Additionally, many white fungi, with the notable exception of

P. chrysosporium, produce extracellular laccases, which catalyze the one-electron oxidation of phenols to phenoxy radicals. Several strains produce laccase and MnP but apparently not LiP, suggesting that they degrade lignin by an oxidative mechanism somewhat different from that of *P. chrysosporium* (Gold and Alic, 1993). Such diversity is also observed in the genetic repertoire of ligninolytic enzymes: *P. chrysosporium* possesses over a dozen of different peroxidase genes but no true laccase sequences, while *Coprinopsis cinerea* encodes an abundant set of laccases but only one peroxidase (Kilaru *et al.*, 2006).

A recent study has classified the enzymes potentially involved in lignin catabolism into sequence-based families and integrated them into a newly developed database, designated fungal oxidative lignin enzymes (FOLy) (Levasseur *et al.*, 2008). These ligninolytic families were divided into the categories of lignin oxidases (LO) and lignin-degrading auxiliary (LDA) enzymes according to their direct or indirect action on lignin degradation. Laccases, LiPs, MnPs and versatile oxidases, which represent a third type of oxidases combining catalytic properties of both LiPs and MnPs enzymes, were found in the LO group. Most enzymes classified as LDAs are H_2O_2 producers. Among H_2O_2-generating enzymes, aryl-alcohol oxidase and glyoxal oxidase are thought to be the main enzymes responsible for the production of H_2O_2 (Kersten and Cullen, 2007). This category regroups also vanillyl-alcohol oxidase, pyranose oxidase, galactose oxidase and glucose oxidase (Fig. 1). *P. chrysosporium* exhibits 16 peroxidases from LO group, 3 aryl-alcohol oxidases, 1 glyoxal oxidase, 1 pyranose oxidase and 1 glucose oxidase.

A. LIGNIN OXIDASES

The LiPs are encoded by a family of 10 structurally related genes designed A to J (Gaskell *et al.*, 1994). Even though the repeated pattern of genomic organization indicates that the LiP family probably arose via a series of duplication events, their differential regulation in response to medium composition suggests specific biological role for individual isozymes (Stewart and Cullen, 1999). In *P. chrysosporium*, one of these proteins has been purified to homogeneity by ion-exchange chromatography. The protein contains one protohaem IX per molecule. It catalyzes, non-stereospecifically, several oxidations in the alkyl side chains of lignin-related compounds: Cα–Cβ cleavage in lignin-related compounds of the type aryl-CαHOH–CβH–R–CγH$_2$OH (R = -aryl or -*O*-aryl), oxidation of benzyl alcohols to aldehydes or ketones, intradiol cleavage in phenylglycol structures and hydroxylation of benzylic methylene groups. It also catalyzes oxidative coupling of phenols, perhaps

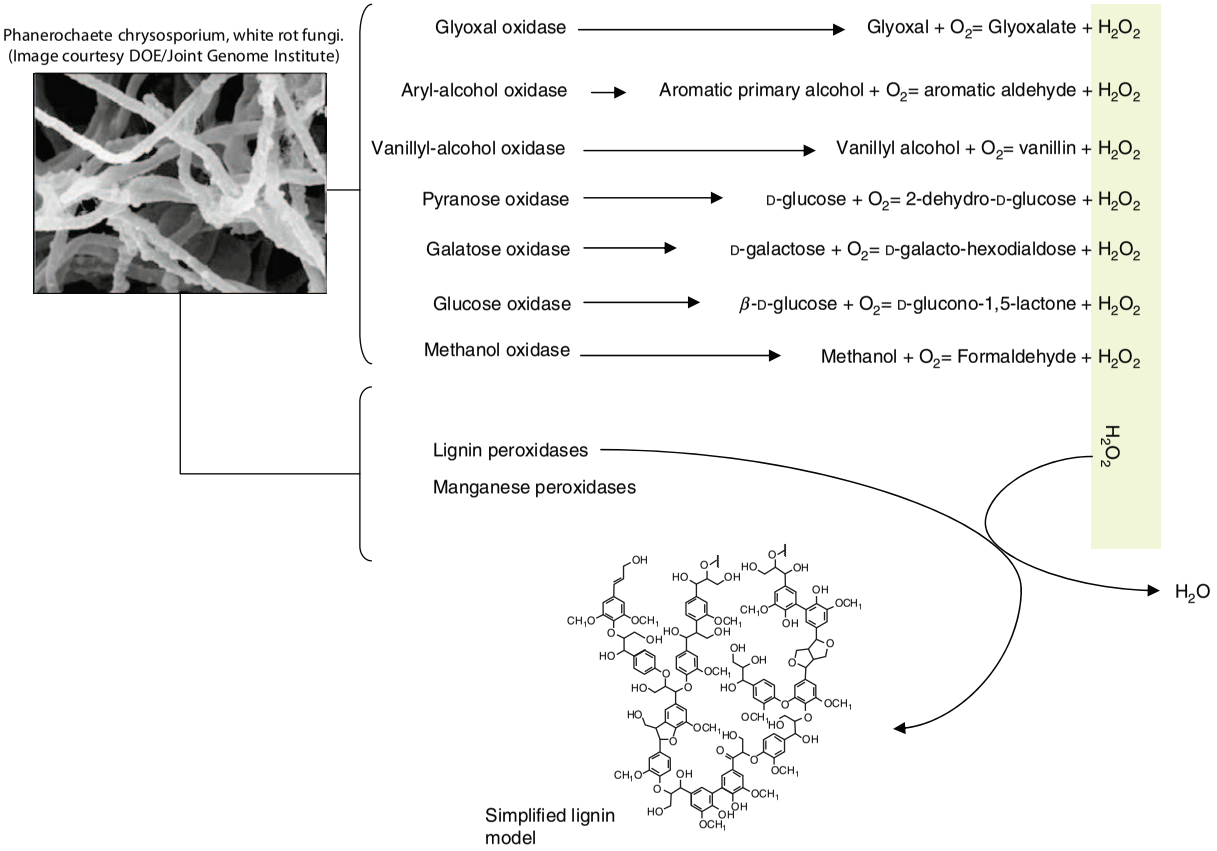

Fig. 1. Extracellular enzymes involved in lignin degradation in *Phanerochaete chrysosporium*. The fungus is able to excrete a large array of oxidases in the extracellular medium. These enzymes generate hydrogen peroxide, which is required for peroxidase activity to cleave lignin linkages.

explaining the long-recognized association between phenol oxidation and lignin degradation. All reactions require H_2O_2. The Cα–Cβ cleavage and the methylene hydroxylation reactions involve substrate oxygenation, the oxygen atom arising from O_2 and not H_2O_2 (Tien and Kirk, 1984). A second lignin peroxidase has been characterized by heterologous production in insect cells (Johnson *et al.*, 1992). The recombinant enzyme was purified to near homogeneity and is capable of oxidizing veratryl alcohol, iodide, and, to a lesser extent, guaiacol.

MnP oxidizes Mn^{2+} to Mn^{3+}, using H_2O_2 as oxidant. In addition to previously characterized MnP genes (*mnp1*, *mnp2*, *mnp3*) (Alic *et al.*, 1997; Orth *et al.*, 1994; Pease *et al.*, 1989; Pribnow *et al.*, 1989), genome analysis revealed two others MnP genes (*mnp4* and *mnp5*). If MnPs do have a major role in lignin degradation, one possibility is that subsequent reactions of Mn^{3+} may generate other oxidants that can cleave non-phenolic structures. For example, Mn^{3+} oxidizes oxalate that chelates it to generate CO_2 and a formate anion radical, which reacts with O_2 to give another molecule of CO_2 and also superoxide ($O_2^{\bullet -}$). At the low pH values in wood undergoing white rot, most of this $O_2^{\bullet -}$ will occur in its protonated form as the perhydroxyl radical ($HOO^{\bullet}$), a strong oxidant that can abstract hydrogen atoms from donors such as unsaturated fatty acids. The result of this chemistry would be lipid peroxidation that generates potentially ligninolytic peroxyl radicals through a radical chain reaction. In agreement with this hypothesis, it has been shown that MnPs catalyze lipid peroxidation in the presence of chelated Mn^{2+} and H_2O_2, that non-phenolic synthetic lignin is cleaved *in vitro* as a result, and that white-rot fungi produce extracellular lipids that could provide the necessary peroxidizable substrates in wood. A role for peroxyl radicals in these reactions is also suggested by data showing that other peroxyl-radical-generating systems are involved in the cleavage of non-phenolic lignin structures (Hammel and Cullen, 2008; Kersten and Cullen, 2007).

The sites of MnP production were localized at different stages of cultivation by an immunolabelling procedure. MnP was mainly concentrated in the chlamydospore-like cells and principally distributed in Golgi-like vesicles located at the periphery of the cytoplasm. The apices of hyphae in the outer layer of the pellets were apparently minor sites of MnP production. Maximal MnP release into the culture supernatant coincided with apparent autolysis of the chlamydospore-like cells (Jimenez-Tobon *et al.*, 2003).

Versatile peroxidases represent a third type of peroxidase, combining catalytic properties of both Lip and MnP enzymes (Heinfling *et al.*, 1998). Other peroxidases were reported in the FOLy database: chloroperoxidases are potential chlorinators of lignin and may thus account for some of the high-molecular-weight organochlorine residues. Cellobiose dehydrogenase plays a

role in carbohydrate metabolism, and some studies also suggest a role in lignin degradation. This haem-containing protein can generate hydroxyl radicals by Fenton-type reactions, thereby oxidizing lignin (Levasseur *et al.*, 2008).

B. LIGNIN-DEGRADING AUXILIARY ENZYMES

Peroxide is required as an oxidant in peroxidative reactions, and several oxidases have been proposed to play a role in this regard. Glyoxal oxidase (GLX) is an extracellular glycoprotein of 68 kDa with two isozymic forms (pI 4.7 and 4.9). It is a copper radical oxidase that catalyzes the oxidation of several simple aldehyde-, α-hydroxycarbonyl- and α-dicarbonyl compounds, coupled to the reduction of O_2 to H_2O_2. During the reaction, GLX is activated by LiP, suggesting a possible extracellular circuit for the control of H_2O_2 production by GLX, and control of Lip activity by H_2O_2 (Kersten and Cullen, 2007; Singh and Chen, 2008). An analysis of the *P. chrysosporium* draft genome had identified six sequences with significant similarity to GLX and designated them *cro1* through *cro6* (Vanden Wymelenberg *et al.*, 2006b). The predicted mature protein sequences diverge substantially from one another, but the residues coordinating copper and constituting the radical redox site are conserved. Transcript profiles, microscopic examination and lignin analysis of inoculated thin wood sections are consistent with differential regulation as decay advances. However, the role of the diverse *cro* genes in lignocellulose degradation is not clearly established.

Fungal glucose oxidase catalyzes the oxidation of D-glucose to β-D-gluconolactone and H_2O_2 in the presence of molecular oxygen. One glucose oxidase was isolated from *P. chrysosporium*. It is a flavoprotein containing two identical polypeptides with a molecular weight of 80 kDa each. The flavin analysis data revealed 1.5 mol of flavin per mol of purified glucose oxidase from *P. chrysosporium* (Kelley and Reddy, 1986). Very few studies have concerned this enzyme and its role remains to be determined. While no evidence supports a role of glucose oxidase in wood degradation, an important role has been proposed for pyranose oxidase (POx) (Daniel *et al.*, 1994; Giffhorn, 2000). POx is preferentially localized in the periplasmic space and the associated membranous materials. The protein has been purified to apparent homogeneity from mycelium extracts of *P. chrysosporium*. It is a homotetrameric flavoprotein with subunits of about 65 kDa, which is not glycosylated compared to POx from other fungi (Artolozaga *et al.*, 1997). A K_m value for O_2 of 0.13 mM has been determined for *P. chrysosporium* POx (Artolozaga *et al.*, 1997).

Structurally related to glucose and pyranose oxidases, three aryl-alcohol oxidases have been identified in *P. chrysosporium* (Varela *et al.*, 2001). The precise role of these enzymes remains uncertain, but they may support a redox

cycle by supplying extracellular peroxide, perhaps coupled to intracellular aryl-alcohol dehydrogenase (Kersten and Cullen, 2007). Among H_2O_2-generating enzymes, aryl-alcohol oxidases and glyoxal oxidase are thought to be the main enzymes responsible for the production of H_2O_2 (Kersten and Cullen, 2007). However, many other oxidases, such as methanol oxidase or vanillyl-alcohol oxidase, could be responsible for providing H_2O_2 for the ligninolytic systems.

Some transcriptomic and proteomic analyses have revealed that MnPs, LiPs and a copper radical oxidase are secreted in the medium when *P. chrysosporium* is cultivated on softwood (Ravalason *et al.*, 2008). In ligninolytic cultures corresponding to C-limited and N-limited conditions, peptides corresponding to LiPs, MnPs and glyoxal oxidase were identified in the culture medium (Vanden Wymelenberg *et al.*, 2006a). Moreover, two MnP coding genes, one LiP coding gene and one glucose-methanol-choline oxidoreductase coding gene, were up-regulated four-fold or greater during the initiation of ligninolytic enzymes production (Minami *et al.*, 2007).

II. INTRACELLULAR ROS FORMATION

The major part of oxygen consumed by aerobic cells is converted into water in mitochondria via a four-electron reduction reaction, catalyzed by cytochrome *c* oxidase (respiratory complex IV). A much smaller part is converted into H_2O_2 as a result of two-electron reduction, catalyzed by a number of enzymes. It should be emphasized that in addition to the most active ensemble of oxidoreductases, known as the respiratory chain, intact mitochondria contain a number of oxidoreductases both in the matrix and in the outer mitochondrial membrane potentially capable of superoxide production. Thus, attributing the generation of hydrogen peroxide solely to the respiratory chain components, something common in the current literature, should be done with caution (Grivennikova and Vinogradov, 2006). Nothing is really known concerning *P. chrysosporium* mitochondrial functioning. Most of the published studies concern mammalian cells; however, it was shown that the structure of the different respiratory complexes is very similar in all forms of life (Joseph-Horne *et al.*, 2001).

A. ROS PRODUCTION IN THE MITOCHONDRIAL INNER MEMBRANE AND MATRIX

In mitochondria, there are several reactions which can potentially produce ROS. The proteins involved contain either a flavin or an iron–sulphur centre with low redox potentials, responsible for the superoxide ion production. It is

assumed that the respiratory chain is the major source of ROS in mitochondria. Indeed, the number of electrons passing through the respiratory chain is much larger than in any other cellular redox system, so large quantities of ROS could be obtained even if a small portion of this electron flow results in formation of $O_2^{-\bullet}$ rather than H_2O (Skulachev, 2006).

Most of the studies have been carried out on mammalian systems and they show that complexes I and III are the components capable of the univalent reduction of oxygen into superoxide (Muller *et al.*, 2004).

Complex I is the entry point for electrons from NADH into the respiratory chain. It is a ca. 1-MDa complex comprising 45 polypeptides in mammalian (Sazanov, 2007). An FMN cofactor accepts electrons from NADH and passes them through a chain of seven iron–sulphur centres to the Coenzyme Q reduction site. The mechanism of $O_2^{-\bullet}$ production by isolated complex I is now reasonably well understood (Murphy, 2009). The isolated complex produces $O_2^{-\bullet}$ from the reaction of O_2 with the fully reduced FMN, and the proportion of the FMN that is fully reduced is set by the NADH/NAD^+ ratio. Consequently, inhibition of the respiratory chain by damage, mutation, ischemia, loss of cytochrome *c* or by the buildup of NADH/NAD^+ ratio will lead to $O_2^{-\bullet}$ formation (Fig. 2A). In contrast, for most situations where mitochondria are respiring normally on NADH-linked substrates and the NADH/NAD^+ ratio is relatively low, only small amounts of $O_2^{-\bullet}$ are produced from complex I. The other mechanism by which complex I produces large amounts of $O_2^{-\bullet}$ is during reverse electron transport. Reverse electron transport occurs for mitochondria when electron supply reduces the CoQ pool, which in the presence of a significant Δp, forces electrons back from $CoQH_2$ into complex I, and can reduce NAD^+ to NADH at the FMN site (Fig. 2B). Although the site of $O_2^{-\bullet}$ production during reverse electron transport is not known, the rate of $O_2^{-\bullet}$ production linked to this electron flow reversal seems to be the highest that occurs in mitochondria (Hurd *et al.*, 2007; Lambert and Brand, 2004a,b).

Complex III is an enzyme complex oxidizing coenzyme Q using cytochrome *c* as an electron acceptor. In mammalian, the monomer is about 240 kDa and comprises 11 polypeptides, three haems, and an FeS centre (Iwata *et al.*, 1998). When supplied with $CoQH_2$ and when the Qi site is inhibited by antimycin, complex III produces large amounts of $O_2^{-\bullet}$ from the reaction of O_2 with an ubisemiquinone bound at the Qo site. This $O_2^{-\bullet}$ is released from complex III to both sides of the inner membrane (Fig. 2C). However, in the absence of antimycin, the Qo site ubisemiquinone is not stabilized and $O_2^{-\bullet}$ production by complex III is low. Although complex III can be induced to produce $O_2^{-\bullet}$ with the inhibitor antimycin, its production in mitochondria under physiological conditions is far lower and is negligible

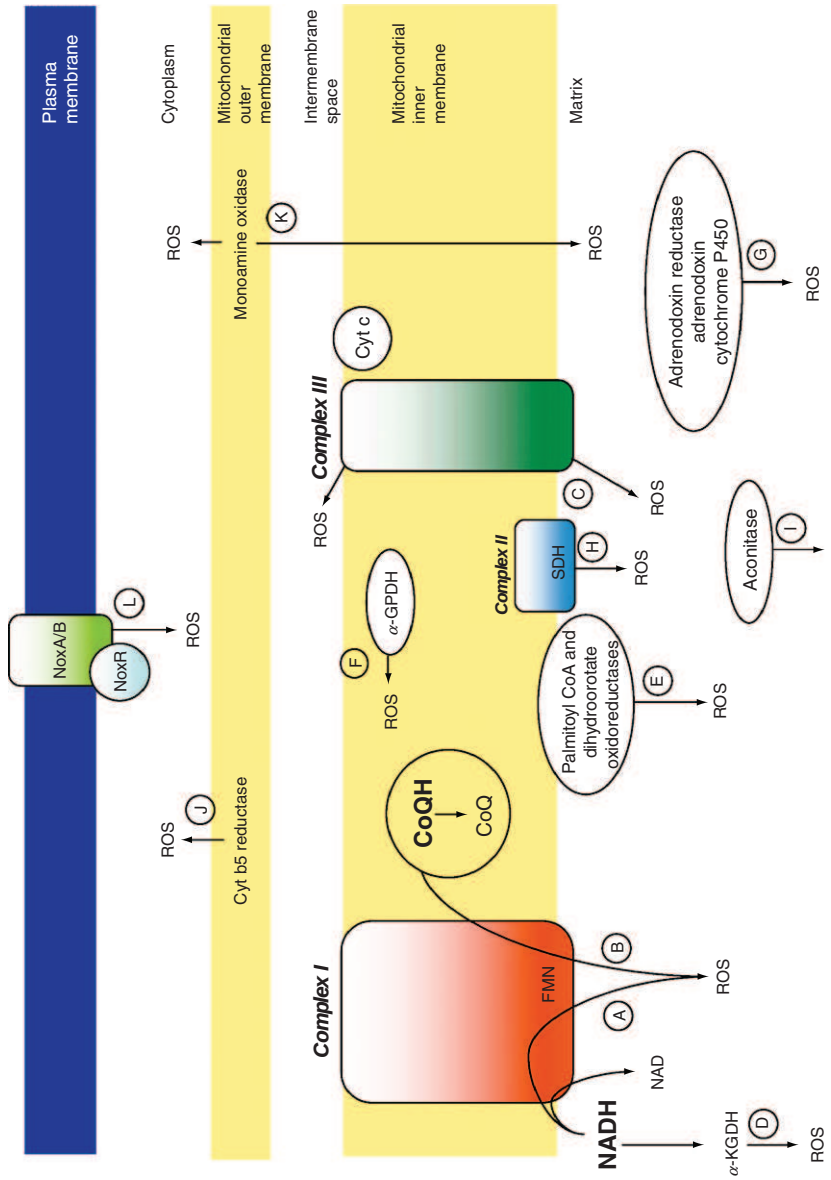

Fig. 2. (Continued)

compared with the maximum rate of $O_2^{-\bullet}$ production from complex I. Moreover, complex I produces superoxide exclusively into the mitochondrial matrix, whereas complex III produces superoxide at both the matrix and cytosolic sides (Muller *et al.*, 2004).

Within mitochondria, other sites of $O_2^{-\bullet}$ production have been listed (Murphy, 2009). Most of these sites have been divided into sites that interact with the matrix NADH pool and those that are connected to the CoQ pool within the inner membrane. For example, the combination of 2-oxoglutarate with a reduced NADH pool may lead to significant ROS production from α-ketoglutarate dehydrogenase (α-KGDH) (Fig. 2D). Indeed, one component of α-KGDH is dihydrolipoamide dehydrogenase, which contains a flavin that can produce ROS when its electron acceptor NAD^+ is limiting (Bunik and Sievers, 2002; Starkov *et al.*, 2004; Tretter and Adam-Vizi, 2004). Some other potential sites interact with the CoQ pool. Oxidation of palmitoyl-CoA or dihydroorotate may produce ROS (Eaton, 2002; Forman and Kennedy, 1976). In particular, in the absence of its natural electron acceptor, CoQ, reduced dihydroorotate dehydrogenase can produce H_2O_2 *in vitro* (Fig. 2E) (Loffler *et al.*, 1996). Moreover, α-glycerophosphate dehydrogenase is a FAD-containing enzyme which takes electrons from α-glycerophosphate to CoQ, and this is associated with ROS production (Fig. 2F) (Tretter *et al.*, 2007). Many other enzymes that can be induced to produce ROS are not connected to the NADH or CoQ pools, such as the adrenodoxin reductase/adrenodoxin/cytochrome P450 system in the matrix that receives electrons from NADPH pool (Fig. 2G) (Hanukoglu, 2006). Succinate dehydrogenase belonging to complex II, when incorporated into liposomes, can produce ROS via its FAD reduced in the absence of electron acceptor (Fig. 2H) (Zhang *et al.*, 1998). Aconitase is located in the mitochondrial matrix. It catalyzes conversion of citrate to isocitrate as part of the tricarboxylic acid cycle. Upon inactivation of the enzyme, due to oxidation of its iron–sulphur cluster by superoxide, production of hydroxyl radical mediated by released Fe^{2+} is induced (Fig. 2I) (Vasquez-Vivar *et al.*, 2000).

Most of the described studies concern mammalian cells; however, it was shown that the structure of the different complexes is very similar in all forms of life (Joseph-Horne *et al.*, 2001). An *in silico* analysis has described, using a

Fig. 2. Reactive oxygen species (ROS) production sites. (A–I) ROS production sites in the mitochondrial inner membrane and matrix. (J, K) ROS production sites in the mitochondrial outer membrane. (L) ROS production by NADPH oxidases (NOX) at the plasma membrane. α-KGDH, α-ketoglutarate dehydrogenase; α-GPDH, α-glycerophosphate dehydrogenase; CoQ, Coenzyme Q; SDH, succinate dehydrogenase; cyt *c*, cytochrome *c*; FMN, flavin mononucleotide.

comparative genomic approach, the oxidative phosphorylation system of many fungi, including *P. chrysosporium* (Lavin *et al.*, 2008). This study demonstrated that *P. chrysosporium* possesses the five complexes of the respiratory chain including complex I, one alternative oxidase (AOX) and three alternative NAD(P)H dehydrogenases. This is not the case for some other fungi. For example, *Saccharomyces cerevisiae, Candida glabrata, Kluyveromyces lactis*, *and Eremothecium gossypii* do not possess complex I or the AOX.

B. ROS PRODUCTION IN THE MITOCHONDRIAL OUTER MEMBRANE

Cytochrome *b*5 reductase oxidizes cytoplasmic NAD(P)H and reduces cytochrome *b*5 in the outer membrane (Fig. 2J). It was reported that mitochondrial cytochrome *b*5 reductase may produce superoxide at a high rate of ca. 300 nmol/min/mg protein (Whatley *et al.*, 1998).

Monoamine oxidase is a flavoprotein ubiquitously expressed in higher eukaryotic organisms, which catalyzes oxidative deamination of biogenic amines. This enzyme contributes to an increase in the steady-state concentrations of reactive species within both the mitochondrial matrix and the cytosol (Fig. 2K) (Cadenas and Davies, 2000).

C. ROS PRODUCTION BY NADPH OXIDASES (NOX) AT THE PLASMA MEMBRANE

Three different subfamilies of NOX are found in the fungal kingdom (Aguirre *et al.*, 2005). Specific isoforms have been shown by genetic analysis to be required for various cellular differentiations, including development of sexual fruiting bodies and ascospore germination (Aguirre *et al.*, 2005; Lara-Ortiz *et al.*, 2003; Malagnac *et al.*, 2004). ROS production catalyzed by NOX has also been proposed to serve for defence against other fungi (Fig. 2L) (Haedens *et al.*, 2005; Silar, 2005). Taken together these findings indicate that ROS production by NOX is a universal signalling system among multicellular organisms. The diffusible nature of superoxide and H_2O_2 makes them ideal second messengers for signalling within the cell, and in the case of H_2O_2, which can cross the cell membrane, inter-cellular signalling. A survey of available fungi genomes revealed considerable variation in NOX gene composition. This gene is completely absent in some species (*S. cerevisiae*, *Schizosaccharomyces pombe*, *Ustilago maydis*, and *Rhizopus oryzae*), but there are up to four copies in *Fusarium solani*, reflecting the diverse morphologies and life cycles of fungal species (Takemoto *et al.*, 2007). Because NOX genes were found in a wide range of fungi from

Chytridomycota to Ascomycota, it is likely that NOX was an ancestral enzymatic function that has been lost during evolution. *P. chrysosporium* possesses the three NOX genes (NOXA, NOXB and NOXR) but not NOXC, which was found in only seven ascomycota fungi (Takemoto *et al.*, 2007).

It should have been implicit that the true source of oxidative stress is not the generation *per se* but spatiotemporal imbalance of ROS production and detoxification. Indeed it is generally accepted that levels of ROS are fine-tuned by the rates of their formation and decomposition by enzymatic and non-enzymatic systems.

III. HOW TO DEAL WITH INTRACELLULAR ROS?

A. SMALL ANTIOXIDANT MOLECULES

1. The glutathione

Glutathione (γ-L-glutamyl-L-cysteinyl-glycine) is synthesized through the activity of two enzymes, the γ-L-glutamyl-L-cysteine ligase (GCL) and the glutathione synthetase (GS). In *P. chrysosporium*, one GCL (PcGCL) and also one GS (PcGS) are predicted from the sequenced genome (Table I). In addition, glutathione has been detected in this fungus (Belinky *et al.*, 2003). Glutathione plays a major role in ROS detoxification, reacting directly with different compounds such as hydrogen peroxide or acting as electron donor to various peroxidases, a point which will be discussed later. In addition, glutathione plays a major role in different detoxification pathways particularly against electrophilic compounds, and also in heavy metal detoxification as a phytochelatin precursor (Noctor and Foyer, 1998; Rouhier *et al.*, 2008). Furthermore, glutathione is involved in post-translational modifications called glutathionylation, this mechanism being involved in different processes such as protein protection against oxidation (Rouhier *et al.*, 2008). These different points will be discussed in the following sections dealing with thiol-dependent systems.

2. Other molecules

L-Ascorbic acid is an important ROS scavenger produced by higher plants (Smirnoff and Wheeler, 2000) and in mammals with few exceptions (Linster and Van Schaftingen, 2007). In fungi, ascorbate is mainly replaced by D-erythroascorbate, a C_5 ascorbate analogue similar in structure and physicochemical properties to ascorbate (Baroja-Mazo *et al.*, 2005). D-Erythroascorbate appears to be the naturally occurring ascorbate analogue in ascomycetes and basidiomycetes, so it is presumed that it may have similar functions to

TABLE I
Proteins involved in the P. chrysosporium antioxidant systems

	Identification number	Localization	Expression studies
Superoxide dismutase			
PcMnSOD1	9267	**M**	Yes
PcMnSOD2	94129	S	Yes
PcMnSOD3	131933	C	No
PcMnSOD4	1323	C	No
PcCu/ZnSOD	128732	S	No
Catalase			
PcCat1	128306	P	Yes
PcCat2	134956	C	Yes
PcCat3	127288	C	Yes
PcCat4	124398	**Mb**	Yes
PcCat5	127266	C	Yes
γ-L-Glutamyl-L cysteine synthase			
PcGCL	133953	C	No
Glutathione synthetase			
PcGS	130608	C	No
Glutathione peroxidase			
PcGpx	130274	C	Yes
Peroxiredoxin			
Pc2-Cys Prx	8807	C	Yes
Pc1-Cys Prx1	10009	**Mb**	Yes
Pc1-Cys Prx2	126313	**Mb**	Yes
PcPrxQ1	6867	C	No
PcPrxQ2	124208	N	No
PcPrxII1	125657	**Mb**	Yes
PcPrxII2	133514	C	No
Thioredoxin			
PcTrx1	7266	C	Yes
PcTrx2	7498	S/**Mb**	Yes
PcTrx3	44586	C	Yes
PcTrx4	122495	M	Yes
NADPH thioredoxin reductase			
PcNTR	8527	C/**M**	Yes
Glutaredoxin			
PcGrx1	10446	N/C	No
PcGrx2	124106	S	No
PcGrx3	127248	M	No
PcGrx4	135692	N	No
Glutathione reductase			
PcGR1	876	M/**C**	Yes
PcGR2	10525	M/C	Yes
PcGR3	135167	C	Yes

(*continues*)

TABLE I (*continued*)

	Identification number	Localization	Expression studies
Methionine sulphoxide reductase			
PcMsrA	122315	M	No
PcMsrB	137333	M	No
Glutathione-*S*-transferase			
PcGTO1	EU791894	M	No
PcGTO2	126388	M	No
PcGTO3	EU791893	C	No
PcGTO4	7168	C	No
PcGTO5	7169	M	No
PcGTO6	3911	C	No
PcGTO7	6880	C	No
PcGTO8	6881	M/C	No
PcURE2p1	503	M	No
PcURE2p2	140156	C	No
PcURE2p3	140271	M	No
PcURE2p4	137250	C	No
PcURE2p5	128511	M	No
PcURE2p6	2269	C	No
PcURE2p7	2266	C	No
PcURE2p8	2268	N	No
PcURE2p9	140259	C	No
PcGTE1	5118	C	No
PcGTE2	5119	C	No
PcGTE3	5122	C	No
PcGTE4	5300	S	No
PcGTE5	7058	M	No
PcGTT2.1	6766	C	No
PcGTT2.2	6683	M/C	No
PcGTT2.2	7971	M	Yes
PcMAK16	137531	N	No
PcEFBγ	39727	C	No
Benzoquinone reductase			
PcBQR1	10307	S/C/M	Yes
PcBQR2	121028	C	Yes
PcBQR3	129887	S/C	Yes
PcBQR4	139901	C	Yes
Alternative dehydrogenase			
PcNDI	3743	M	No
PcNDE1.1	134180	C	No
PcNDE1.2	123031	M	No
PcNDE2	6157	M	No
Alternative oxidase			
PcAOX	40093	M	No

Localizations in bold have been determined according to experimental studies; the others are based on software predictions (psort, target and mitoprot). M, mitochondria; C, cytosol; N, nucleus; S, secreted; Mb, membrane.

L-ascorbate in these organisms. Usually, erythroascorbate in fungi appears to be glycosylated at the C_5 position by a range of sugars including glucose, galactose and xylose. In *P. chrysosporium*, metabolomic experiments have failed to identify both ascorbate and erythroascorbate in this fungus; however, threonate and erythronate were identified as oxygen-stress responsive metabolites (Miura *et al.*, 2004). The significance and the role of this metabolite accumulation during oxidative stress remain unexplained. In the same experiments, intracellular accumulation of veratryl alcohol (VA) has been reported when *P. chrysosporium* was submitted to oxidative stress (Miura *et al.*, 2004). VA is a well-characterized secondary metabolite involved in LiP protection and lignin degradation (Kersten and Cullen, 2007). Again, the significance of this accumulation remains to be explored.

B. ENZYMATIC ROS DETOXIFICATION

Concerning *P. chrysosporium* and more generally basidiomycetes, studies devoted to enzymes involved in ROS detoxification are particularly scarce. By comparison with other fungi and by mining genomic, transcriptomic and proteomic databases, different genes coding for proteins involved in the cellular redox balance could be identified in *P. chrysosporium* (Table I). The predicted proteins are putatively distributed in several subcellular compartments.

1. Superoxide dismutases

Superoxide dismutases (SODs) have an antioxidant function by catalyzing the disproportionation of superoxide anion to hydrogen peroxide. The SOD activity requires the presence of active metal ions in the active site allowing to classify them as Cu/ZnSODs and MnSODs. In *P. chrysosporium*, the presence of at least three MnSOD genes has been reported: MnSOD1 and two additional putative MnSOD genes: MnSOD2, which is located in scaffold 8 (nucleotides 478804 to 479683) and MnSOD3, which is located in scaffold 9 (nucleotides 1861632 to 1862495) (Matityahu *et al.*, 2008). On the other hand, the same authors indicated in a previous study that no Cu/ZnSOD activity or homologous sequence has been detected in this organism (Belinky *et al.*, 2002; Matityahu *et al.*, 2008). Nevertheless, additional sequences encoding putative SODs could be found in *P. chrysosporium* genome: one related to a Cu/ZnSOD located in scaffold 8 (nucleotides 464475 to 465167) and one related to a Mn SOD in scaffold 2 (nucleotides 883075 to 884217) that we named MnSOD4. MnSOD1 has been shown to be localized in mitochondria (Belinky *et al.*, 2002), whereas the Cu/ZnSOD and MnSOD2 are predicted to be secreted. As confirmed by RNAi experiments, the main SOD activity observed in *P. chrysosporium* cultivated in liquid conditions is

due to MnSOD1 (Belinky *et al.*, 2002; Matityahu *et al.*, 2008). The expression of MnSOD1 has been shown to be downregulated at the transcriptional level by the presence of exogenous benzoic acid (Matsuzaki *et al.*, 2008). In addition, MnSOD2 has also been shown to be down-regulated during initiation of ligninolytic enzyme production (Minami *et al.*, 2007). MnSOD1 expression has been particularly studied in Mn-depletion conditions, since these culture conditions induce an oxidative stress in *P. chrysosporium* and also induce lignin peroxidase production (Belinky *et al.*, 2003; Rothschild *et al.*, 1999). This relationship between oxidative stress, MnSOD1, and lignin peroxidase production is discussed in Section IV.

2. Catalases

Catalases reduce hydrogen peroxide using the redox properties of a haem group. It has been shown for long time that *P. chrysosporium* possesses catalases, four different isoforms having been detected (Kwon and Anderson, 2001). At least five encoding genes: PcCat1, PcCat2, PcCat3, PcCat4 and PcCat5 could be found in the sequenced genome (Table I). PcCat2, PcCat3 and PcCat 5 are clearly related together and belong to clade 2 as defined for *Histoplasma capsulatum* catalases (Johnson *et al.*, 2002). These three proteins are predicted to be cytosolic. On the other hand PcCat1 is related to small-subunit catalase protein with greatest similarity to known peroxisomal catalases (Johnson *et al.*, 2002).

Previous cytological studies have shown that a catalase activity is present in the periplasmic space and is up-regulated during ligninolytic metabolism (Forney *et al.*, 1982). More recently, PcCat4 has been shown to be the main isoform associated with the fungal outer membrane. The production of this isoform is strongly induced under ligninolytic conditions (up to 35-fold), suggesting a role in surveillance against extracellular ROS (Shary *et al.*, 2008). Furthermore, this isoform has also been shown to be upregulated after addition of vanillin in the culture medium (Shimizu *et al.*, 2005). Nevertheless, the detection of catalase activity in the whole cells does not seem to be directly correlated with the production of LiPs (Kwon and Anderson, 2001). It is particularly true during induction of LiPs by intracellular oxidative stress due to Mn depletion (Belinky *et al.*, 2003), suggesting that the regulation of intracellular isoforms is not linked to the lignin metabolism.

3. Thiol peroxidases

SODs and catalases are peroxidases which use metal ions to catalyze the reduction of ROS. Cells have also developed other enzymatic systems able to reduce oxidative molecules in the absence of a metal. These proteins possess often cysteinyl residue(s) in their catalytic site, these latter being involved in

the reduction of peroxides to the corresponding alcohols. The catalytic efficiently of such enzymes is lower in comparison to metal-containing enzymes. Depending on the system used to regenerate these cysteine-containing enzymes, two classes of thiol peroxidases can be distinguished: the glutathione peroxidases and peroxiredoxins.

a. Glutathione peroxidases. Even though they are named Glutathione peroxidases (Gpxs), only mammalian Gpxs used glutathione for their regeneration. Their bacterial, fungal, and plant counterparts use thioredoxins as reductants (Rouhier and Jacquot, 2005). In *P. chrysosporium*, only one gene related to Gpx could be found, the corresponding protein (PcGpx) being predicted as cytosolic (Table I) (Morel *et al.*, 2008). This protein is related to the phospholipid hydroperoxide glutathione peroxidase 3 (Gpx3) from yeast and could have a function in the reduction of lipid hydroperoxides. Furthermore, the yeast Gpx3 acts as a sensor of oxidative stress and is involved in the oxidation of the AP1 transcriptional factor (Delaunay *et al.*, 2002). PcGpx has been shown to be slightly up-regulated after addition of exogenous vanillin (Shimizu *et al.*, 2005). Such aromatic compounds induce oxidative stress suggesting a function of this protein in the cellular redox control.

Besides the so-called Gpx, other fungal proteins use glutathione to reduce organic and non-organic peroxides. These proteins are related to glutathione-*S*-transferases (GSTs). In yeast, at least five isoforms are involved in cellular redox control (Herrero *et al.*, 2008): ScGTT1, ScGTT2 and three GST belonging to the omega class. The function(s) of these proteins remain unclear, but ScGTT1 is able to reduce *in vitro* organic hydroperoxides, whereas omega GSTs act as 1-cys thiol transferase (Garcera *et al.*, 2006). In *P. chrysosporium*, at least 27 genes encoding GST-related proteins have been detected (Morel *et al.*, 2009). First attempts have been performed in our laboratory to characterize this superfamily at the protein level. Particularly, PcGTT2, an isoform related to ScGTT2, has been shown to reduce very efficiently cumene hydroperoxides ($k_{cat} = 141\ s^{-1}$) (Morel *et al.*, unpublished data). This efficiency suggests that this isoform could be involved in the reduction of oxidized lipids and also in the membrane repair after lipid peroxidation.

b. Peroxiredoxins. Among the thiol-peroxidase superfamily, four groups of peroxiredoxins (Prxs) are present in fungi based on sequence homology and also on biochemical data : 2-Cys Prxs, 1-Cys Prx, Prx Q and type II Prx (Rouhier and Jacquot, 2005). In *P. chrysosporium*, the distribution of Prxs is the following one: one 2-Cys Prx (Pc2-CysPrx), two 1-Cys Prx (Pc1-CysPrx1 and Pc1-CysPrx2), two Prx Q (PcPrxQ1 and PcPrxQ2) and two type II Prx

(PcPrxII1 and PcPrxII2) (Table I) (Morel *et al.*, 2008). To date, biochemical and physiological data concerning these proteins remain particularly scarce. The 2-Cys peroxiredoxin has been heterologously produced and the resulting purified protein exhibits a typical activity against hydrogen peroxide. Nevertheless, potential physiological reductants (i.e., thioredoxins) have not been tested in this study (Jiang *et al.*, 2005). The 2-Cys Prx has been identified in the cytosolic fraction by proteomics (Shimizu *et al.*, 2005) and shown to be down-regulated in the presence of benzoic acid (Matsuzaki *et al.*, 2008). PcPrxII1, Pc1-CysPrxs and Pc2-CysPrx have been found in *P. chrysosporium* microsomal preparations (Shary *et al.*, 2008). The localization of these proteins in microsomal fraction is in accordance with the hypothesis that peroxiredoxins and more generally thiol peroxidases are involved in ROS sensing and signalling mechanisms linked in particular to lipid peroxidation (Rouhier and Jacquot, 2005). Among the Prxs not identified in this proteomic study, one is predicted nuclear (PcPrx Q2) by protein localization software. It is interesting to note that no Prx is predicted as mitochondrial in *P. chrysosporium* (Morel *et al.*, 2008).

C. REPAIR OF OXIDATIVE PROTEIN DAMAGE

Besides the enzymatic network involved in ROS detoxification, cells possess also enzymatic mechanisms involved in repairing ROS-damaged proteins. The sulphur containing amino acids, cysteine and methionine, are the major targets of oxidation, their level of oxidation being also used by the cells as signalization mechanisms. Two thiol-related systems are mainly involved in the redox regulation/protein repair mechanisms, namely the thioredoxin system (Gelhaye *et al.*, 2005) and the glutathione/glutaredoxin system (Rouhier *et al.*, 2004). In this volume, the papers by Hagglund *et al.*, Nishiyama and Hisabori, Li and Zachgo, Selles *et al.* and Gao *et al.* describe various aspects of the thioredoxin and glutaredoxin systems in plants and cyanobacteria and they are a complement to this study in fungi.

1. The thioredoxin system

Thioredoxins are small proteins, which possess two cysteines in their catalytic site. They catalyze dithiol/disulphide exchange interacting with more or less specific targets as Gpxs or Prxs (Gelhaye *et al.*, 2005), and besides their function in redox signalization/ detoxification, they are involved in various cellular processes (Gelhaye *et al.*, 2005; Herrero *et al.*, 2008). In *P. chrysosporium*, four genes encoding thioredoxins have been identified: PcTrx1, PcTrx2, PcTrx3 and PcTrx4 (Morel *et al.*, 2008). PcTrx4 is predicted to be mitochondrial, whereas PcTrx1 and PcTrx3 are probably

cytosolic (Table I). A putative secretion signal has been detected in the N-terminal part of PcTrx2 (Morel *et al.*, 2008). Interestingly, PcTrx2 has been shown to be present in *P. chrysosporium* microsomal preparations (Shary *et al.*, 2008). Since phylogenetic studies suggest that the presence of PcTrx2 orthologs is restricted to basidiomycetes (Morel *et al.*, 2008), further work will be required to functionally characterize this isoform. PcTrx1 and PcTrx4 have been produced heterologously in our laboratory; the resulting proteins are able to reduce efficiently dithionitrobenzene, a compound classically used to characterize thioredoxins (Gelhaye *et al.*, unpublished data). The regeneration of Trxs is usually catalyzed by NADPH thioredoxin reductase (NTR). In *P. chrysosporium*, only one gene encoding an NTR could be found (PcNTR). Nevertheless, an N-terminal extension recognized as a transit peptide has been predicted suggesting an alternative splicing leading to either a cytosolic or a mitochondrial targeting of the resulting proteins (Morel *et al.*, 2008,). This dual targeting has been previously shown for instance in the case of *Cryptococcus neoformans* NTR or for the *Arabidopsis* protein (Missall and Lodge, 2005; Reichheld *et al.*, 2005). After production of PcNTR in *Escherichia coli*, the resulting flavoprotein was able to reduce PcTrx1 and PcTrx4, confirming that *P. chrysosporium* possesses a functional thioredoxin system (Gelhaye *et al.*, unpublished data). In the presence of aromatic hydrocarbons, which have been shown to increase the mitochondrial production of ROS, PcNTR production was up-regulated at the protein level (Shimizu *et al.*, 2005). In contrast, the induction of the Trx system does not seem to be not correlated with the lignin metabolism, since the expression of PcTrx1 is downregulated at the transcriptional level during the initiation of ligninolytic enzyme production (Minami *et al.*, 2007). Similar results have been obtained in our laboratory when the whole Trx system expression was studied under Mn depletion, conditions which also trigger LiP production (Morel *et al.*, unpublished data).

2. The glutathione/glutaredoxin system

Glutaredoxins are also small proteins involved in various cellular reactions mainly due to the presence of one or two cysteinyl residues in their active site (Rouhier *et al.*, 2008). In *P. chrysosporium*, four genes encoding glutaredoxins have been reported: PcGrx1, PcGrx2, PcGrx3 and PcGrx4 (Table I) (Morel *et al.*, 2008). Based on phylogenetic analysis and sequence comparison with previously characterized proteins, it has been suggested that PcGrx3 should be involved in mitochondrial formation of Fe–S cluster (Morel *et al.*, 2008; Rouhier *et al.*, 2004). Concerning PcGrx4 and PcGrx1, a nuclear localization is predicted for both proteins. Nevertheless, PcGrx1 could be also targeted to the cytosol (Morel *et al.*, 2008). On the other hand, PcGrx2 is

predicted to be secreted. To date, among the different transcriptomic and proteomic studies (Matsuzaki *et al.*, 2008; Minami *et al.*, 2007; Shary *et al.*, 2008; Shimizu *et al.*, 2005), no glutaredoxin has been identified. Grxs could act as thiol oxidoreductases, reducing protein disulphides or glutathione–protein mixed disulphides, with reduced glutathione acting as hydrogen donor. The system includes also NADPH and glutathione reductase (GR). In *P. chrysosporium*, three genes encoding GRs have been found: PcGR1, PcGR2 and PcGR3. PcGR1 and PcGR2 are predicted to be mitochondrial, whereas PcGR3 should be cytosolic (Table I) (Morel *et al.*, 2008). A putative alternative translation initiation site containing the consensus sequence AXXAUG is found in the genes encoding GR 876 and GR 10525 (Morel *et al.*, 2008). The dual targeting has been confirmed for GR 876 which has been found in the cytosolic fraction (Shimizu *et al.*, 2005). Overall, five isoforms (two mitochondrial and three cytosolic) of GR should be present in *P. chrysosporium*, PcGR1, PcGR2 and PcGR3 genes being expressed (Morel *et al.*, 2008). The physiological significance of such high number of isoforms remains unclear and requires further investigations.

3. *Methionine sulphoxide reductases*

Methione sulphoxide reductases (Msr) are enzymes able to reduce methione sulphoxide (MetSO) resulting from methionine oxidation occurring under oxidative conditions (Agbas and Moskovitz, 2009; Tarrago *et al.*, 2009; Vlamis-Gardikas, 2008). Two classes of Msr could be identified, MsrA and MsrB, specific for the *S*- and *R*-diastereomers of MetSO, respectively. In *P. chrysosporium*, one MsrA (PcMsrA) and one MsrB (PcMsrB) have been identified, both proteins being predicted as mitochondrial (Table I) (Morel *et al.*, 2008). No additional data are available about expression and activity of these proteins.

D. PREVENTING ROS FORMATION AND SUBSEQUENT OXIDATIVE DAMAGES

Various compounds are able to initiate redox reactions leading to the production of ROS. Among them, phenolic compounds are known as being able to produce toxic ROS molecules. In fact, these aromatic compounds could be oxidized to *ortho*- and *para*-related benzoquinones. Quinones, beside being potential strong electrophilic molecules (Huyen *et al.*, 2009), also act as catalysts in the generation of ROS. In fact, quinones can undergo one-electron reduction resulting in the generation of semiquinone radicals, which reduce molecular oxygen, producing ROS such as superoxide anion and then leading to redox-cycling reactions. Since white-rot fungi and particularly *P. chrysosporium* are able to completely mineralize lignin, they are

exposed to various quinones resulting in particular from the lignin oxidation. Since benzoquinones are generated by the peroxidase-catalyzed oxidation of lignin and appear to be key intermediates in the degradation of aromatic compounds (Valli and Gold, 1991; Valli *et al.*, 1992), *P. chrysosporium* is also thought to possess extensive systems for preventing ROS formation from quinones. Besides the quinone catabolic pathways (Valli and Gold, 1991; Valli *et al.*, 1992), which will be not discussed here, we will give later an overview of benzoquinone reductases (BQR) and conjugation systems present in *P. chrysosporium.* In addition, alternative pathways preventing ROS production in mitochondria, mainly due to incomplete reduction of ubiquinone or menaquinone by NADH oxidoreductases will be discussed in this section.

1. Benzoquinone reductases

As previously discussed, a wide variety of oxidized metabolic intermediates are generated during the oxidative degradation of lignin, including in particular substituted quinones and hydroquinones. Some quinones are potent redox active compounds generating a redox cycling with their corresponding semiquinone radical, thus producing superoxide anion radicals. To prevent this first one-electron reduction step, the presence of intracellular enzymes involved in the reduction of benzoquinones has been shown in *P. chrysosporium* (Akileswaran *et al.*, 1999; Brock and Gold, 1996; Brock *et al.*, 1995). These enzymes are not able to directly reduce ROS, but they prevent ROS generation. Mining the genome suggests that at least four genes encode 1,4 BQR-related proteins: PcBQR1, PcBQR2, PcBQR3 and PcBQR4 (Table I). PcBQR1 has been previously thoroughly studied and has been shown to be cytosolic (Akileswaran *et al.*, 1999; Brock and Gold, 1996). Nevertheless, an export peptide signal is present in the N-terminal part of the protein suggesting the possibility of alternative splicing (Akileswaran *et al.*, 1999). PcBQR3 is predicted to be also secreted, whereas PcBQR2 and PcBQR4 seem to be cytosolic. The expression of PcBQR1 has been shown to be highly regulated both at the transcriptional and the translational levels induced by the presence of benzoquinone-related compounds (Akileswaran *et al.*, 1999; Shimizu *et al.*, 2005). As other BQRs (Andrade *et al.*, 2007; Lee *et al.*, 2007; Turley and Taliercio, 2008), PcBQR1 is a NADPH-dependent quinone reductase using FMN as cofactor, able to reduce various quinone-containing compounds (Brock *et al.*, 1995). Moreover, quinones can be reduced via other flavoproteins as NTR (Bironaite *et al.*, 1998).

2. Glutathione-S-transferases

Aromatic phenolic compounds, which result from the activation of xenobiotics by various oxidative enzymes as P450 monooxygenases and peroxidases, can be conjugated to biomolecules, that is, sugars via glycosyltransferases or

to the tripeptide glutathione via glutathione *S*-transferases (GSTs). Since nothing is known about glycosyltransferases in *P. chrysosporium*, only an overview of GSTs is given as follows.

The GST main chemistry is to catalyze the conjugation of the tripeptide glutathione with compounds containing an electrophilic centre, in particular on quinone-derived compounds, to form more soluble, non-toxic peptide derivatives, ready to be degraded, excreted, or compartmentalized by phase III enzymes (Hayes *et al.*, 2005). In addition, during oxidative stress, lipid peroxidation leads to the formation of electrophilic compounds as 4-hydroxy-2-nonenal (4-HNE) (Forman *et al.*, 2008). 4-HNE can bind covalently to proteins forming stable adducts and leading to their inactivation. GSTs catalyze Michael addition of GSH to these various substrates and are important in preventing ROS damages.

In *P. chrysosporium*, at least 27 genes encoding related-GSTs have been detected in the genome (Table I) and six classes could be identified. The URE2p, GTT2, EFBγ, MAK16 and Omega classes have been identified in agreement with a previous study on ascomycetes (McGoldrick *et al.*, 2005). A phylogenetic analysis has revealed a new class, enclosing proteins which are related to bacterial LIG proteins and which could also be involved in the degradation of internalized lignin-derived compounds (Masai *et al.*, 2007). *P. chrysosporium* possesses five isoforms of this class that we named GTE (glutathione transferase etherase-related) (Morel *et al.*, 2009). The functions of these different GSTs remain to be elucidated; nevertheless, some isoforms have been shown to be involved in pentachlorophenol catabolism pathway (Valli *et al.*, 1992). The first attempts made in our laboratory to characterize *P. chrysosporium* GSTs have shown that some isoforms (omega class), harbouring a cysteine in their catalytic site, are able to catalyze deglutathionylation reactions cleaving in particular C–S links. Further studies will be required to determine the structural features of such enzymes and the physiological significance of such activities.

3. Alternative pathways in mitochondria

a. Alternative dehydrogenases. In most animals, complex I provides the sole mechanism for entry of electrons from NADH into the respiratory chain. By contrast, in fungi as in plants it seems that alternatives to complex I are widely distributed. Three classes of NADH:ubiquinone oxidoreductases are now recognized. Complex I is assigned to class 1, while alternative dehydrogenases of plant and fungal mitochondria belong to class 2. In class 3, electron transfer is coupled to Na^+ pumping, but this is restricted to bacteria (Joseph-Horne *et al.*, 2001). *S. cerevisiae* lacks complex I. Instead, reducing equivalents are delivered to the respiratory chain via three NADH

dehydrogenases (NADH-dh): NDE1, NDE2 (both external) and NDI1 (internal) (De Vries *et al.*, 1992). The other fungus in which alternative NADH-dh have been extensively studied is *Neurospora crassa* (Joseph-Horne *et al.*, 2001). These enzymes use NAD(P)H from either the cytosol (external enzymes) or the mitochondrial matrix (internal enzymes). They do not pump protons and may be useful as a system that keeps reducing equivalents at physiological levels, but their precise role is not completely clear. The variation in number and specificity among species suggests that they fulfil specific needs of different organisms.

The proton-pumping complex I and the alternative NADH-dh have overlapping roles and both activities are probably required for the optimal functioning of the cells. In *N. crassa*, it seems that complex I and the NDE1 protein are constitutively expressed throughout the fungal life cycle (Melo *et al.*, 2001). The complexity of the enzyme, its involvement in several biological processes, such as fungal and plant development, and different metabolic pathways in microorganisms and, its contribution to energy conservation argue against the possibility of the complete substitution of complex I with alternative enzymes (Archer *et al.*, 1993; Claas *et al.*, 2000; Dupuis *et al.*; 1998, Laval-Favre *et al.*, 1997; Rasmusson *et al.*, 1998; Videira, 1998; Zambrano and Kolter, 1993). For instance, the introduction of the *E. coli NDH-2* gene in *Pseudomonas denitrificans* allowed the disruption of complex I genes in the latter organism (Finel, 1996). Moreover, the *NDI1* gene of *S. cerevisiae* was used to complement complex I defects in mammalian cells (Seo *et al.*, 1998, 1999, 2000).

Four sequences have been identified as putative NADH-dh in *P. chrysosporium*. One sequence (PcNDI) exhibits homology with internal NADH-dh, and three sequences could correspond to external NADH-dh (PcNDE1.1, PcNDE1.2, PcNDE2) (Table I). However nothing is known concerning their regulation.

b. Alternative oxidase. AOX is present in the mitochondria of plants, fungi and many types of yeast. This protein exists in the inner membrane as a homodimer. Two distinct states of the dimer can be identified: an oxidized state in which the dimer is covalently cross-linked by a disulphide bridge and a reduced state which is maintained through non-covalent interactions (Sluse and Jarmuszkiewicz, 1998). The reduced form can be four-to five-fold more active than the oxidized form and the ratio of oxidized to reduced protein varies considerably between species and tissues (Umbach and Siedow, 1993). This enzyme transfers electrons from the ubiquinol pool directly to oxygen without contributing to the proton transfer across the mitochondrial membrane. Electron flow through the alternative pathway bypasses two of the

three sites along the cytochrome chain where electron transport is coupled to ATP synthesis.

AOX is involved in stress responses, programmed cell death, and maintenance of the cellular redox balance. Its activity is controlled by several parameters among which we can distinguish regulatory events and substrate availability. Regulation of AOX activity can occur at different levels: (i) gene expression that affects the amount of the protein in the membrane and differential gene expression that modifies the ratio between isoforms; (ii) post-translational modifications of the protein (i.e. its redox status that affects the nature of the dimer); (iii) the action of allosteric effectors like pyruvate (Sluse and Jarmuszkiewicz, 1998).

Interestingly, stimulation of AOX by AMP and respiratory rates obtained after inhibition of the cytochrome pathway showed that fungal/protist AOX is activated under low-energy conditions, in contrast to plant AOX, which is activated under high-energy conditions (Juarez *et al.*, 2006).

In *Aspergillus fumigatus*, both AOX activity and mRNA expression were induced with menadione or paraquat, suggesting an important role of AOX under oxidative stress (Magnani *et al.*, 2007). Similarly, knock-out AOX mutants are more susceptible both to an imposed *in vitro* oxidative stress condition and to macrophage killing, suggesting that AOX is required for the *A. fumigatus* pathogenicity, mainly for the survival of the fungus conidia during host infection and resistance to ROS generated by macrophages (Magnani *et al.*, 2008). It has been proposed that AOX may serve a more general function by limiting mitochondrial ROS formation (Maxwell *et al.*, 1999).

P. chrysosporium exhibits one sequence coding for AOX (PcAOX). Nothing is known concerning its regulation in *P. chrysosporium*.

IV. RELATIONSHIP BETWEEN INTRACELLULAR ROS AND LIGNIN DEGRADATION

The formation of LiP is particularly dependent on exposure of cultures to high oxygen tensions; indeed, cultures of *P. chrysosporium* which are oxygen starved at the pellet centre produce much lower levels of LiPs and MnPs (Dosoretz *et al.*, 1990). It has been thus proposed that a high partial pressure of oxygen in the culture headspace is needed to make sufficient oxygen available to the submerged hyphae (Michel *et al.*, 1992). Oxygen has, however, the potential to give rise to toxic oxygen-free radicals that are capable of oxidizing, fragmenting, and cross-linking proteins, carbohydrates, lipids, and nucleic acids. Zacchi *et al.* (2000) suggested that cultures of

P. chrysosporium exposed to O_2 to trigger LiP synthesis are subjected to oxygen toxicity, which leads to disorganization of the cellular ultrastructure and chlamydospore development, probably in response to accumulating ROS. This ROS accumulation has been also confirmed by ROS concentration measurements and the enhancement of the antioxidant defence system in *P. chrysosporium* (Belinky *et al.*, 2003). It has been thus proposed that the induction of LiP by oxygen may result from reactions of ROS with the cell surface or from the entry of these species into the cells.

The LiP formation normally induced by high oxygen concentrations can be replaced by manganese deficiency, and this substitution is effective under both nitrogen limitation and excess (Rothschild *et al.*, 1999). The question arising from this result concerns the mechanism by which Mn deficiency can

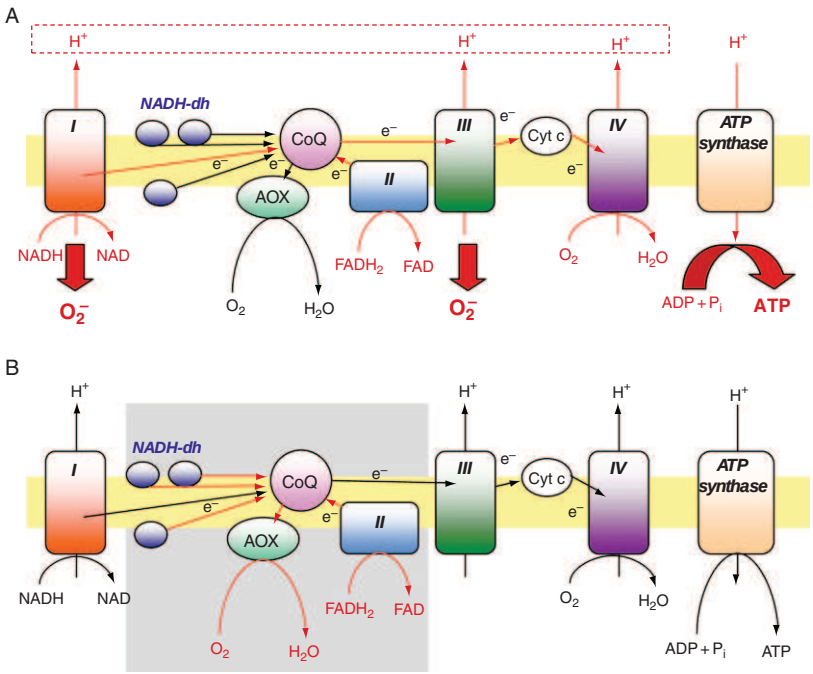

Fig. 3. Regulation of the mitochondrial electron transport chain of *P. chrysosporium* grown in a ligninolytic condition compared to a control condition. (A) When the intracellular oxidative stress is low, all the classical mitochondrial complexes are involved in creating the proton gradient required to produce ATP. Complexes I and III are responsible for most of the superoxide production. To maintain a nontoxic amount of superoxide inside the cell, some detoxifying enzymes function in their degradation. (B) When there is a high oxidative stress, another level of regulation involves alternative mitochondrial pathways to bypass complexes I and III and limit ROS production. Red arrows indicate the preferential pathway in conditions (A) and (B).

replace the high level of oxygen needed for LiP formation. A hypothetical explanation may be that both Mn deficiency and a high level of oxygen can increase the level of oxygen radicals generated within the fungal cell. The fact that no Mn-dependent superoxide dismutase (Mn-SOD) activity was observed when there was a Mn deficiency is consistent with results reported for other non-fungal organisms (Borrello *et al.*, 1992; Hassan and Schrum, 1994). Because of the importance of Mn-SOD and high Mn^{2+} levels as antioxidants, it may be assumed that Mn-deficient cultures of *P. chrysosporium* are more susceptible to oxidative stress than non-deficient cultures. An antioxidant role of LiP has consequently been proposed (Morpeth, 1987), since LiP formation was initiated only after a decrease in the levels of the antioxidant enzymes, catalase and SOD.

Another aspect of LiP induction has been studied. We focused on the bioenergetic state of LiP production in *P. chrysosporium*. Although no significant difference in total respiration has been observed between lignino-lytic and non-ligninolytic conditions, some differences in mitochondrial functioning have been highlighted (Fig. 3) (Morel *et al.*, unpublished data). The participation of complex I is reduced in the ligninolytic conditions and all or almost all the activity is assured by the NADH-dehydrogenases (NADH-dh) (Fig. 3B). Moreover, oxidative activity is rather due to AOX than to complex IV. By reducing the activity of complexes I, III, and IV, the cells reduce the proton gradient required for ATP synthesis, leading to a lower energetic state of the fungus grown in a ligninolytic conditions. This supports the hypothesis that a reduced bioenergetic state of fungal cells could be involved in LiP induction (Morel *et al.*, unpublished data).

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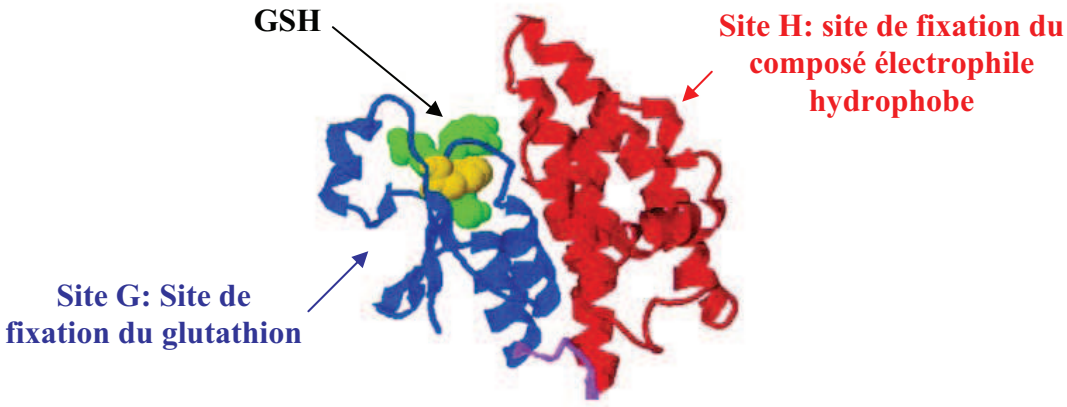
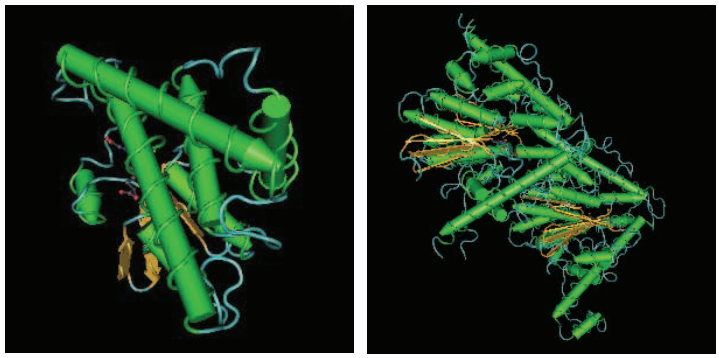
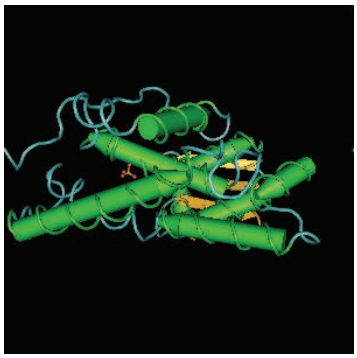


Figure 10 : Structure classique des protéines de la famille des GSTs. Les protéines sont composées de deux domaines, le site G (en bleu) fixant le GSH représenté en vert et jaune, et le site H (en rouge) qui permet la fixation de la molécule xénobiotique.

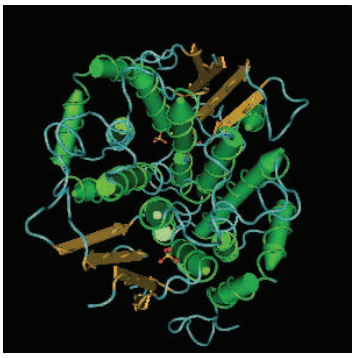


ScGTT2
MMDB ID:
77349

ScUre2p
MMDB ID:
18313



Sc EF1B
MMDB ID:
21878



Coccidioides immitis GST
MMDB ID:
82862

Figure 11 : Structures des GSTs fongiques disponibles dans les bases de données. ScGTT2, ScUre2p et ScEF1b sont des protéines de *Saccharomyces cerevisiae*. La structure d'une autre GST de *Coccidioides immitis* a également été déposée. Les identifiants de ces structures sont reportés (MMDB: Molecular Modeling Database, PDB: Protein Data Bank).

3. Les glutathion S-transférases (GSTs)

Les glutathion S-transférases (GSTs) sont des enzymes de phase II comme décrit précédemment (2.3.2) qui interviennent dans la conjugaison du glutathion (GSH) sur des molécules toxiques, en général hydrophobes et présentant un centre électrophile (carbone, azote ou soufre), pour les rendre plus hydrosolubles et favoriser leur séquestration dans la vacuole ou leur excrétion par des enzymes de phase III (Coleman et al., 1997).

3.1 Structure

Le point commun des enzymes de la superfamille des glutathion transférases est la présence de deux domaines, chacun spécifique d'un substrat (Figure 10). La partie N-terminale appelée site G est impliquée dans la fixation du GSH sur l'enzyme au niveau de résidus conservés (Lys59, Val72, Glu85, Ser86 (numérotation basée sur la séquence de la GST humaine HsGTO1-1) (Vararattanavech et Ketterman, 2007). Le site catalytique à proprement parlé diffère en fonction de la classe de GSTs et contient soit une serine, une tyrosine, une phénylalanine ou une cystéine. La partie C-terminale appelée site H est plus variable et représente le site de fixation des molécules hydrophobes. Cette variabilité reflète le vaste spectre de substrats toxiques ou non que peut prendre en charge ce type d'enzymes. Généralement, les GSTs sont actives sous forme d'homodimères ou d'hétérodimères mais dans tous les cas les sous-unités des GSTs d'une classe ne sont capables de s'associer qu'avec des sous-unités de GSTs de la même classe (Hayes et Pulford, 1995). Peu de structures de GSTs fongiques sont disponibles dans les bases de données. La base de données structurales de NCBI liste les structures de 4 protéines fongiques : 3 GSTs de *Saccharomyces cerevisiae* des classes GTT2 (Ma et al., 2009), EF1γ (Jeppesen et al., 2003) et Ure2p (Bousset et al., 2001) et une GST de *Coccidioides Immitis* (Figure 11). Toutes ces protéines présentent une structure classique des GSTs avec les deux domaines N et C terminal bien distincts. Elles forment toutes des dimères. Pour ScUre2p par exemple, le domaine N-terminal est composé de 4 feuillets beta puis 4 hélices alpha, 2 de chaque côté. Le domaine C-terminal lui, est entièrement composé d'hélices alpha. De manière surprenante, cette protéine possède un site de fixation du GSH alors qu'elle ne présente pas l'activité classique des GSTs, à savoir la conjugaison du GSH sur une molécule xénobiotique. En ce qui concerne ScGTT2, c'est une molécule d'eau stabilisée par des résidus Ser et His qui permet la déprotonation de l'atome de

Tableau 4 : Classification des GSTs microsomales (MAPEG) (Frova, 2006).

Ancienne classification (Jakobsson et al., 2000) (groupes)	Nouvelle classification (classes)	Sous-classes
IV	1	MGST1; PGES1
I	2	MGST2 ; LTC_4S ; FLAP
II	3	MGST3
III	B1	
Synecosystis	B2	

Tableau 5 : Classification des GSTs cytosoliques (Frova, 2006; McGoldrick et al., 2005).

Taxon	**Classes communes**	**Classes spécifiques**
Mammifères	Zeta (Z) Theta (T) Omega (O) Sigma (S)	Alpha (A) Mu (M) Pi (P)
Insectes	Zeta (Z) Theta (T) Omega (O) Sigma (S)	Delta (D)
Plantes	Zeta (Z) Theta (T)	Phi (F) Tau (U) Lambda (L) DHAR
Bactéries	Theta (T) ?	Beta (B)
Champignons	Omega (O)	EF1Bγ MAK16 GTT Ure2p Cluster1 Cluster2

soufre du GSH et non pas des résidus catalytiques classiques comme les Ser, les Tyr ou les Cys.

3.2 Classification

Les GSTs ont été classées en 3 groupes majeurs: les GSTs microsomales, les GSTs mitochondriales et les GSTs cytosoliques qui représentent le groupe le plus important.

3.2.1 GSTs microsomales (MAPEG)

Ces isoformes ont été appelées MAPEG pour « membrane-associated proteins in eicosanoid and glutathione metabolism » (Jakobsson *et al*., 1999). Elles sont présentes dans de nombreux organismes, couvrant toute l'échelle de l'évolution (Bresell et al., 2005). La plupart de ces enzymes sont impliquées dans la synthèse des eicosanoïdes, leukotriènes et prostaglandines (Frova, 2006). En considérant les alignements de séquences primaires, 4 groupes avaient initialement été identifiés, nommés I, II, III et IV (Jakobsson et al., 2000). Six isoformes qui se répartissent à la fois dans les groupes I, II et IV ont été identifiés chez l'homme. Le groupe III est exclusivement bactérien regroupant des séquences d'*Escherichia coli* et *Vibrio cholerea*. Une troisième protéine bactérienne de *Synecosystis* n'a pas de localisation claire et semble être davantage liée au groupe I. Le groupe II est le plus hétérogène incluant à la fois une protéine humaine et des protéines de plantes et de champignons. La répartition hétérogène des protéines humaines au sein de ces différents groupes a suggéré une nouvelle classification basée sur des classes et non plus des groupes (Frova, 2006). Le groupe IV est devenu classe 1, le groupe I est devenu classe 2 et le groupe II est devenu classe 3, les 3 nouvelles classes regroupant les protéines humaines (Tableau 4). Le troisième groupe a été renommé classe B1 (B pour bactérie) et la séquence particulière de *Synecosystis* fait partie de la classe B2. Cette classification a été proposée en considérant un set original de 13 protéines. Depuis, grâce au développement des outils informatiques, un total de 131 séquences ont été identifiées, 52 chez les procaryotes, 79 chez les eucaryotes et un set de 24 séquences qui sont spécifiques des plantes (Bresell et al., 2005).

3.2.2 GSTs mitochondriales

Ces protéines ont initialement été isolées dans la mitochondrie (Harris et al., 1991) mais peuvent également être présentes dans les peroxysomes, mais pas dans le cytoplasme (Jowsey et al., 2003; Morel et al., 2004). Les GSTs mitochondriales sont référencées dans la classe des GSTs Kappa et sont spécifiques des mammifères. La souris, le rat et l'humain possèdent seulement un représentant de ce groupe de GSTs. Elles avaient d'abord été groupées avec d'autres GSTs (de la classe théta) présentant des similarités de poids moléculaire, d'état d'oligomérisation et de séquence N-terminale. Cependant, la structure tridimensionnelle de la protéine de rat a révélé qu'il s'agissait bien d'un groupe à part des autres déjà identifiées (Pemble et al., 1996).

3.2.3 GSTs cytosoliques

Les GSTs cytosoliques ont été classées en fonction de l'identité de leur séquence nucléotidique et protéique, de la structure physique de leurs gènes (nombre d'introns et position) et de leurs propriétés immunoréactives (Pickett et Lu, 1989). Les différentes classes sont répertoriées dans le Tableau 5. Chez les mammifères, 7 classes ont été identifiées et nommées Alpha, Mu, Pi, qui sont spécifiques des mammifères et Sigma, Theta, Zeta et Omega que l'on retrouve également chez d'autres organismes. Les plantes possèdent 6 classes, dont 4 spécifiques des plantes : Lambda, Phi, Tau, DHAR (dehydroascorbate reductase), et 2 également retrouvées chez les insectes et les mammifères : Theta et Zeta. Il existe 5 classes chez les insectes : Sigma, Theta, Zeta, Omega et une seule classe spécifique, Delta. Concernant les bactéries, la classification est moins bien décrite. Il existe une classe spécifique nommée Beta et les autres protéines s'apparentent davantage aux Theta, voire même à d'autres classes. La classification pour les GSTs fongiques a été décrite plus tard. Les séquences fongiques bien connues de *Issatchenka orientalis*, *Saccharomyces cerevisiae* et *Cunninghamella elegans* ne suivent absolument pas la classification classique des GSTs. De nouvelles classes ont alors été désignées : EFIBγ, MAK16, GTT, Ure2p et une classe déjà existante, la classe omega. Cependant, en considérant un plus grand nombre de séquences, McGoldrick et al. (2005) ont montré que cette classification était incomplète et on désigné 2 nouvelles classes qu'ils ont appelées cluster 1 et cluster 2. Les GSTs de champignons étant beaucoup moins étudiées que celles des autres organismes, la classification n'est encore pas définitive et risque d'être modifiée avec l'extension des données génomiques.

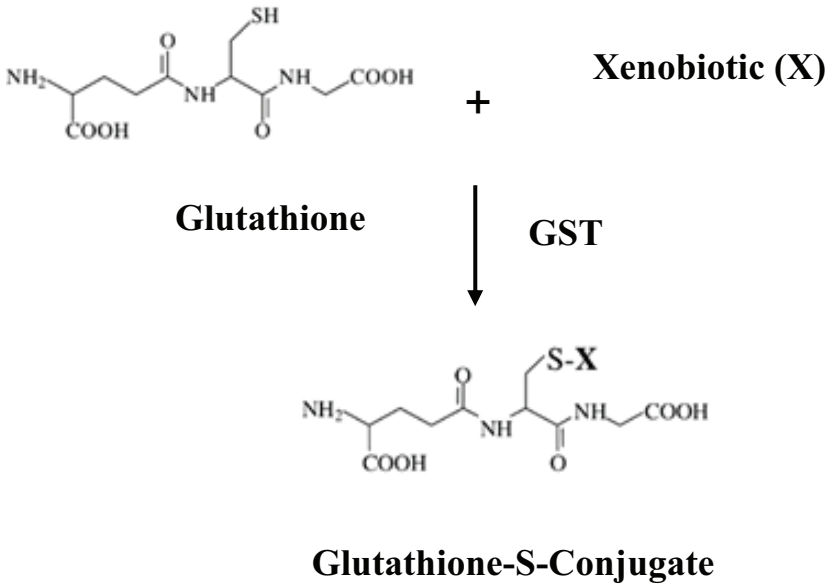


Figure 12 : Activité de conjugaison du GSH sur des molécules xénobiotiques par les GSTs.

3.3 Fonctions

Les GSTs sont des enzymes impliquées dans les mécanismes de défense de la cellule, grâce notamment à leur action coordonnée avec le GSH. Comme leur nom l'indique, leur fonction première et de complexer le GSH sur des molécules toxiques pour favoriser leur élimination (Figure 12). Cependant, le nombre croissant d'études concernant la caractérisation de ces enzymes chez de nombreux organismes a révélé qu'elles pouvaient avoir d'autres propriétés enzymatiques.

3.3.1 Les GSTs de plantes

3.3.1.1 Activité de conjugaison du GSH sur les xénobiotiques

Concernant les plantes, la réaction de conjugaison la plus souvent décrite est la substitution nucléophile d'un halogène au niveau d'un site électrophile sur un cycle aromatique ou un groupement alkyle. Ces réactions de conjugaison ont été mises en évidence pour la détoxification d'insecticides et d'herbicides tels que l'atrazine, le fluorodifène, le pentachloronitrobenzène, le propachlore, l'éthyl de chlorimuron (Lamoureux et Rusness, 1986; Brown, 1990). Généralement la fixation du GSH permet au xénobiotique d'être moins réactif et par conséquent moins toxique pour la cellule. Cependant, dans certains cas, l'effet inverse peut être obtenu. La conjugaison du GSH sur des haloalka(e)nes forme des ions episulfonium ou du formaldéhyde qui sont très instables et plus réactifs que le composé de départ (Eaton et Bammler, 1999). L'élimination de ces conjugués est particulièrement importante pour éviter leur activité génotoxique sur l'ADN (Ishikawa et al., 1994). Une telle activité a été décrite chez le fraisier où deux étapes de conjugaison du GSH sur le fongicide dichlofluanide résultent en la formation de dérivés thiosphosgenes qui sont toxiques pour la cellule (Schuphan et al., 1981).

3.3.1.2 Activité de conjugaison du GSH sur des produits endogènes

Chez les plantes, les GSTs peuvent également avoir une activité sur des substrats endogènes tels que l'acide caftarique, la gibberthione, une forme d'acide gibbérellique contenant du soufre, et des alkenals cytotoxiques liés aux dommages cellulaires engendrés par un stress oxydant comme le 4-hydroxynonenal (Cummins et al., 1997; Gronwald et

Plaisance, 1998). Les anthocyanes ont également été proposées comme substrats endogènes des GSTs puisque la séquestration de ces pigments chez le mais et le pétunia fait intervenir ces enzymes (Alfenito et al., 1998). Cependant, ces formes glutathionylées d'anthocyanines n'ont jamais pu être isolées, ce qui a permis d'énoncer l'hypothèse que les GSTs pourraient plutôt avoir un rôle de « ligand » pour la stabilité des flavonoïdes à l'intérieur de la cellule (Mueller et al., 2000) et leur transport dans la vacuole (Kitamura et al., 2004).

3.3.1.3 Activité « ligandine »

Cette activité non-catalytique de fixation des GSTs sur des composés endogènes comme mentionné ci-dessous concernant les anthocyanes, a été également montrée pour la bilirubine, des hèmes, des stéroïdes, des colorants, des carcinogènes et des drogues (Litwack et al., 1971) mais leur fonction exacte n'a pas encore été déterminée. En intervenant dans le stockage et le transport de ces molécules, les GSTs pourraient limiter les dommages cellulaires causés par des composés cytotoxiques et génotoxiques qui peuvent oxyder les protéines et altérer l'ADN (Axarli et al., 2004).

3.3.1.4 Activité peroxydase

Certaines GSTs de plantes peuvent catalyser des réactions de réduction d'hydroperoxydes cytotoxiques en monohydroxy alcools. En fonctionnant comme des peroxydases, ces enzymes protègent la cellule des effets des espèces oxygénées réactives produites lors d'un stress oxydatif (Marrs, 1996). De par cette activité, elles peuvent également avoir un rôle dans la régulation de l'apoptose (Kampranis et al., 2000) et la signalisation cellulaire en réponse au stress (Loyall et al., 2000).

3.3.1.5 Activité isomérase

La structure tridimensionnelle d'une GST Zeta d'*Arabidopsis thaliana* a révélé que l'enzyme fixait de manière réversible du GSH au niveau d'une double liaison du maleylacetoacetate pour produire du fumarylacetoacetate (Thom et al., 2001).

3.3.1.6 Fixation d'acides gras

Certaines GSTs d'*A. thaliana* ont une très forte affinité pour les thioesters (Dixon et Edwards, 2009). La fixation forte et spécifique de divers acides gras oxygénés sur les GSTs recombinantes à la fois d'*A. thaliana* et de *Nicotiana Benthamiana* suggère que ces protéines pourraient avoir un rôle dans la fixation et la conjugaison *in vivo* de ces métabolites instables. Il a été suggéré qu'elles pourraient alors transporter ces conjugués dans un compartiment approprié grâce à une interaction avec des transporteurs de composés glutathionylés.

3.3.2 Les GSTs humaines

Les GSTs ont été largement étudiées chez l'homme parce qu'elles présentent un intérêt en pharmacologie et toxicologie. En effet ce sont des cibles pour les thérapies anti-asthmatique et antitumorale (Evans et al., 1991; Matsushita et al., 1998; Jakobsson et al., 1999; Ruscoe et al., 2001). Elles peuvent métaboliser des agents chimiothérapiques utilisés dans les traitements contre le cancer, des insecticides, des herbicides et d'autres agents cancérigènes (Hayes et al., 2005). Outre les activités de détoxication cellulaire déjà décrites ci-dessus, les GSTs humaines présentent des activités spécifiques à l'espèce.

3.3.2.1 Dégradation des acides aminés aromatiques

Chez les mammifères, la phénylalanine est dégradée en acétoacétate et acide fumarique. Certaines GSTs ont une activité maleylacétoacétate et catalysent la pénultième étape du catabolisme de la phénylalanine et la tyrosine (Fernández-Canon et al., 1998).

3.3.2.2 Synthèse d'hormones stéroïdiennes et d'eicosanoïdes

La testostérone et la progestérone sont synthétisées à partir d'un métabolite du cholestérol, le 3β-hydroxy-5-pregnene-20-one. Ce métabolite est clivé et oxydé pour former des intermédiaires, ce processus faisant intervenir des GSTs. En particulier, il a été montré qu'une GST, présente seulement dans les tissus steroïdogéniques, possédait une forte activité d'isomérisation des 3-céto-stéroides (Johansson et Mannervik, 2001).

Les GSTs sont également impliquées dans la biosynthèse de métabolites issus de l'acide arachidonique qui ont un intérêt pharmacologique important, les prostaglandines et les

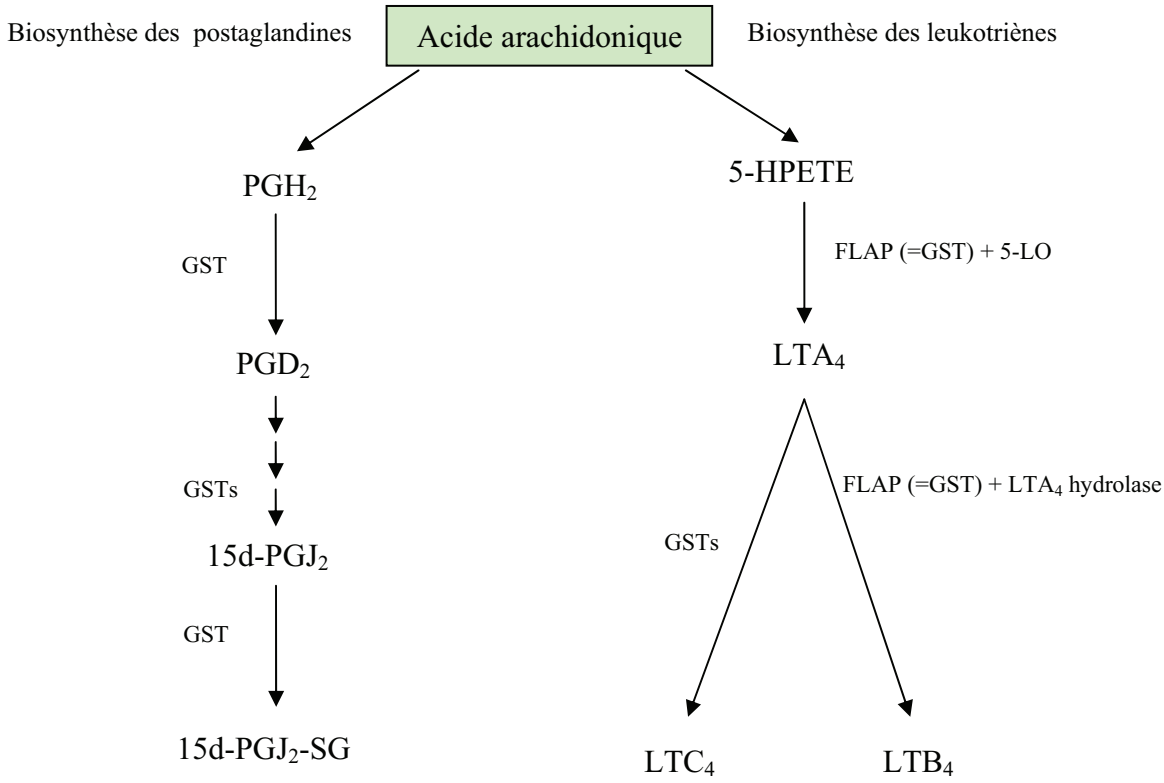


Figure 13 : Rôle des GSTs dans la biosynthèse des prostagalandines et des leukotriènes. PG: prostaglandine, SG: glutathion oxydé; 5-HPETE: acide 5-hydroxyeicosatetraeonique; LT:Leukotriène, FLAP: 5-lipoxigenase activating protein; 5-LO: 5-lipoxigénase (Frova, 2006).

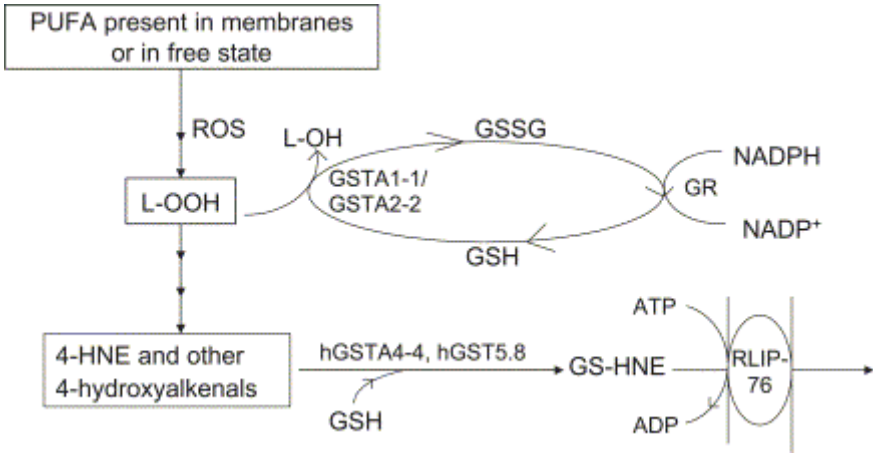


Figure 14 : Rôle des GST dans l'atténuation de la peroxydation lipidique (Awasthi et al., 2004). Les GSTA1-1/A2-2 réduisent ces L-OOH en alcools (L-OH) and limitent la production de HNE. hGSTA4-4 et hGST5.8 conjuguent le HNE avec le GSH formant un complexe qui est excrété des cellules par les transporteurs RLIP76 or RALBP1 (Barrera et al., 1991). PUFA: acides gras polyinsaturés, L-OOH: hydroperoxydes lipidiques, HNE: 4-Hydroxynonenal, GR: glutathion réductase.

leukotriènes (Figure 13). Ces GSTs peuvent avoir une activité prostaglandine D_2 (PGD_2) synthase, prostaglandine $F_{2\alpha}$ ($PGF_{2\alpha}$) synthase, prostaglandine E synthase (PGES1) ou encore être impliquées dans la formation de 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) (Beuckmann et al., 2000; Nakashima et al., 2003). Concernant la synthèse des leukotriènes, plusieurs GSTs ont été identifiées avec des activités leukotriène C4 synthase (LTC4S), 5-lipoxigénase activating protein (FLAP) (Mandal et al., 2004). FLAP est une protéine non enzymatique qui se fixe sur l'acide arachidonique et active la 5-lipoxigénase (5-LO).

3.3.2.3 Signalisation cellulaire

Les médiateurs lipidiques endogènes influencent diverses voies de signalisation, la modulation de leur métabolisme par les GSTs a donc de nombreuses conséquences biologiques. En particulier, la quantité de 15d-PGJ_2 peut réguler l'expression du peroxisome proliferor-activated receptor γ (PPARγ) qui a un rôle dans la différentiation des adipocytes. Elle peut également avoir un effet sur l'expression du facteur nucléaire erythroïde 2 p45-related factor 2 (Nrf2) qui va induire l'expression de gènes liés au système antioxydant (Itoh et al., 2004). De la même manière, la 15d-PGJ_2 peut avoir une action sur le facteur nucléaire κB (NF-κB) (Rossi et al., 2000). Les GSTs, en intervenant à la fois dans la synthèse de la molécule et sa glutathionylation sont directement impliquées dans ces mécanismes de régulation.

Sous l'action d'un stress oxydatif, les acides gras polyinsaturés peuvent générer des hydroperoxydes lipidiques qui vont générer du 4-Hydroxynonenal (HNE). Cet aldéhyde toxique a un rôle important dans la signalisation cellulaire concernant les processus d'apoptose, de prolifération et de différentiation cellulaires (Awasthi et al., 2004). Les GSTs peuvent moduler la concentration intracellulaire de HNE en réduisant les hydroperoxydes grâce à leur activité glutathion peroxydase et en le glutathionylant grâce à leur activité transférase (Figure 14). Le complexe ainsi formé est excrété des cellules par des transporteurs spécifiques de type RLIP76 or RALBP1 (Figure 14) (Barrera et al., 1991). En accord avec ce mécanisme, la surexpression des GSTs humaines hGSTA1-1/A2-2, hGSTA4-4 ou hGST5.8 réduit fortement la quantité de HNE intracellulaire (Awasthi et al., 2004).

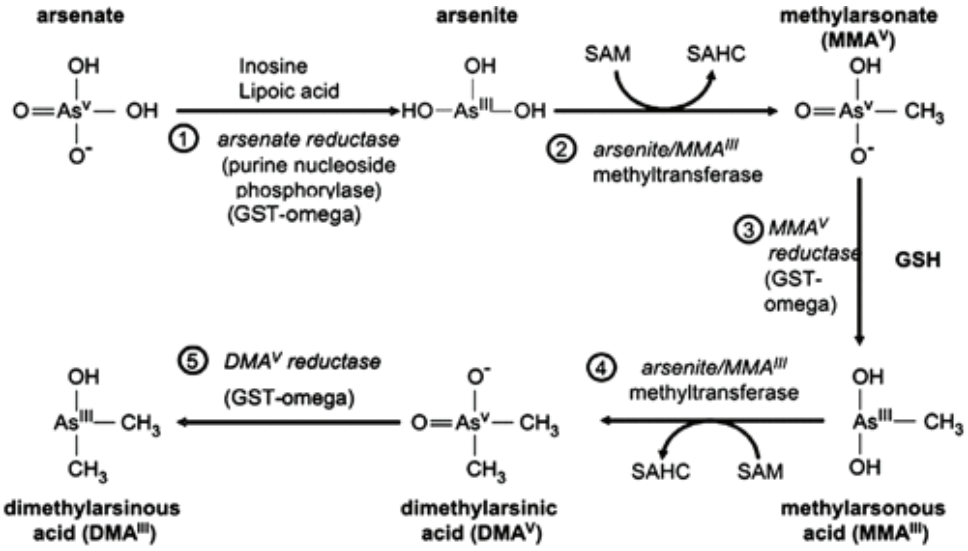


Figure 15 : Biotransformation de l'arsenate (Zakharyan et al., 2005). Cette voie est catalysée par les enzymes arsenate réductase, arsenite méthyltransférase et monométhylarsenate méthyltransférase. La GST humaine hGSTO1-1 intervient dans cette voie en catalysant la réduction du MMA (V) et du DMA (V) (Zakharyan et Aposhian 1999, Aposhian et al., 2004). DMA: dimethylarsenate; GST: glutathione transferase; MMA: monomethylarsenate; GSH: glutathione, SAM: S-adenosyl méthionine.

3.3.2.4 Activité CLIC

Une GST humaine présente une activité similaire aux protéines CLIC (chloride ion channels) (Dulhunty et al., 2001). La famille des protéines CLIC est constituées de protéines assez inhabituelles puisqu'elles existent à la fois sous forme soluble et membranaire. Ces protéines présentent le repliement caractéristique de la famille des GSTs (Harrop et al., 2001). Deux isoformes ont été identifiées chez l'humain, CLIC1 et CLIC2. CLIC1 ne présente aucune activité catalytique alors que CLIC2 possède une activité GSH peroxydase et est capable de moduler l'activité des RyR (ryanodine receptor) qui sont des canaux calciques dans le réticulum endoplasmique de nombreuses cellules (Board et al., 2004). Il a donc été suggéré que cette GST humaine pouvait jouer un rôle dans la régulation de la concentration intracellulaire de calcium, protégeant ainsi la cellule contre des dommages dus aux radiations et contre l'apoptose induite par la mobilisation du calcium issu des réserves intracellulaires (Dulhunty et al., 2001).

3.3.2.5 Séquestration du complexe dinitrosyl-fer

Les complexes dinitrosyl-fer se forment lorsque les cellules sont exposées à de l'oxyde nitrique. Ils peuvent engendrer des dommages cellulaires importants puisqu'ils se fixent sur les protéines et inhibent ainsi leur activité. Certaines GSTs, en séquestrant ce complexe peuvent protéger l'inhibition de certaines enzymes. Cela a été démontré pour la glutathion reductase (GR) par exemple (Pedersen et al., 2007). Les auteurs ont montré qu'une GST fixait le complexe avec une affinité extrêmement forte (K_D= 10^{-10} M) et que l'activité de la GR était maintenue en présence d'oxyde nitrique jusqu'à des concentrations qui correspondent à la limite de capacité de fixation du complexe par la GST.

3.3.2.6 Réduction de l'arsenate

Deux GSTs humaines de la classe oméga présentent des activités arsenate reductase, monométhyl arsenate réductase (V) et diméthyl arsenate réductase (Zakharyan et Aposhian, 1999; Aposhian et al., 2004). L'intervention de ces enzymes dans ces mécanismes de réduction est présentée en Figure 15.

3.3.3 Les GSTs bactériennes

Les GSTs bactériennes sont spécialisées dans de nombreux processus de détoxification d'agents antimicrobiaux et comme pour les autres organismes, elles ont un rôle dans la protection cellulaire lors d'un stress oxydant. Il a été montré que certaines d'entre elles étaient induites au niveau transcriptionnel en présence de peroxyde d'hydrogène (Allocati et al., 2003) et d'autres voyaient leur quantité de protéines augmenter en présence de composés phénoliques (Favaloro et al., 2000). Ces derniers résultats ont d'ailleurs été appuyés par la présence de la protéine en grande quantité dans l'espace périplasmique lorsque la bactérie a été exposée à du 4-chlorophénol (Tamburro et al., 2004).

3.3.3.1 Activité thiol transférase

Certaines isoformes catalysent des réactions « glutaredoxine-like » encore qualifiées de thiol transférase avec des substrats tels que le cystéine S-sulfate et l'hydroxyéthyl disulfide (Caccuri et al., 2002). La cystéine présente dans le domaine de fixation du glutathion est essentielle à ce type d'activité.

3.3.3.2 Activité de déhalogénation

Les composés halogénés sont couramment utilisés en industrie et constituent un groupe important de polluants. Les déhalogénases microbiennes jouent un rôle clé dans la biodégradation de plusieurs xénobiotiques chlorés qu'ils soient aliphatiques ou aromatiques (Copley, 1998; Janssen et al., 2005). Certaines GSTs bactériennes présentent de fortes homologies avec ces enzymes et sont capables de catalyser les mêmes réactions en utilisant le GSH comme cofacteur. Par exemple, les GSTs de la classe phi sont proches des dichlorométhane déhalogénases, présentant une sérine catalytique conservée dans leurs séquences (Vuilleumier et Leisinger, 1996). Elles peuvent également avoir une activité tétrachlorohydroquinone (TCHQ) déhalogénase en catalysant l'attaque nucléophile du GSH sur le TCHQ et la réduction des conjugués ainsi formés, grâce à un résidu cystéine conservé (McCarthy et al., 1996).

3.3.3.3 Dégradation de molécules aromatiques complexes

Certaines GST bactériennes sont impliquées dans le métabolisme de base et plus précisément la mobilisation du carbone, en catalysant des réactions dégradation de composés aromatiques complexes. En particulier, les protéines LigE, LigG et LigF identifiées chez *Shingomonas paucimobilis* sont des GSTs capables de cliver des liaisons β-aryl ethers (Masai et al., 1993). Cette activité est une étape fondamentale de la dégradation de la lignine sachant que ces liaisons sont les plus abondantes au sein de la molécule. LigE et LigF clivent les liaisons β-aryl ethers de manière énantiomère-sélective en consommant du GSH alors que LigG est une GSH lyase qui permet de déglutathionyler le conjugué formé par LigF (Masai et al., 1999; Masai et al., 2003).

Les GSTs bactériennes peuvent également dégrader des composés aromatiques monocycliques tels que le toluène, les xylènes, les phénols et l'atrazine (Santos et al., 2002) ainsi que des hydrocarbures aromatiques polycycliques (HAPs), des composés chimiques très toxiques à la fois pour l'homme et l'environnement (Hofer et al., 1994; Kanaly et Harayama, 2000).

3.3.3.4 Dégradation d'antibiotiques

Cette propriété semble être spécifique aux GSTs bactériennes. Il a été montré qu'une GST de *Proteus mirabilis* pouvait interagir avec des antibiotiques. En effet, l'efficacité antimicrobienne de plusieurs antibiotiques est diminuée en présence de la protéine recombinante PmGST dans le milieu. De plus, cette protéine, dont la quantité augmente lorsque la bactérie est cultivée en présence d'antibiotiques, lui confère une résistance significative contre ces composés (Perito et al., 1996; Allocati et al., 2003).

Une autre protéine nommée FosA et présente chez plusieurs souches bactériennes est impliquée dans la modification enzymatique d'un antibiotique à large spectre efficace contre les bactéries Gram positives et négatives: la fosfomycin (Mendoza et al., 1980). Cette protéine a été qualifiée de GST puisqu'elle est capable de fixer du GSH sur le cycle oxirane de l'antibiotique et ainsi l'inactiver. Cependant, elle présente des propriétés différentes des GSTs classique puisque l'enzyme recombinante ne se fixe pas sur des matrices d'affinité couplée au GSH et elle ne catalyse pas la réaction classique de conjugaison du GSH sur

le substrat classique utilisé pour mesurer l'activité GDH transférase, le 1-chloro 2,4 dinitrobenzene (CDNB) (Arca et al., 1988).

3.3.4 Les GSTs fongiques

3.3.4.1 Détoxication et lutte contre le stress oxydant

La plupart des données disponibles sur les fonctions des GSTs fongiques concernent les ascomycètes et en particulier les levures. Trois GSTs ont été étudiées chez *Schizosaccharomyces pombe* (Veal et al., 2002). Les 3 protéines ont une activité de conjugaison du GSH sur le CDNB et une d'entre elles possède une activité GSH peroxydase. Le fait que les 3 gènes correspondants soient surexprimés en présence de peroxyde d'hydrogène et soient régulés par la protéine kinase STy1 activée en condition de stress, suggère un rôle de ces GSTs dans la réponse au stress oxydant. Ce rôle a également été montré pour *Aspergillus nidulans* (Burns et al., 2005) et *S. cerevisiae* qui possède des GSTs capables de réaliser des activités thiol transférases à la manière des glutarédoxines (Garcera et al., 2006). D'autres pourraient avoir un rôle dans l'élimination des métabolites toxiques qui s'accumulent pendant la phase stationnaire de croissance (Choi et al., 1998). Chez *Glomus intraradices*, un champignon endomycorhizien, l'expression de gènes codant pour des GSTs est fortement augmentée en présence de cadmium, zinc et cuivre (Waschke et al., 2006), probablement en réponse à la production d'espèces oxygénées réactives produites en présence de ces métaux lourds.

3.3.4.2 Activités spécifiques des GSTs fongiques

Certaines GSTs fongiques présentant l'organisation et la structure classique de cette famille d'enzymes ont cependant été qualifiées d'orphelines puisque leur activité ne correspond pas aux activités déjà décrites concernant les GSTs d'autres organismes. En particulier, ces enzymes ont un rôle de facteur de transcription impliqué dans la régulation du métabolisme azoté ou un rôle de facteur d'élongation (Oakley, 2005). La relation entre la structure caractéristique des GSTs de ces protéines et leur rôle dans la cellule est inexpliquée. Une hypothèse consiste à penser que la nature a utilisé les propriétés structurales des GSTs pour la formation de dimères nécessaires à cette activité d'élongation qui serait alors un vestige évolutif (Oakley, 2005). De la même manière, une autre protéine identifiée comme

étant une GST, est impliquée dans la progression du cycle cellulaire et la biogénèse des sous unités ribosomales 60S (McGoldrick et al., 2005).

Objectifs

L'objectif global de cette thèse était d'améliorer les connaissances concernant les GSTs fongiques. En effet, au début de ce travail, très peu de données étaient disponibles dans la littérature. De plus, les études réalisées portaient essentiellement sur des ascomycètes et en particulier des levures. *P. chrysosporium* représente un bon modèle pour cette étude. En effet, c'est un basidiomycète saprophyte connu pour ses propriétés ligninolytiques et de dégradation de xénobiotiques. Ceci sous-entend qu'il possède des systèmes de dégradation et des systèmes antioxydants efficaces lui permettant de résister à un environnement très oxydant. L'intérêt des GSTs est alors double chez ce champignon car ces enzymes peuvent intervenir dans ces deux processus.

RESULTATS

RESUME DES RESULTATS

The overall goal of this work concerned the characterization of *P. chrysosporium* GSTs belonging to two different classes: omega and ure2p. Three papers have been written from the obtained results, two of them being published, whereas the third is a project of publication still awaiting complementary data (see last section).

The first article was accepted in Cellular and Molecular Life Sciences Journal. It highlights the fungal GSTs classification using a genomic and phylogenetic analysis from various sequenced fungal genomes. It is entitled: **The fungal glutathione S-transferases system. Evidence of new classes in the wood-degrading basidiomycete *Phanerochaete chrysosporium*. Cell and Molecular Life Sciences. 66 (23): 3711-3725. Mélanie Morel, Andrew A. Ngadin, Michel Droux, Jean-Pierre Jacquot and Eric Gelhaye.** In this paper, the high diversity of fungal GSTs has been studied. The phylogenetic study also includes other fungi with different ways of life (pathogen and symbiotic), demonstrating the extension of particular GST classes in saprophytic fungi. In addition, a new class of etherase-related GSTs has been detected in fungi.

The second paper deals with the biochemical and structural characterization of omega GSTs in *P. chrysosporium*. The investigation showed that these enzymes are involved in deglutathionylation, this catalysis being due to the presence of a cysteinyl residue in the catalytic site. The paper is entitled "**Glutathione transferases of *Phanerochaete chrysosporium*: A S-glutathionyl-P-hydroquinone reductase belongs to a new structural class. Edgar Meux, Pascalita Prosper, Andrew A. Ngadin, Claude Didierjean, Mélanie Morel, Stéphane Dumarçay, Tiphaine Lamant, Jean-Pierre Jacquot, Frederique Favier, Eric Gelhaye",** and is published in Journal of Biological Chemistry.

The third part concerns isoforms belonging to the ure2p class, focusing particularly on the putative involvement of these enzymes in polycyclic aromatic hydrocarbons catabolism. The paper, still in preparation, would be entitled: "**Specificities of Ure2p-like glutathione transferases from *Phanerochaete chrysosporium*. Andrew A. Ngadin, Cécile Caupert, Patrick Billard, Jean-Pierre Jacquot, Eric Gelhaye and Mélanie Morel.**" and describes that two members of this class (PcUre2p4 and PcUre2p6) could be involved in PAH degradation, these results being mainly based on gene expression analysis.

Article II

The fungal glutathione S-transferases system. Evidence of new classes in the wood-degrading basidiomycete *Phanerochaete chrysosporium*

Introduction

The glutathione S-transferases (GSTs) is a multigenic family, probably correlated with the diversity of their putative functions dealing with signaling, cell defense and foreign or xenobiotic compounds degradation. These enzymes exhibit two binding sites called G-site and H-site. The G-site is located in the N-terminal part of the protein and is involved in the interactions with GSH. It is relatively well conserved and contains essential aminoacyl residues involved in the catalysis, the nature of these residus varying depending of the considered GST class (Ser, Tyr, Phe, or Cys) (Vararattanavech and Ketterman, 2007). The H-site found in the C-terminal part of the proteins is less conserved and is usually involved in the interaction of the proteins with hydrophobic substrates. In comparison with other organisms, funal GSTs are poorly characterized. The aim of this study was to investigate the diversity and evolution of this multigenic family in fungi.

Results

A phylogenetic analysis is reported in this article, showing the high diversity of fungal GSTs. This study includes Ascomycetes, Zygomycetes and Basidiomycetes, allowing the identification of six different classes. Those include GTT1, GTT2, URE2p, Omega, EFBγ, MAK16 and GTE. The saprophytic fungi exhibit more GST-coding sequences than other fungi and this is mainly due to an extension of the omega, GTT, Ure2p, and GTE classes. In addition, proteins from Ure2p, Omega and GTE classes are highly represented in *P. chrysosporium*. Two subclasses of the Ure2p-like have been identified. The first subclass contains the well-characterized Ure2p from *Saccharomyces cerevisiae* which has been shown to be involved in nitrogen catabolite repression. On the other hand, the second subclass contains an isoform from *Aspergillus nidulans* which is involved in heavy metal and xenobiotic resistance. In omega class, three subclasses have been identified related to plant tau and lambda GSTs and to human GTOs. HsGTO1-1 has been previously studied in details and is able to remove glutathione adducts from different substrates, this deglutathionylation activity being dependant of a cysteinyl residue in the G-site. The GTE class is related to bacterial Lig proteins which are involved in *β*-etherase activity. These GTE-like are of great interest since they could play a role in the organic matter degradation particularly in white rot-fungi.

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Cellular and Molecular Life Sciences

REVIEW

The fungal glutathione S-transferase system. Evidence of new classes in the wood-degrading basidiomycete *Phanerochaete chrysosporium*

Mélanie Morel · Andrew A. Ngadin · Michel Droux · Jean-Pierre Jacquot · Eric Gelhaye

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Abstract The recent release of several basidiomycete genome sequences allows an improvement of the classification of fungal glutathione S-transferases (GSTs). GSTs are well-known detoxification enzymes which can catalyze the conjugation of glutathione to non-polar compounds that contain an electrophilic carbon, nitrogen, or sulfur atom. Following this mechanism, they are able to metabolize drugs, pesticides, and many other xenobiotics and peroxides. A genomic and phylogenetic analysis of GST classes in various sequenced fungi—zygomycetes, ascomycetes, and basidiomycetes—revealed some particularities in GST distribution, in comparison with previous analyses with ascomycetes only. By focusing essentially on the wood-degrading basidiomycete *Phanerochaete chrysosporium*, this analysis highlighted a new fungal GST class named GTE, which is related to bacterial etherases, and two new subclasses of the omega class GSTs. Moreover, our phylogenetic analysis suggests a relationship between the saprophytic behavior of some fungi and the number and distribution of some GST isoforms within specific classes.

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M. Morel (✉) · A. A. Ngadin · J.-P. Jacquot · E. Gelhaye
IFR 110 Ecosystèmes Forestiers, Agroressources, Bioprocédés et Alimentation, Unité Mixte de Recherches INRA UHP 1136 Interaction Arbres Microorganismes, Université Nancy I BP 239, 54506 Vandoeuvre-lès-Nancy Cedex, France
e-mail: mmorel@scbiol.uhp-nancy.fr

M. Droux
Laboratoire de Génomique Fonctionnelle des Champignons Pathogènes des Plantes, Unité Mixte de Recherches 5240, CNRS-UCB-INSA-Bayer CropScience Microbiologie, Adaptation et Pathogénie, Bayer CropScience, 69263 Lyon cedex 9, France

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Introduction

Glutathione S-transferases (GSTs; EC 2.5.1.18) constitute a complex and widespread enzyme superfamily that has been subdivided into an ever-increasing number of classes based on a variety of criteria, including amino acid/nucleotide sequence comparisons, and immunological, kinetic, and structural properties. Four main subfamilies are generally recognized: the cytosolic GSTs, the microsomal GSTs (MAPEG), the mitochondrial (also known as kappa class GST), and the bacterial fosfomycin-resistance GSTs [1, 2]. A common feature of this superfamily is the ability of these proteins to bind a broad range of ligands, and particularly hydrophobic ligands, explaining their potential role as class II biotransformation enzymes that function in the detoxification of xenobiotics and endogenous toxicants [2, 3]. In plants and animals, GSTs are the principal phase II enzymes involved in metabolic detoxification processes. Their main chemistry is to catalyze the conjugation of the tripeptide glutathione (GSH) with compounds containing an electrophilic center (carbon, nitrogen, or sulfur) to form more soluble, non-toxic peptide derivatives, ready to be excreted or compartmentalized by phase III enzymes [4]. A recent remarkable study has highlighted the capacity of the tau class GSTs to bind glutathionylated lipids with high specificity and affinity and also to catalyze the linkage of the glutathione moiety to the acyl group [5].

Some GSTs, however, possess functions which overlap with those of thiol-dependent peroxidases (peroxiredoxins and glutathione peroxidases), reducing peroxides and other

products resulting from oxidative stress. In human, a GST has been recognized as one of the predominant enzymes responsible for the metabolism of both 4-hydroxy-2-nonenal (HNE) enantiomers [6]. HNE is a toxic aldehyde generated upon lipid peroxidation, and its GST-dependent conjugation with GSH influences many signal transduction pathways and modulates the activity of transcription factors [2]. Other GSTs can function in prostaglandin and steroid synthesis, or degradation of aromatic amino acids [2], and many of them have been considered as biomarkers of several human diseases, mainly cancer [7–9]. A rat liver membrane-bound microsomal GST contributes to transition pore opening controlling the mitochondrial permeability [10]. In bacteria, GSTs could display various functions such as protection against antibiotics [11] or lignin degradation by cleaving β-aryl ether linkages [12]. They could also be potential useful markers for polycyclic aromatic hydrocarbon (PAH) pollution [13]. In *Saccharomyces cerevisiae*, the role of ScGTO1 in the peroxisomes could be related to the redox regulation of the Str3 cystathionine β-lyase protein [14].

Cytosolic GSTs are soluble dimeric proteins with a relatively conserved N-terminal thioredoxin-like domain bearing a $\beta\alpha\beta\alpha\beta\beta\alpha$ topology that is responsible for GSH binding and a more variable C-terminal domain. This canonical GST fold is observed extensively in nature, being sometimes associated with biological functions unlinked to GSTs. This is the case for instance of the bacterial stringent starvation protein A (SspA) or the intracellular chloride ion channel (CLIC1) [15].

A common feature in the GST superfamily is the presence of two binding sites for each of the substrates, a G-site for GSH and an H-site for the hydrophobic electrophile. Generally, GSTs are functionally active as homodimers or heterodimers, but in any case GST subunits are able to interact only with subunits of the same class [16]. Usually, within the classes, the N-terminal part of the proteins is the most conserved since it encloses an important part of the active site, namely the glutathione binding area, the G-site [17]. On the other hand, the H-site is found primarily in the C-terminal domain and its structure varies among GSTs. The large diversity among GSTs could thus be explained by the vast spectrum of electrophilic toxic or non-toxic compounds. Usually, the binding of GSH to the enzyme induces a lowering of the pKa value of its thiol group from 9 to about 6.2–6.6 [18], allowing a facilitated deprotonation of the thiol to form the thiolate required for the nucleophilic attack. The catalytic residue of the G-site differs depending on the class considered and it is usually either Ser, Tyr, Phe, or Cys. Furthermore, in the URE2p class, an Asn seems to be essential for the activation of the thiolate of GSH [19]. In addition, a model for GSH activation which involves a water molecule and the GSH glutamyl α-carboxylate group has been proposed [20].

Bacterial and fungal GSTs are so far poorly characterized in terms of functions and diversification in comparison with their plant and animal counterparts [21, 22]. Although nearly 10 isoforms can be found in proteobacteria for instance, the functions of these enzymes have remained obscure until now. The bacterial GSTs that have been investigated are usually involved in degradation pathways of recalcitrant chemicals which can be used for growth by host bacteria [23, 24]. Fungal GSTs have also been poorly studied, the few available data, concerning mainly yeast, indicate that they are potentially involved in protecting cells against damage resulting from oxidative stress, heavy metals, and antifungal compounds, thus highlighting the functional diversity of these enzymes [25–27]. Taking advantage of the recent release of several basidiomycete and ascomycete genome sequences, this review will deal with the description of the main fungal cytosolic GSTs classes focusing mostly on the wood-degrading basidiomycete *Phanerochaete chrysosporium*. In addition to making an overview of GSTs in fungi, this study aims at establishing a relationship between the way of life of the fungi and the occurrence of specific GSTs, searching for putative environmental biomakers.

Glutathione S-transferases diversity in fungi

We have investigated the diversity of GSTs in fungi focusing to the genomes of *P. chrysosporium*, *Postia placenta*, *Trichoderma reseei*, *Neurospora crassa*, *Aspergillus* sp., *Magnaporthe grisea*, *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Laccaria bicolor*, *Coprinus cinereus*, *Ustilago maydis*, *Melampsora larici-populina*, *Fusarium* sp., *Mycosphaerella* sp., *Chaetomium globosum*, *Stagonospora nodorum*, *Schizosaccharomyces pombe*, *Candida albicans*, *Cryptococcus neoformans*, *Sporobolomyces roseus*, *Rhizopus oryzae* and *Phycomyces blakesleeanus*. The methodology used for mining the different genomes as well as the different accession numbers are given in the electronic supplementary material. The choice of these fungi has been made in order to investigate, in a phylogenetic analysis, different ascomycetes, zygomycetes, and basidiomycetes and different ways of life, saprophytic, symbiotic, and pathogenic, in comparison with the well-known yeast *S. cerevisiae*. Indeed, it appears in this phylogenetic analysis that the total number of GST-related sequences differs strongly from one organism to another (from 7 to 46 sequences), these differences not being related to the evolutionary tree, to the size of the genome or to the gene content, but rather to the physiology of the fungi (Table 1). Organisms containing a high number of GSTs are those corresponding to ascomycetes and basidiomycetes of high complexity that are able to degrade many

Table 1 Comparative analysis of glutathione S-transferase genes in various sequenced zygomycetes, ascomycetes and basidiomycetes

		Genome size (Mb)	Gene models	GTT1	GTT2	URE2p	Omega	EFBγ	MAK16	GTE	Others	Total
Schizosaccharomyces pombe	Asc (yeast)	12.50	5,027	1	0	2	1	2	1	0	0	7
Cryptococcus neoformans	Bas (pathogen)	18.87	6,967	0	1	1	3	1	1	0	2	9
Sporobolomyces roseus	Bas	21.20	5,536	0	1	3	3	1	1	1	0	10
Melampsora laricis-populina	Bas (pathogen)	101.10	16,694	0	1	4	1	1	1	0	2	10
Ustilago maydis	Bas (pathogen)	19.68	6,522	2	0	2	3	1	1	1	0	10
Saccharomyces cerevisiae	Asc (yeast)	11.74	5,695	1	1	1	3	4	1	0	0	11
Neurospora crassa	Asc	39.23	9,826	1	0	2	3	3	1	1	1	12
Chaetomium globosum	Asc (saprophyte)	34.89	11,124	1	0	5	3	2	1	1	0	13
Magnaporthe grisea	Asc (pathogen)	41.70	11,074	1	0	2	3	3	1	1	3	14
Rhizopus oryzae	Zyg	45.26	17,459	0	1	0	2	2	2	2	5	14
Mycospherella fijiensis	Asc (pathogen)	73.40	10,327	2	1	4	6	1	0	2	0	16
Candida albicans	Asc (yeast)	14.30	6,177	5	2	2	1	5	1	0	0	16
Phycomyces blakesleeanus	Zyg	55.90	14,792	0	1	0	2	2	1	9	1	16
Mycosphaerella graminicola	Asc (pathogen)	39.70	10,952	2	0	6	4	1	1	3	0	17
Aspergillus clavatus	Asc (pathogen)	27.86	9,121	2	1	4	5	3	1	2	0	18
Aspergillus nidulans	Asc (pathogen)	30.07	10,701	1	0	3	5	5	1	3	0	18
Sclerotinia sclerotiorum	Asc (pathogen)	38.33	14,522	3	1	6	5	1	1	1	0	18
Stagonospora nodorum	Asc (pathogen)	37.10	15,983	3	1	6	5	1	1	2	0	19
Aspergillus terreus	Asc (pathogen)	29.33	10,406	1	2	5	4	3	1	4	0	20
Botrytis cinerea	Asc (pathogen)	42.66	16,448	4	1	5	5	2	1	3	1	22
Fusarium graminearum	Asc (pathogen)	36.45	13,332	2	2	4	5	2	1	3	3	22
Laccaria bicolor	Bas (mycorrhizal)	64.90	20,614	1	11	1	3	2	1	3	1	23
Aspergillus fumigatus	Asc (pathogen)	29.38	9,887	4	1	4	5	3	1	2	3	23
Fusarium verticillioides	Asc (pathogen)	41.78	14,179	3	4	3	5	2	1	3	3	24
Phanerochaete chrysosporium	Bas (saprophyte)	35.10	10,048	0	3	9	8	1	1	5	0	27
Fusarium oxysporum	Asc (pathogen)	61.36	17,735	3	6	4	6	1	1	4	2	27
Coprinus cinereus	Bas (saprophyte)	36.29	13,392	4	5	2	4	1	1	14	1	32
Trichoderma reesei	Asc (saprophyte)	34.10	9,129	1	2	5	7	3	1	4	10	33
Postia placenta	Bas (saprophyte)	90.90	17,173	2	5	17	8	2	1	11	0	46

The classification into the different classes is based on a phylogenetic analysis using MEGA4 software

Details and accession numbers are given in the electronic supplementary material

Asc Ascomycete, *Bas* basidiomycete, *Zyg* zygomycete

organic compounds (*P. chrysosporium*, *F. oxysporum*, *C. cinereus*, *T. reesei and P. placenta*), while yeasts exhibit few GSTs (*S. pombe*, *S. cerevisieae* and *C. albicans*). These differences in GST number could be explained by an overrepresentation of GSTs inside a class and/or the appearance of new classes or subclasses.

In *S. cerevisiae*, omega [26], GTT [27], Ure2p [28], MAK16 [29], and EFBγ [30] classes have been independently identified and characterized. Moreover, by screening 67 GST-like sequences from 21 fungal species, essentially ascomycetes, McGoldrick and coauthors have identified three well-known GST classes (EFBγ, URE2p, and MAK16) and two clusters that they named clusters 1 and 2 [22]. Cluster 1 includes GTT1 from *S. cerevisieae* and cluster 2 includes GSTA from *A. nidulans*, which could be related to the URE2p class.

Based on a phylogenetic analysis and sequence comparisons with *S. cerevisiae* GSTs, six known classes have been extrapolated to all the fungi considered in our study: URE2p-like, GTT2, EFBγ, GTT1, omega, and MAK16.

Interestingly, we have highlighted a new class that we named GTE (glutathione transferase etherase-related) since the sequences show homology with bacterial etherases [12].

The number of isoforms in each class differs considerably according to the investigated genome. For instance, in *C. albicans* and *A. nidulans*, five sequences are related to EFBγ class, whereas only one is detected in *P. chrysosporium* and *C. cinereus*. In contrast, the URE2p-like class is overrepresented in *P. chrysosporium* (nine sequences) whereas only one is present in *L. bicolor and C. neoformans* for example.

Some fungi, especially saprophytic fungi, exhibit more GST-coding sequences than other fungi and this is mainly due to an extension of the omega, GTT, Ure2p-like, and the newly identified GTE classes. We have thus focused our analysis on these classes.

The GTT classes (GTT1 and GTT2)

Saccharomyces cerevisiae possesses one of the simplest GST-related equipment of the investigated fungi. Among the different identified genes, two GTTs (glutathione transferase) have been characterized [27], one GTT1 and one GTT2. Strains lacking *GTT1* and *GTT2* are viable and unaffected in growth during normal aerobic conditions [31]. ScGTT1 is associated with the endoplasmic reticulum [27]. Both proteins exhibit activity against classical GST substrates as 1-chloro-2,4-dinitrobenzene (CDNB), but they probably have different physiological functions. Involved in cadmium detoxication, ScGTT2 catalyzes the formation of glutathione-Cd conjugates [32], while ScGTT1 catalyzes the reduction of hydroperoxides, in particular cumene hydroperoxide [33]. ScGTT1 and ScGTT2 have overlapping functions with glutaredoxins (ScGrx1 and ScGrx2), these latter also exhibiting classical glutathione transferase activities [31]. The involvement of glutaredoxins in xenobiotic conjugation could explain why the *gtt1 gtt2* mutant does not show any increased sensitivity to CDNB. Moreover, ScGTT1 and ScGTT2 seem to be crucial in the response to H_2O_2 stress [34], and ScGTT2 acts as a general protective factor involved in quinone detoxification [35].

Among the investigated fungi, the number of ScGTT1-related genes ranges from 0 to 5 isoforms depending on the fungus, this class representing one-third of the total GSTs of *C. albicans*. In contrast, no GTT1 isoform has been detected in *P. chrysosporium*.

The number of GTT2 isoforms varies from 0 for some ascomycetes to 11 for *L. bicolor* (Table 1). Although *L. bicolor* possesses the highest gene content among the fungi studied, the striking difference in the number of GTT2 isoforms in this fungus might not be related to that property, since GTT2 isoforms represent nearly half (48%) of its total GST content. It is not due either to a difference in the thiol-dependent antioxidant systems. In fact, for instance, five and four Grxs could be found in *L. bicolor* and *A. nidulans*, respectively [36], while no GTT2 was detected in *A. nidulans*. *P. chrysosporium* possesses 3 GTT2-related sequences with few identities between each other and compared to yeast GTT2 (Fig. 1a). In a phylogenetic analysis, Phchr6683 and Phchr6766 sequences cluster with ScGTT2 (Fig. 1b). The recombinant Phchr6766 protein has been produced in *Escherichia coli*. It exhibits a strong activity with organic peroxides ($k$cat = 181 s^{-1} and $k$cat = 141 s^{-1} using ter-butyl peroxide and cumene peroxide, respectively) (Morel et al., unpublished). The RasMol representation of Phchr6766 was based on the crystal structure of the glutathione S-transferase-like domain of EF1Bγ from *S. cerevisiae* [37]. The structure is overall very similar to the glutathione S-transferase proteins and contains a binding pocket highly homologous to those observed in glutathione S-transferase enzymes (data not shown). The third *Phanerochaete* GTT2-related isoform (Phchr7971) clusters with plant phi GSTs (Fig. 1b). Accordingly, its RasMol structure model could be built from the 3D structure of a phi class GST from *Arabidopsis thaliana* in complex with an herbicide [38] (data not shown). Phi class is a plant-specific class, which is one of the largest in *A. thaliana* with 13 members. The proteins possess GST activity against 4-hydroxynonenal (HNE), a naturally occurring lipid peroxidation product [39]. A phi class GST from *Oryza sativa* (OsGSTF5) appears to have a role in herbicide conjugation and to possess glutathione peroxidase activity [40]. By selectively co-reducing the expression of several of the major phi class GSTs, their role in limiting metabolic changes that arise from oxidative stress has been proposed [41]. The *P. chrysosporium* isoform (Phchr7971) is newly expressed upon the addition of benzoic acid in a proteomic analysis carried out on *P. chrysosporium* [42]. Nothing else is known concerning this protein in fungi.

The URE2p class

In the wood-decomposing fungi *P. chrysosporium* and *P. placenta*, the URE2p-like class represents about one-third of the total identified GSTs, corresponding to 9 and 17 sequences, respectively (Table 1). *P. chrysosporium* sequences are quite homologous between each other showing between 25.1 and 83.2% identity (Table 2). In particular, PcURE2p4, 6 and 7 exhibit between 62 and 83% identity suggesting recent duplication events of these sequences. A comparison analysis using blast search suggests that these 3 sequences are related to GSTII of *S. pombe* (SPCC965.07). A GSTII-lacZ fusion has been constructed in *S. pombe* and shows that (1) GSTII is

A

	Phchr6683	Phchr7971	YLL060c (ScGTT2)
Phchr6766	24.1	21.7	16.7
Phchr6683		17.4	17.6
Phchr7971			21.2

B

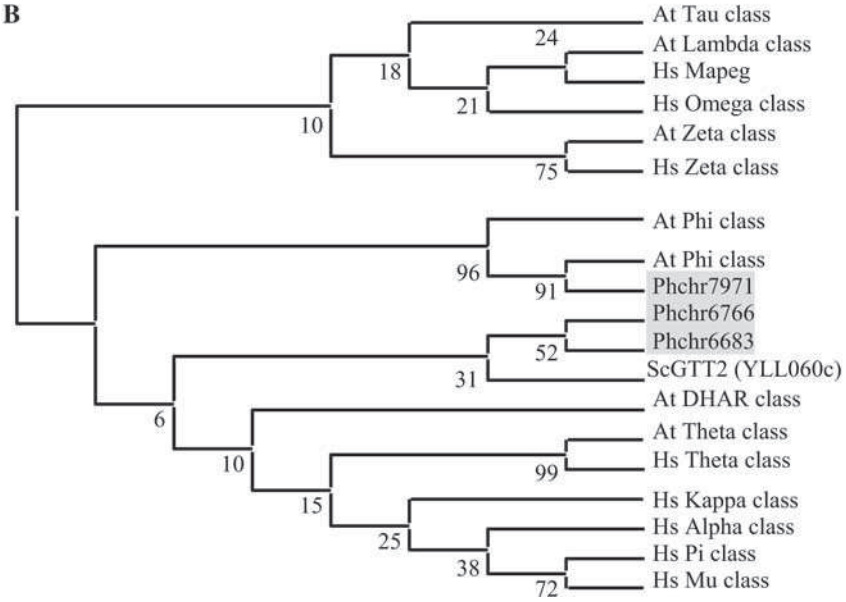


Fig. 1 GTT2-related sequence comparisons. **a** Percentage of identity between GTT2-related sequences from *P. chrysosporium* and *S. cerevisiae* (ScGTT2), based on global alignment determined by Lalign software. (http://www.ch.embnet.org/software/LALIGN_form.html). **b** Phylogenetic distribution of GTT2-related sequences of *P. chrysosporium* among GST classes from *Homo sapiens*, *A. thaliana*, and *S. cerevisiae*. The diagram has been drawn based on a phylogenetic analysis carried out with ClustalW and MEGA4 software. The analyses were conducted using the neighbor-joining (NJ) method implemented in MEGA, with the pairwise deletion option for handling alignment gaps, and with the Poisson correction model for distance computation. Bootstrap tests were conducted using 1,000 replicates. Branch lengths are proportional to phylogenetic distances. Sequences from human, *A. thaliana* and *S. cerevisiae* were obtained from NCBI databases (http://www.ncbi.nlm.nih.gov/). Sequences from *P. chrysosporium* were obtained from JGI (http://genome.jgi-psf.org). *Hs Homo sapiens*, *At A. thaliana*, *Sc S. cerevisiae* and *Pc P. chrysosporium*. Accession numbers for *A. thaliana* proteins are: At5g41210; At5g41240; NP_198938; X68304; At4g02520; At2g02930; At1g02950; At1g02940; At1g02930; At1g02920; At2g47730; At2g30860; At2g30870; At3g03190; At5g17220; At3g62760; At1g49860; Q9ZVQ3; Q9ZVQ4; At2g29490; At2g29480; At2g29470; At2g29460; At2g29450; At2g29440; At2g29420; At3g09270; At5g62480; At1g74590; At1g69930; At1g69920; At1g27130; At1g27140; At1g59670; At1g59700; At1g10370; At1g10360; At1g78380; At1g78370; At1g78360; At1g78340; At1g78320; At1g17170; At1g17180; At1g17190; At3g43800; At1g53680; At5g02790; At5g02780; At3g55040; and for human proteins are: AAB96392; AAC13317; AAA60963; AAA70226; NP_899062; NP_665735; CAA33508; Q9Y2Q3

Table 2 Percentage of identity between URE2p and cluster 2 sequences of *Phanerochaete*, based on global alignment determined by Lalign software

	PcUre2p3	PcUre2p4	PcUre2p5	PcUre2p6	PcUre2p7	PcUre2p8	PcUre2p9	Phchr 503
PcUre2p2	64.3	65.1	43.0	65.4	53.3	40.6	52.8	29.9
PcUre2p3		62.6	41.3	60.9	50.2	39.3	49.3	24.9
PcUre2p4			45.8	83.2	61.9	45.8	53.3	26.2
PcUre2p5				42.1	29.4	25.1	27.2	20.9
PcUre2p6					68.3	47.7	52.9	29.0
PcUre2p7						57.8	58.4	34.6
PcUre2p8							43.9	29.5
PcUre2p9								34.8

basically more expressed than the 2 other GSTs of the fungus, and (2) GSTII gene expression is increased by various stress agents such as sodium nitroprusside, terbutylhydroquinone, and L-buthionine-[S,R]-sulfoximine [43].

All of the nine URE2p-like sequences of *P. chrysosporium* could have a specific role in the fungus physiology since they are all transcribed (data not shown). One can wonder how this expanded class in *P. chrysosporium* and *P. placenta* is linked to the saprophytic properties of these fungi.

The phylogenetic analysis of the sequences belonging to the URE2p-like class revealed the presence of two subclasses (Fig. 2): one containing the yeast URE2p homologue and URE2p2 to URE2p9 from *P. chrysosporium*, and a second one containing GSTA (AN4905) from *A. nidulans*. This protein appeared in a cluster defined by McGoldrick et al. [22] and named cluster2. We have kept this name to define the corresponding subclass.

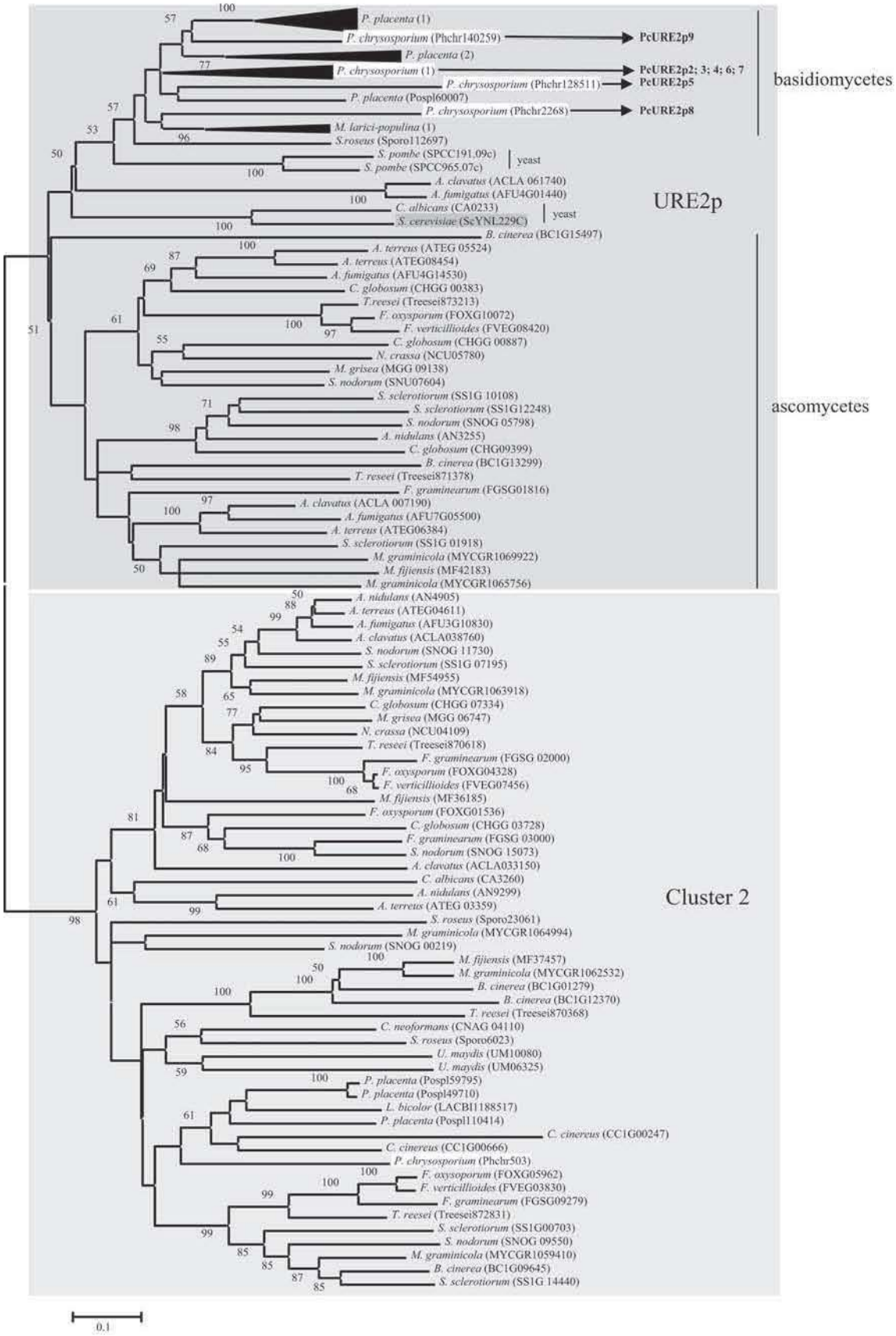
For GST classification, it has been admitted for mammalian sequences that, in the same class, GSTs share at least 60% identity in the primary structure and that those with less than 30% identity are assigned to a different class [44]. For bacterial sequences, it has been proposed that proteins belong to the same class with >40% identity, whereas GSTs of different classes share <25% identity [45]. Based on these criteria, we cannot consider cluster2 being part of an independent class from URE2p.

In *S. cerevisiae*, it has been known for a long time that ScURE2p from the URE2p subclass is involved in the nitrogen catabolite repression (NCR) by preventing gene expression by ScGLN3p in cells grown on the preferred source of nitrogen. Indeed, ScURE2p is able to bind GLN3p and retains it in the cytoplasm. The shift from the preferred source of nitrogen (ammonia or glutamine) to the nonpreferred source of nitrogen (proline) results in the release of ScGLN3p and its entry into the nucleus where it activates the transcription of nitrogen-regulated genes. It is therefore likely that ScURE2p senses the decline in the intracellular glutamine concentration [46]. Yeast URE2p possesses prion-like characteristics. The prion module in the N-terminal domain contributes to the function and stability of the ScURE2p protein and also to the transformation of Ure2p into the Ure3p proteolysed prion form. In addition, it may also influence the interactions between ScURE2p and other nitrogen regulatory proteins [47]. Moreover, ScURE2p exhibits primary sequence and three-dimensional homologies to known glutathione S-transferases. It participates in heavy metal ion and oxidant detoxification when ammonia is used as sole nitrogen source [28]. The protein is composed of two clearly divergent domains: the prion domain is in the N-terminal position and the GST-like domain on the C-terminal side [48]. By comparing URE2p sequences from

Fig. 2 Phylogenetic analysis of URE2p-like proteins. Fungi analysed are those described in Table 1. The alignment was performed with CLUSTALW and phylogenetic tree with MEGA4 software. *P. placenta* (1) sequences are Pospl121705; Pospl120102; Pospl86808; Pospl9572; Pospl9064; Pospl97355; Pospl91142; Pospl10142; Pospl91146. *P. placenta* (2) sequences are Pospl93639; Pospl88909; Pospl 27082; Pospl96818. Sequences from *P. chrysosporium* (1) are: PcURE2p2: Phchr140156; PcURE2p3: Phchr140271; PcURE2p4: Phchr137250; PcURE2p6: Phchr2269; PcURE2p7; Phchr2266. Sequences from *M. larici-populina* (1) are: Mellp72597; Mellp40377; Mellp90288

various species of the genus *Saccharomyces*, it has been noticed that the N-terminal ~40 residues are largely conserved, but there are a number of differences in the following ~50 residues of the prion domain. Conversely, the C-terminal parts of the molecule are nearly invariant [49]. ScURE2p displays GSH-dependent peroxidase activity and Asn124 has a key role in the catalytic mechanism by functioning in a way similar to that of the catalytic Ser of typical GST enzyme in activation of GSH [19]. The mutation of this Asn into Ala or Val (but not into a Ser, Tyr, or Cys) restores the GST activity of URE2p towards CDNB. The authors thus suggest that mutations have allowed URE2p to diversify and acquire additional functions as a prion and a repressor of nitrogen catabolism. It has been recently shown that ScUREp also shows thiol-disulfide oxidoreductase activity similar to that of glutaredoxins even if it does not possess cysteine residue [50]. Concerning the investigated fungi of our study, only *C. albicans* and *S. cerevisiae* exhibit the N-terminal prion domain; however, many other fungal sequences with shorter URE2p and deprived of the prion domain cluster are present in this class (Fig. 2). The distribution of ScURE2p seems to follow the evolution tree since both ascomycete and basidiomycete groups can be distinguished. Surprisingly, yeast sequences are closer to basidiomycetes sequences rather than to ascomycetes.

In our study, cluster 2 is an expanded subclass (Fig. 2) containing *A. nidulans* GSTA (AN4905) and a sequence from *P. chrysosporium* (Phchr 503). GSTA lacks the nitrogen metabolite repression activity of URE2p, but contributes to heavy metal and xenobiotic resistance [51]. Additionally to GSTA, another sequence of *A. nidulans* has been identified (AN9299) showing 50.7% identity with it. Up to now nothing is known about it. The sequence of *P. chrysposporium* (Phchr503) exhibits 44% identity with GSTA from *A. nidulans* and between 29.9 and 40.6% identity with the other *P. chrysosporium* URE2p sequences (Table 2). The characterization of this protein in relation with its putative role in oxidative stress response is an interesting point, which is currently under investigation in our laboratory. The first results show that the recombinant protein exhibits thioltransferase and reductase activities using β-hydroxyethyl disulphide (HED) and dehydroascorbate (DHA) as substrates, suggesting a putative role in stress response (Anak-Ngadin et al., unpublished).


P. placenta (1)
P. chrysosporium (Phchr140259)
PcURE2p9
P. placenta (2)
P. chrysosporium (1)
PcURE2p2; 3; 4; 6; 7
P. chrysosporium (Phchr128511)
PcURE2p5
P. placenta (Pospl60007)
P. chrysosporium (Phchr2268)
PcURE2p8
M. larici-populina (1)
S.roseus (Sporo112697)
S. pombe (SPCC191.09c)
S. pombe (SPCC965.07c)
yeast
A. clavatus (ACLA 061740)
A. fumigatus (AFU4G01440)
C. albicans (CA0233)
S. cerevisiae (ScYNL229C)
yeast
URE2p
basidiomycetes
B. cinerea (BC1G15497)
A. terreus (ATEG 05524)
A. terreus (ATEG08454)
A. fumigatus (AFU4G14530)
C. globosum (CHGG 00383)
T.reesei (Treesei873213)
F. oxysporum (FOXG10072)
F. verticillioides (FVEG08420)
C. globosum (CHGG 00887)
N. crassa (NCU05780)
M. grisea (MGG 09138)
S. nodorum (SNU07604)
S. sclerotiorum (SS1G 10108)
S. sclerotiorum (SS1G12248)
S. nodorum (SNOG 05798)
A. nidulans (AN3255)
C. globosum (CHG09399)
B. cinerea (BC1G13299)
T. reseei (Treesei871378)
F. graminearum (FGSG01816)
A. clavatus (ACLA 007190)
A. fumigatus (AFU7G05500)
A. terreus (ATEG06384)
S. sclerotiorum (SS1G 01918)
M. graminicola (MYCGR1069922)
M. fijiensis (MF42183)
M. graminicola (MYCGR1065756)
ascomycetes
A. nidulans (AN4905)
A. terreus (ATEG04611)
A. fumigatus (AFU3G10830)
A. clavatus (ACLA038760)
S. nodorum (SNOG 11730)
S. sclerotiorum (SS1G 07195)
M. fijiensis (MF54955)
M. graminicola (MYCGR1063918)
C. globosum (CHGG 07334)
M. grisea (MGG 06747)
N. crassa (NCU04109)
T. reseei (Treesei870618)
F. graminearum (FGSG 02000)
F. oxysporum (FOXG04328)
F. verticillioides (FVEG07456)
M. fijiensis (MF36185)
F. oxysporum (FOXG01536)
C. globosum (CHGG 03728)
F. graminearum (FGSG 03000)
S. nodorum (SNOG 15073)
A. clavatus (ACLA033150)
C. albicans (CA3260)
A. nidulans (AN9299)
A. terreus (ATEG 03359)
Cluster 2
S. roseus (Sporo23061)
M. graminicola (MYCGR1064994)
S. nodorum (SNOG 00219)
M. fijiensis (MF37457)
M. graminicola (MYCGR1062532)
B. cinerea (BC1G01279)
B. cinerea (BC1G12370)
T. reesei (Treesei870368)
C. neoformans (CNAG 04110)
S. roseus (Sporo6023)
U. maydis (UM10080)
U. maydis (UM06325)
P. placenta (Pospl59795)
P. placenta (Pospl49710)
L. bicolor (LACBI1188517)
P. placenta (Pospl110414)
C. cinereus (CC1G00247)
C. cinereus (CC1G00666)
P. chrysosporium (Phchr503)
F. oxysoporum (FOXG05962)
F. verticillioides (FVEG03830)
F. graminearum (FGSG09279)
T. reesei (Treesei872831)
S. sclerotiorum (SS1G00703)
S. nodorum (SNOG 09550)
M. graminicola (MYCGR1059410)
B. cinerea (BC1G09645)
S. sclerotiorum (SS1G 14440)
0.1

The omega class

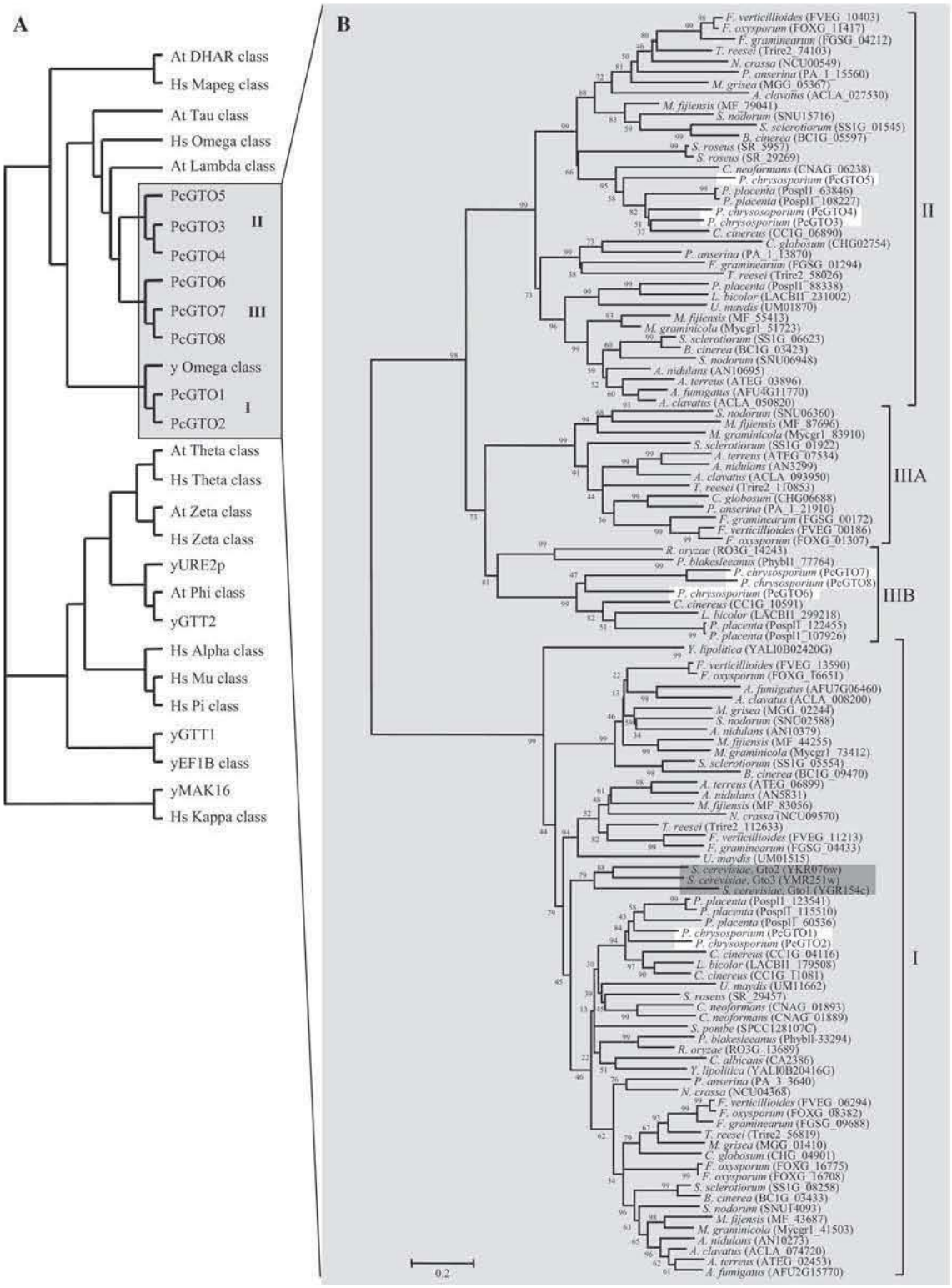
The omega class of GSTs has been identified only recently in fungi, most investigations having been conducted on human proteins [52]. This class is represented in humans by two functional genes, named *GTO1* and *GTO2*. Both proteins exhibit thioltransferase, dehydroascorbate reductase, and monomethylarsonate reductase activities, and their activity is dependent on an active-site cysteine residue [53]. In addition to its ability to act as a glutathione-dependent thioltransferase, it was proposed that HsGTO1-1 can reduce the S-thiol adduct formed between GSH and cysteine residues of proteins under stress, restoring their enzymatic functions [52]. HsGTO1-1 catalyzes the rate-limiting step in the biotransformation of arsenic [54], and it is also involved in various important biological processes. Indeed, human HsGTO1-1 can modulate ryanodine receptors, which are calcium channels in the endoplasmic reticulum of various cells [55], or catalyze the reduction of *S*-(Phenacyl) glutathione, an intermediate in the degradation of the toxic α-haloketone, to yield non-toxic acetophenones [56]. Moreover, S-(4-nitrophenacyl) glutathione (4NPG) has a high turnover with GSTO1-1 but negligible activity with GSTO2-2 and other members of the glutathione transferase superfamily [57]. Transgenic *Caenorhabditis elegans* overexpressing GSTO-1 were generated exhibiting an increased resistance to juglone-, paraquat-, and cumene hydroperoxide-induced oxidative stress, while specific silencing of the GSTO-1 by RNAi created worms with an increased sensitivity to several prooxidants, arsenite, and heat shock [58].

The *P. chrysosporium* genome possesses eight sequences, named PcGTO1 to PcGTO8, which exhibit homology with GSTs of the omega class. Based on a phylogenetic analysis and the percentage of identity between the omega-related sequences, we show that these eight proteins cluster into three distinct subclasses: The yeast GTO-related subclass (subclass I), and 2 new subclasses, that we named subclass II and III (Figs. 3 and 4a). If we take into account the bacterial classification, which determined that proteins belong to the same class with >40% identity, whereas GSTs of different classes share <25% identity, these 3 subclasses could be considered as independent classes. However, nothing has been established concerning fungal GST classification and since the sequences from subclasses II and III are clearly related to human GTO, we choose to qualify them as subclasses from the omega class.

The subclass I encloses PcGTO1 and PcGTO2, subclass II encloses PcGTO3 to PcGTO5, and subclass III encloses PcGTO6 to PcGTO8. Sequences from subclass I are close to yeast GTOs, while subclasses II and III are related to plant tau and lambda proteins, and to human GTOs. Interestingly, while the *S. cerevisiae* GTO orthologues (subclass I) are identified in all ascomycete and basidiomycete genomes analyzed, sequences from subclasses II and III were not identified in *S. cerevisiae*, *S. pombe*, and *C. albicans* (Fig. 3b). These results suggest acquisition of new functions for the omega-related proteins within the evolution of this phylum.

Subclass I is composed of proteins related to the three *S. cerevisiae* GTOs (ScGTO1, ScGTO2, and ScGTO3). Two *P. chrysosporium* GSTs (PcGTO1 and PcGTO2) belong to this subclass. Both display a CPWATR active site compatible with the CP(W/F)(A/T)(H/Q)R motive found in ScGTOs (Fig. 4b). In yeast, the GTOs have been partially characterized. The three ScGTOs exhibit glutaredoxin-like activities, being able to reduce HED and DHA, and all three *GTO* genes are induced by agents causing oxidative stress suggesting a function in the defense against oxidants [33]. ScGTO1 has been shown to be located in peroxisomes and involved in sulfur metabolism. The other fungal orthologs, and in particular PcGTO1 and PcGTO2, clustering in this omega class differ from their yeast counterparts in particular at the H-site and are, in contrast, strongly related to bacterial GSTs. Moreover, according to the prediction softwares, both *P. chrysosporium* orthologs (PcGTO1 and PcGTO2) possess a signal peptide targeting

Fig. 3 Phylogenetic distribution of omega proteins. **a** Distribution of omega GSTs of *P. chrysosporium* among GSTs from *H. sapiens*, *A. thaliana*, and *S. cerevisiae*. The diagram has been drawn based on a phylogenetic analysis carried out with ClustalW and MEGA4 software. Sequences were obtained as described in Fig. 1 and accession numbers are given in the legend of Fig. 1. Accession numbers for *S. cerevisiae* are given in brackets in Fig. 3b and JGI accession numbers of *P. chrysosporium* sequences are: PcGTO2: 126388; PcGTO4: 7168; PcGTO5: 7169; PcGTO6: 3911; PcGTO7: 6880; PcGTO8: 6881. PcGTO1 and PcGTO3 have been manually corrected and deposited to NCBI under the accession numbers: EU791894 and EU791893, respectively. **b** Phylogenetic tree of omega sequences from various ascomycetes (*Aspergillus clavatus*, *Aspergillus fumigatus*, *Aspergillus terreus*, *A. nidulans*, *Mycosphaerella graminicola*, *Mycosphaerella fijiensis*, *Fusarium oxysporium*, *Fusarium verticillioides*, *Fusarium graminearum*, *Podospora anserina*, *Chaetomium globosum*, *Magnaporthe grisea*, *Botrytis cinerea*, *Trichoderma reesei*, *Sclerotinia sclerotiorum*, *Septoria nodorum*, *Schizosaccharomyces pombe*, *Yarrowia lipolytica*, *Candida albicans*, *Neurospora crassa*, *Saccharomyces cerevisiae*), basidiomycetes (*Phanerochaete chrysosporium*, *Laccaria bicolor*, *Coprinus cinereus*, *Postia placenta*, *Cryptococcus neoformans*, *Ustilago maydis*, *Sporobolomyces roseus*) and zygomycetes (*Rhizopus oryzae* and *Phycomyces blakesleeanus*). Sequences were obtained and identified using the transcript or protein accession number, in genomes from the BROAD institute (http://www.broad.mit.edu/annotation/), the JGI (http://genome.jgi-psf.org/euk_home.html), *Podospora anserina* (http://podospora.igmors.u-psud.fr/index.html), *Schizosaccharomyces pombe* (http://www.sanger.ac.uk/Data Search/blast.shtml), *Yarrowia lipolitica* (http://www.ncbi.nlm.nih.gov/projects/genome/seq/) using alternatively the *S. cerevisiae* or the *P. chrysosporium* sequences as template. The alignment was performed with CLUSTALW and phylogenetic tree with MEGA4 software



to mitochondria (respectively 92 and 97% of probability). Surprisingly, we found that PcGTO1 and PcGTO2 exhibit strong homology with some plant lambda and bacterial proteins (Fig. 5a). In *E. coli*, the homologue of PcGTO1 (yqjG) belongs to an operon containing a gene coding for a quinol oxidase, which is a key energy-transducing respiratory enzyme in microorganisms. This is of particular interest since PcGTO1 is predicted to be localized in the mitochondria. In bacteria, a tetrachlorohydroquinone dehalogenase appeared to be a member of the GST

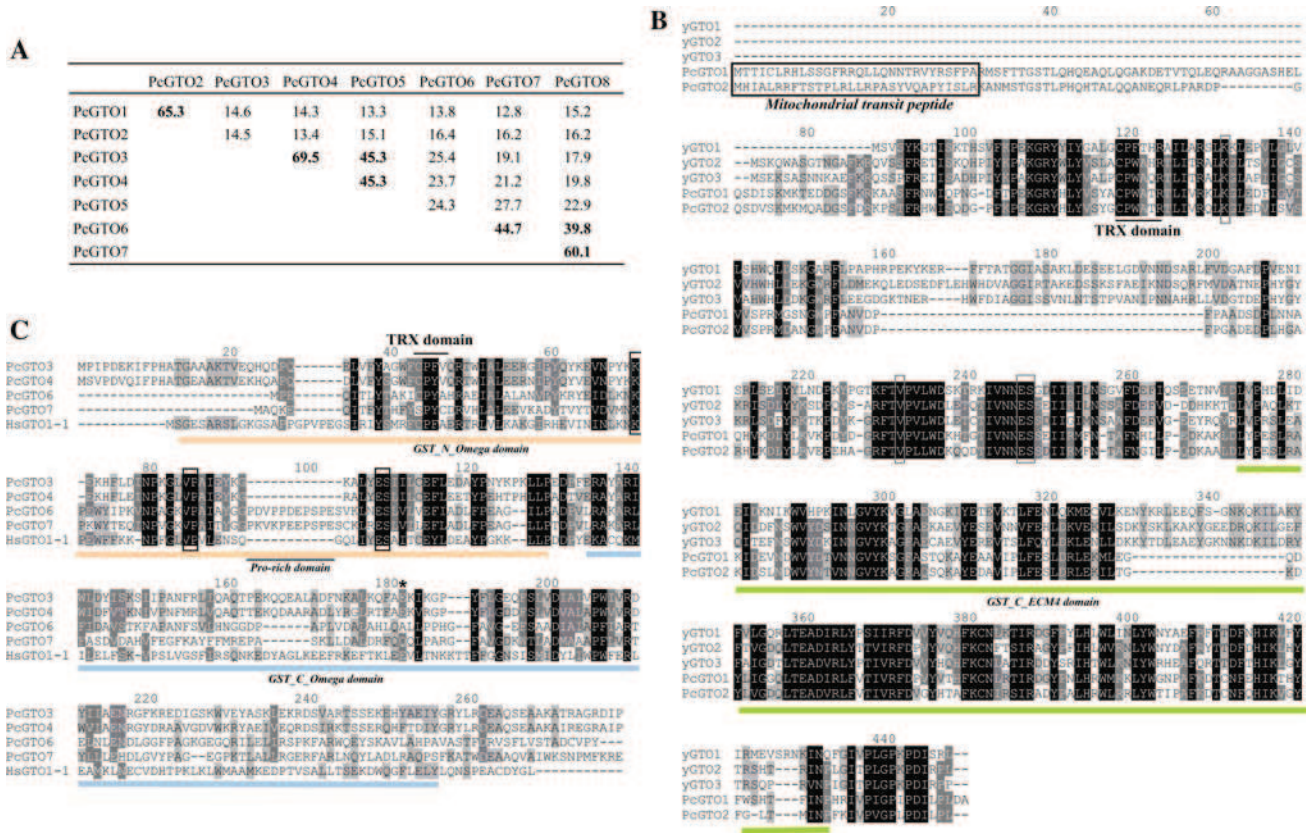


Fig. 4 Omega GST sequence comparisons. **a** Percentage of identity between omega GST sequences of *P. chrysosporium*, based on global alignment determined by Lalign software. **b** Alignment of *P. chrysosporium* GST omega proteins from subgroup I in comparison to *S. cerevisiae* GTOs. Alignments have been performed using ClustalW. Amino acids involved in GSH binding and mitochondrial targeting sequences are *framed*. **C**. Alignment of *P. chrysosporium* GSTs from subclasses II and III previously defined in Fig. 3, in comparison with human GTO1-1. PcGTO5 and PcGTO8 are not represented in the figure because the sequences are not firmly delineated. The conserved amino acids involved in GSH binding are *framed*. The *asterisk* localizes Glu155 of HsGTO1-1 sequence

superfamily and, particularly, related to PcGTO1. This enzyme converts tetrachlorohydroquinone to dichlorohydroquinone using GSH as reducing equivalent [59]. In *P. chrysosporium*, this reductive dehalogenation requires two enzymes, a glutathione S-transferase for glutathionylation and an enzyme called glutathione conjugate reductase for deglutathionylation (GCR) [60]. Subsequent results have shown that some GSTs are able to deglutathionylate aromatic compounds [56], and thus one can wonder whether this GCR might also belong to the GST superfamily. Nothing is known concerning the *Arabidopsis* homologue (At4G19880). Data from Geneinvestigator (https://www.genevestigator.com/gv/index.jsp) [61] show that the gene is essentially expressed in roots of young plantlets, and during hypoxia and hormonal stress. The multiple alignment of these fungal, bacterial, and plant sequences (Fig. 5b) shows different amino acid identities especially at the active site and the residues important for the interaction with GSH. Similarly to PcGTO1 and PcGTO2, the *A. thaliana* sequence exhibits a signal peptide for mitochondria (92.5% probability). Moreover, it exhibits a lysine-rich extension at the H-site compared to the other sequences.

The analyzed fungi exhibit 1–3 sequences belonging to subclass II, except *S. pombe*, *M. larici-populina*, *S. cerevisiae*, *R. oryzae*, *C. albicans* and *P. blakesleeanus*, which do not possess any sequences of this subclass. Similarly, proteins from subclass III are not represented in all fungi and their distribution follows evolution, i.e. sequences from ascomycetes and basidiomycetes cluster independently (Fig. 3b). In our study, one sequence was identified in the two zygomycetes, some pathogenic ascomycetes and all basidiomycetes, with *P. chrysosporium* and *P. placenta* exhibiting 3 and 2 sequences, respectively.

GTOs are known to contain the G-site, which contains a conserved cysteinyl residue essential for activity. Amino-acid alignments of sequences from subclass I (Fig. 4b) and subclasses II and III (Fig. 4c) show the presence of this conserved residue in all described PcGTOs except in PcGTO7. This conserved cysteine aligns with Cys-32 of human GTO1-1, which is located at the beginning of the

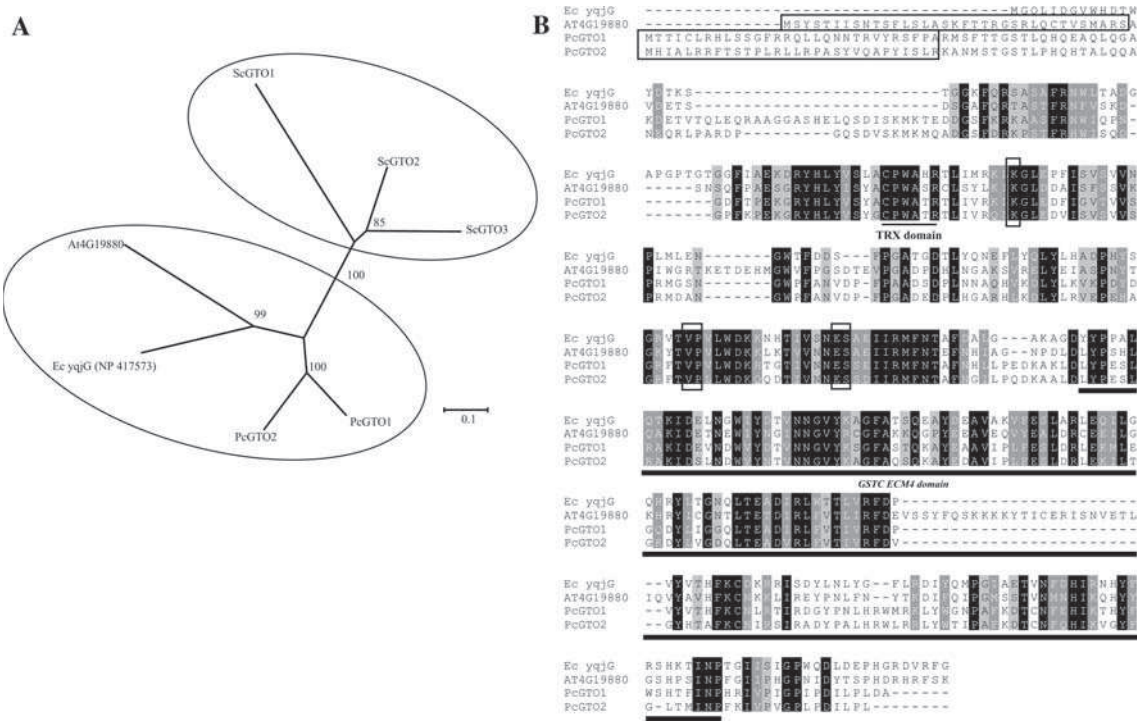


Fig. 5 Subgroup I omega GSTs. **a** Phylogenetic relationship between omega GST proteins from *P. chrysosporium*, *S. cerevisiae*, and *A. thaliana* (At4G19880) and *E. coli* (yqjG) homologues. The alignment was performed with CLUSTALW and phylogenetic tree with MEGA4 software. **b** Alignment of *P. chrysosporium* GTO1 and GTO2, *E. coli* (yqjG) and *A. thaliana* homologues (At4G19880). Amino acids involved in GSH binding and mitochondrial targeting sequences are *framed*

first α-helix, in a position similar to the thiol group of the catalytic cysteine of the CxxC motif of thioredoxin and glutaredoxin. This suggests that GSTs from subclasses II and III, except PcGTO7, could exhibit the same glutathionylation and deglutathionylation properties exhibited by HsGTO1-1. Moreover, the adjacent proline, which promotes optimal positioning of the Cys32 thiol for stabilisation of the thiolate form in HsGTO1-1, is absolutely conserved [62]. Other residues required for the interaction with GSH in HsGTO1-1 (Lys59, Val72, Glu85, Ser86) [52] are also conserved in the different PcGTOs. The C-terminal part of the proteins, corresponding to the hydrophobic H-site involved in xenobiotic binding in the different GSTs classes [52], exhibits a larger variability. PcGTO6 and PcGTO7 possess a proline rich domain not present in PcGTO3 and PcGTO4. This sequence could play a role either in its own folding or else be required for catalytic activity. It has been recently shown that the deletion of Glu155 of the human GTO1-1 causes a deficiency of the protein possibly due to an induced instability of the variant protein [63]. However, this mutation does not alter sensitivity to arsenic trioxide and other cytotoxic drugs. This residue (marked by an asterisk in Fig. 4c) is conserved in PcGTO3 and PcGTO4 but not in PcGTO6 nor in PcGTO7. PcGTO3 and PcGTO4, but also PcGTO7 and PcGTO8, are present in tandem in the *P. chrysosporium* genome. They show 69.5% and 60.1% amino acid identity, respectively (Fig. 4a) suggesting quite recent duplication events.

The GTE class

A new class of GSTs that we have called GTE has been highlighted by this phylogenetic analysis. These proteins are related to bacterial Lig proteins. In *Sphingomonas paucimobilis*, three genes, LigF, LigE, and LigG, are localized in an operon called *LigDFEG*. They are all members of the GST superfamily. However, a weak activity with the universal substrate for GST (CDNB) was detected in *E. coli* extracts overexpressing Lig proteins [64]. LigE and LigF are enantioselective GSTs involved in cleavage of *β*-aryl ether compounds, which is the most important step in lignin degradation. LigG is the glutathione lyase for the glutathione conjugate produced by LigF [12]. In a phylogenetic analysis using representative prokaryotic and eukaryotic members of the GST superfamily, *S. paucimobilis* LigF and LigG group together, not so far from the omega class, but distant from LigE which is more closely related to zeta class enzymes [45].

A

	PcGTE2	PcGTE3	PcGTE4	PcGTE5	LIG-E	LIG-G	LIG-F
PcGTE1	**74.0**	**59.1**	38.9	29.5	23.7	19.1	11.4
PcGTE2		**58.4**	38.8	32.3	24.7	17.4	14.9
PcGTE3			38.0	33.5	23.4	13.8	13.9
PcGTE4				27.9	16.2	13.5	12.4
PcGTE5					17.1	16.2	14.1
LIG-E						20.3	16.0
LIG-G							16.7

B

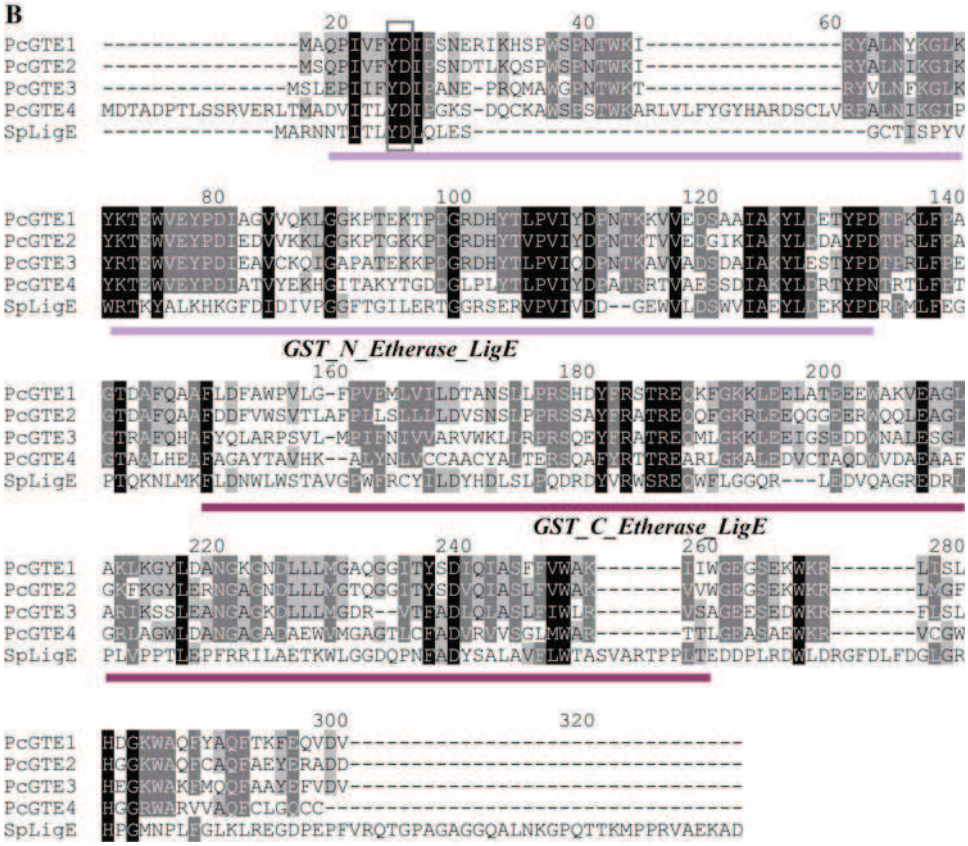


Fig. 6 GTEs and Lig GST sequence comparisons. **a** Percentage of identity between GTE sequences of *P. chrysosporium* and Lig sequences from *S. paucimobilis* (LigE: BAA02032; LigG: BAA77216; LigF: BAA02031), based on global alignment determined by Lalign software. **b** Amino acid sequence alignments of GTEs and LigE proteins from *Sphingomonas paucimobilis*. Alignments have been performed using ClustalW. Amino acids putatively involved in catalysis are identified by a *frame*

The homologies between the four *P. chrysosporium* GTE members and bacterial Lig proteins are quite weak, ranging from 11.4 to 23.7% identity (Fig. 6a). PcGTE1, PcGTE2, and PcGTE3 are rather related to LigE, and sequence analysis revealed that PcGTEs possess the specific GST-N and GST-C domains of the LigE sequence (Fig. 6b). These sequences exhibit a conserved tyrosine in the N-terminal region (G-site) putatively involved in catalysis as postulated for their bacterial counterparts [24]. LigE catalyzes the reductive cleavage of the β-aryl ether linkage of guaiacylglycerol β-O-4-methylumbelliferone (GOU) to produce β-hydroxypropiovanillone and 4-methylumbelliferone in vitro [65]. The recombinant GTE3 of *P. chrysosporium* is not active using this substrate (Morel et al., unpublished results). However, other substrates have to be tested, since a high substrate specificity of the β-aryl ether cleavage activity has been proposed [65]. In *S. paucimobilis*, LigE is tightly associated with cell membranes [66]. Conversely, the predictions of *P. chrysosporium* enzymes localization revealed that one could be excreted (PcGTE4), the others being cytosolic (PcGTE1, PcGTE2, and PcGTE3) or mitochondrial (PcGTE5).

Fungal genome analysis revealed that hemiascomycetes such as *S. cerevisiae*, *C. albicans*, and *S. pombe* do not possess GTE proteins. In contrast, depending on their physiology, all filamentous fungi analyzed present a variable number of GTE-like sequences as evidenced by their analogy to the LigE sequence. Most animal and plant pathogenic fungi exhibit 1–3 putative GTEs, while 3–14 genes were found in saprophytic fungi (Table 1). In particular, the basidiomycetes *P. placenta* and *C. cinereus*

exhibit 11 and 14 GTE sequences, respectively. Moreover, 3 or 4 sequences were identified in necrotroph plant pathogens such as *Aspergillus* sp., *Fusarium* sp., or *B. cinerea*. These fungi are predominately saprophytes and grow on dead or live plant and animal tissues in the soil. For this reason, these enzymes could be very important in nutrient recycling, or for their infectious cycle. These observations highlight an apparent relationship between these GTEs and the capacity of the fungi to degrade organic matter. This is in accordance with their homology with the bacterial Lig proteins. A better characterization of these enzymes is of great interest in understanding wood and xenobiotic degradation processes. Moreover, using a complete expression dataset of *L. bicolor* genes available at (http://www.ncbi.nlm.nih.gov/geo/) as series GSE9784 [67], it appeared that an etherase-like gene (LbGTE3) is upregulated by about 20-fold in ectomycorrhizas compared to free-living mycelium. This suggests putative functions of GTE proteins, both saprophytic and mycorrhizal.

A surprising result is the occurrence of nine sequences related to GTEs in the zygomycete fungus *P. blakesleeanus,* corresponding to more than half of its total GST number. This fungus is known for the variety and sensitivity of its responses to light and the regulation of the biosynthesis of the pigment beta-carotene. However, it does not seem to have saprophytic properties, and thus the physiological significance of these GTE-like sequences in this fungus is still an open question.

Conclusion

In summary, this study has highlighted the occurrence of new GST-proteins in fungi, belonging to the omega class (subclasses II and III) and a newly identified GTE class. Globally, saprophytic fungi exhibit more GST-coding sequences than the other fungi, and this is mainly due to an extension of the omega, GTT, Ure2p, and GTE classes.

Proteins from omega, Ure2p, and GTE classes are highly represented in *P. chrysosporium.* This opens many perspectives to better understand how the fungus is able to oxidize and detoxify a broad range of toxic chemical pollutants. This fungus uses both an extracellular oxidative system (Lignin peroxidases) and GSTs and/or cytochrome P450 mono-oxygenases. Cytochrome P450 mono-oxygenases are involved in phase I detoxification metabolism. Interestingly, *P. chrysosporium* possesses the highest number of P450 sequences among fungi (1% of the total genome) [68]. Thus, the high diversity of GSTs could be related to this very large number of cytochrome P450 mono-oxygenases. This study also shows for the first time that some basidiomycetes possess GSTs known to have β-etherase activity in bacteria (GTE class). Moreover, we can make a link between the representation of this class among total GSTs and the saprophytic properties of the fungi. These proteins could thus have a role in organic matter degradation and in particular in wood degradation for *P. chrysosporium* and *P. placenta*. Through these properties, they can be good actors in xenobiotic oxidation processes and represent a versatile tool with a variety of biotechnological applications such as bioremediation. So far, all GST engineering experiments have been carried out with bacteria [45], mouse, and *Drosophila melanogaster* (for a review, see [2]). Recently, the effectiveness of gene silencing by RNA interference has been demonstrated in *P. chrysosporium* [69], opening new perspectives, by using this genetic tool, to study the function of GSTs in detoxification purposes.

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Article III

Glutathione-S-transferases of *Phanerochaete chrysosporium*: A S-Glutathionyl-P-Hydroquinone reductase belongs to a new structural class

Introduction

The fungal omega GSTs have been poorly characterized in comparison with their human counterparts. The phylogenetic analysis previously described showed that this class is extended in *P. chrysosporium* since eight sequences encoding putative omega GSTs are found in the genome of this fungus (Morel et al., 2009a). Among this omega class, three subclasses could be defined. A subclass I containing two putative isoforms is related to plant GST (called lambda) and also to bacterial GSTs. The subclasses II and III each containing three putative isoforms are more related to mammals omega GSTs. A major feature of GTOs is the presence of a cysteinyl residue in the catalytic site (in the glutathione binding site), which could explain the main activity of the previously characterized isoforms. GTOs are indeed involved in deglutathionylation reactions, leading to the removal of glutathione from various substrates. At the beginning of this study, fungal GTOs were only weakly characterized, the main studies having been conducted on *S. cerevisiae,* showing that the three isoforms detected in this organism exhibit glutaredoxin-like activities. In this study, we have focused on the biochemical and structural characterizations of two isoforms: PcGTO1 belonging to the subclass I and PcGTO3 belonging to the subclass II.

Results

The comparison of the biochemistry and three-dimensional structure data of the omega isoforms in *P. chrysosporium* were performed related to other GTO-like proteins. The PcGTO1 belonging to the subclass1, possesses a signal peptide targeting to mitochondria. The structure of PcGTO1 has been resolved showing the presence of a dimer. Biochemical data suggest that PcGTO1 is in fact a S-glutathionyl-p-hydroquinone reductase (GHR), catalyzing the removal of glutathione from quinone related substrates. The deglutathionylation activity is due to the presence a catalytic cysteine in the G site (domain CPWA). In summary, this paper describes a new structural class of GSTs linked to a new functional class, this class being found in bacteria, fungi and plants.

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Glutathione Transferases of *Phanerochaete chrysosporium*

S-GLUTATHIONYL-*p*-HYDROQUINONE REDUCTASE BELONGS TO A NEW STRUCTURAL CLASS*[S]

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Edgar Meux[‡], Pascalita Prosper[§], Andrew Ngadin[‡], Claude Didierjean[§], Mélanie Morel[‡], Stéphane Dumarçay[¶], Tiphaine Lamant[‡], Jean-Pierre Jacquot[‡], Frédérique Favier[§1], and Eric Gelhaye[‡2]

From the [‡]UMR 1136 INRA-UHP "Interactions Arbres/Micro-Organismes," IFR110 "Ecosystèmes Forestiers, Agroressources, Bioprocédés et Alimentation," the [§]CRM2, Equipe Biocristallographie, UMR 7036 CNRS-UHP, Institut Jean Barriol, and the [¶]Laboratoire d'Études et de Recherches sur le Matériau Bois, EA UHP 4370, Nancy Université, Faculté des Sciences et Techniques, BP 70239, 54506 Vandoeuvre-les-Nancy, France

The white rot fungus *Phanerochaete chrysosporium*, a saprophytic basidiomycete, possesses a large number of cytosolic glutathione transferases, eight of them showing similarity to the Omega class. PcGSTO1 (subclass I, the bacterial homologs of which were recently proposed, based on their enzymatic function, to constitute a new class of glutathione transferase named *S*-glutathionyl-(chloro)hydroquinone reductases) and PcGSTO3 (subclass II related to mammalian homologs) have been investigated in this study. Biochemical investigations demonstrate that both enzymes are able to catalyze deglutathionylation reactions thanks to the presence of a catalytic cysteinyl residue. This reaction leads to the formation of a disulfide bridge between the conserved cysteine and the removed glutathione from their substrate. The substrate specificity of each isoform differs. In particular PcGSTO1, in contrast to PcGSTO3, was found to catalyze deglutathionylation of *S*-glutathionyl-*p*-hydroquinone substrates. The three-dimensional structure of PcGSTO1 presented here confirms the hypothesis that it belongs not only to a new biological class but also to a new structural class that we propose to name GST xi. Indeed, it shows specific features, the most striking ones being a new dimerization mode and a catalytic site that is buried due to the presence of long loops and that contains the catalytic cysteine.

Phanerochaete chrysosporium is considered as the model organism to study the physiology of white rot fungi, the only known microorganisms able to completely break down lignin to carbon dioxide and water (1–3). Analysis of the genome of this basidiomycete revealed a complex superfamily of cytochrome P450 monooxygenases representing around 1% of this genome (1, 4). The large size of this gene family is correlated with the extraordinary ability of this fungus to degrade and metabolize numerous recalcitrant compounds such as pesticides, polyaromatic hydrocarbons, halogenated aromatics, and textile dyes. Besides extracellular oxidative systems, including lignin and manganese peroxidases, cytochrome P450 monooxygenases are thought to be involved in the phase I of detoxication processes catalyzing the oxidation of various substrates (5–7). The oxidation of recalcitrant compounds is often followed by glutathione conjugation catalyzed by glutathione transferases (GSTs). The glutathione conjugates formed are less toxic and lipophilic than the parent compounds (8).

The distribution of GSTs in fungi has been recently investigated (9), highlighting that *P. chrysosporium* exhibits a large subfamily of cytosolic GSTs (at least 27 isoforms) in comparison with *Saccharomyces cerevisiae* for instance (11 isoforms). Based on primary structure similarity, these cytosolic GSTs belong to seven different classes as follows: Ure2p-like, GTT1, GTT2, EFBγ, MAK16, etherases, and Omega (GSTO)[3] (9). Among the cytosolic GSTs, *P. chrysosporium* exhibits eight putative proteins showing homology with GSTs of the Omega class, named PcGSTO1 to PcGSTO8. In a previous phylogenetic analysis of fungal GSTs, we found that GSTOs fall into three different subclasses. Subclass 1 encloses PcGSTO1 and PcGSTO2, these latter being related to uncharacterized plant GST Lambda and bacterial GSTs (9). Members of subclasses II and III are related to the well characterized human HsGSTO1-1 (10, 11).

A major feature of GSTOs is the presence of a cysteinyl residue in the catalytic site present in the glutathione binding domain (G-site), explaining the deglutathionylation activities exhibited by these proteins. For instance, HsGSTO1-1 has been shown to remove glutathione from *S*-(phenacyl)glutathione (12), as this activity requires the presence of the catalytic Cys^{32} (13). In addition, GSTOs have been shown to be involved in the reduction of methyl and dimethyl arsonate, an intermediate in

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This work is dedicated to the memory of the late Dr. André Aubry.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1, Figs. 1 and 2, and additional references.

The atomic coordinates and structure factors (code 3PPU) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

[1] To whom correspondence may be addressed: CRM2, UMR 7036 CNRS-UHP, Nancy Université, Faculté des Sciences et Techniques, BP 70239, 54506 Vandoeuvre-les-Nancy Cedex, France. Tel.: 33-3-83-68-48-79; Fax: 33-3-83-40-64-92; E-mail: Frederique.Favier@crm2.uhp-nancy.fr.

[2] To whom correspondence may be addressed: UMR 1136 INRA-UHP, IFR110, Nancy Université, Faculté des Sciences et Techniques, BP 70239, 54506 Vandoeuvre-les-Nancy, France. Tel.: 33-3-83-68-42-28; E-mail: Eric.Gelhaye@lcb.uhp-nancy.fr.

[3] The abbreviations used are: GSTO, glutathione transferase Omega; DHA, dehydroascorbate; GHR, *S*-glutathionyl-(chloro)hydroquinone reductase; HED, hydroxyethyldisulfide; PDB, Protein Data Bank; PAP-SG, *S*-(phenacylacetophenone)glutathione; Se, selenium; CDNB, 1-chloro-2,4-dinitrobenzene.

the arsenic biotransformation (14, 15). Bacterial GSTOs, which belong to subclass I, have been shown to be involved in pentachlorophenol catabolism acting as *S*-glutathionyl-(chloro)hydroquinone reductase; this activity was also related to the presence of a catalytic cysteine (16, 17). In fungi, few studies have been devoted to this GST class except in *S. cerevisiae* (ScGSTO). The three ScGSTOs belong to the subclass I and have been partially characterized particularly at the protein level. The three proteins exhibit glutaredoxin-like activities because they are able to reduce both hydroxyethyldisulfide and dehydroascorbate in the presence of glutathione. In addition, the transcription of the three genes is induced by oxidative stress (18, 19). Moreover, ScGSTO1 has been shown to be involved in sulfur metabolism and located in peroxisomes. PcGSTO1 and PcGSTO2, nevertheless, differ from their yeast counterparts particularly at the H-site (the domain thought to interact with the hydrophobic substrate).

From phylogenetic analysis and biochemical analysis, Xun *et al.* (17) recently proposed that the Omega subclass I members constitute a new class of GSTs named *S*-glutathionyl-(chloro)hydroquinone reductase (GHR) due to their activity with *S*-glutathionyl-hydroquinones.

The aim of this study was the investigation of the PcGSTOs, a class of GSTs that is over-represented in *P. chrysosporium*. Recombinant proteins of isoforms that belong to the GSTO subclasses I and II have been biochemically characterized, exhibiting substrate specificity. Furthermore, the three-dimensional structure of PcGSTO1 has been solved, allowing the first description of a *S*-glutathionyl-*p*-hydroquinone reductase at the structural level. From these biochemical and structural data, the potential functions of the different isoforms are discussed.

EXPERIMENTAL PROCEDURES

Materials—Hydroxyethyldisulfide (HED) and 5,5′-dithiobis-2-nitrobenzoic acid were from Aldrich and Pierce, respectively. All other reagents were from Sigma.

Cloning and Construction of Pcgsto1 and Pcgsto3 Mutants by Site-directed Mutagenesis—The open reading frame sequences encoding *P. chrysosporium* PcGSTO1 and PcGSTO3 were amplified from *P. chrysosporium* cDNA using *Pcgsto1* and *Pcgsto3* forward and reverse primers (see supplemental Table 1) and cloned into the NcoI and BamHI restriction sites (underlined in the primers) of pET3d (Novagen). For PcGSTO3, the amplified sequence encoded a protein in which an alanine has been added during cloning. Using two complementary mutagenic primers, the two cysteines of PcGSTO3 were individually substituted into serines, and the primers are listed in supplemental Table 1. For PcGSTO1, due to the presence of the signal peptide, a first construction has been made, and the resulting protein starts with the N-terminal sequence MATTICLRH. Because we obtained a mixture of different proteins after production and purification (see under "Results"), a new construction has been made. The resulting protein with an additional alanine started thus with the N-terminal sequence MASFTTGST (see supplemental Fig. 1). PcGSTO1 used for structure determination consists also of the 352 last residues of the full-length protein.

Expression and Purification of the Recombinant Proteins—For protein production, the *Escherichia coli* BL21(DE3) strain that contained the pSBET plasmid was co-transformed with the different recombinant plasmids (20). Cultures were progressively amplified up to 2.4 liters in LB medium supplemented with ampicillin and kanamycin at 37 °C. Protein expression was induced at exponential phase by adding 100 μM isopropyl β-D-thiogalactopyranoside for 4 h at 37 °C. The cultures were centrifuged for 15 min at 4400 × *g*. The pellets were resuspended in 30 ml of TE NaCl buffer (30 mM Tris-HCl, pH 8.0, 1 mM EDTA, 200 mM NaCl), and the suspension was stored at −20 °C.

Cell lysis was performed by sonication (three times for 1 min with intervals of 1 min), and the soluble and insoluble fractions were separated by centrifugation for 30 min at 27,000 × *g*. The soluble part was then fractionated with ammonium sulfate in two steps, and the protein fraction precipitated between 40 and 80% of the saturation contained the recombinant protein, as estimated by 15% SDS-PAGE. The protein was purified by size exclusion chromatography after loading on an ACA44 (5 × 75 cm) column equilibrated in TE NaCl buffer. The fractions that contained the protein were pooled, dialyzed by ultrafiltration to remove NaCl, and loaded onto a DEAE-cellulose column (Sigma) in TE buffer (30 mM Tris-HCl, pH 8.0, 1 mM EDTA). The proteins were eluted using a 0–0.4 M NaCl gradient. Finally, the fractions of interest were pooled, dialyzed, concentrated by ultrafiltration under nitrogen pressure (YM10 membrane; Amicon), and stored in TE buffer at −20 °C. Purity was checked by SDS-PAGE. Protein concentrations were determined spectrophotometrically using a molar extinction coefficient at 280 nm of 64,860 $M^{-1}\cdot cm^{-1}$ for the PcGSTO1 WT, C86S, and 51340 $M^{-1}\cdot cm^{-1}$ for PcGSTO3 WT and C37S.

E. coli strain B121 containing the pET-*Pcgsto1* plasmid was cultured as described previously (21) and was used as a source of selenomethionine-substituted form of PcGSTO1 (SeMet-PcGSTO1). The purification of SeMet-PcGSTO1 was performed following a procedure similar to that described for PcGSTO1.

Mass Spectrometry Analysis—PcGSTOs WT and mutated were analyzed by quadrupole-TOF MS as described by Koh *et al.* (22).

Fluorescence Properties of Wild-type and Mutated PcGSTOs—The fluorescence characteristics of PcGSTOs in the reduced and oxidized forms were recorded with a spectrofluorometer (Cary Eclipse; VARIAN) in TE buffer at a protein concentration of 10 μM.

Determination of Free Thiol Groups—The number of free thiol groups in either untreated, denatured, or reduced conditions was determined spectrophotometrically with 5,5′-dithiobis-2-nitrobenzoic, as described by Koh *et al.* (22). All thiol titrations were performed on enzymes either as purified or after SDS treatment or after dithiothreitol (DTT) reduction. In the latter case, proteins were first reduced by incubation with 10 mM DTT for 30 min at room temperature, then precipitated with 1 volume of 20% (w/v) trichloroacetic acid (TCA), and finally stored on ice for 30 min. The mixture was then centrifuged for 10 min at 13,000 × *g*, and the pellets were washed twice with 2% (w/v) TCA. The precipitate was resuspended in 30 mM Tris-HCl, pH 7.0, 1 mM EDTA, and 1% (w/v) SDS. The

concentration was estimated by UV spectrophotometry. For denatured proteins, samples were incubated in the presence of 2% SDS treatment during 30 min before 5,5′-dithiobis-2-nitrobenzoic addition.

Activity Measurements—The activity measurements of WT or mutant PcGSTOs in the HED assay or for reduction of dehydroascorbate (DHA) were performed as described by Couturier *et al.* (23). Dimethylarsinate reductase and glutathionyl-phenylacetophenone assay were performed as described previously (12, 13). The GST activity was measured with 0.2 mM CDNB as the substrate in 30 mM Tris buffer, pH 8.0, and the continuous absorbance at 340 nm was monitored. *S*-Glutathionyl-*p*-hydroquinone reductase activity was monitored as follows: 1 mM 1,4-benzoquinone in 30 mM Tris-HCl, pH 8, was used as base line for an absorbance spectrum (230–400 nm). After 2 min, 1 mM reduced glutathione (GSH) was added to the reaction mixture, and the spectra were monitored every minute during 5 min. At last, purified recombinant proteins were added in the reaction mixture, and spectra were recorded every minute. In complementary experiments, 1 mM 1,4-benzoquinone in 30 mM Tris-HCl, pH 8, was incubated in the presence of 1 mM GSH, 200 μM NADPH, 0.5 IU glutathione reductase before the addition of the protein of interest. The activity was followed by monitoring the decrease in absorbance arising from NADPH oxidation in this coupled enzyme assay system that showed the formation of glutathione disulfide (GSSG).

In complementary experiments, *S*-(phenacylacetophenone)-glutathione (PAP-SG) and 2-methyl-*S*-glutathionyl-naphthoquinone (thiodione) were prepared as described previously (24, 25), and the purity and the nature of the products were verified by mass spectrometry. Activities of the WT and variant enzymes were assayed by following the appearance of deglutathionylated menadione (at 425 nm) and phenylacetophenone (at 290 nm). The reactions were performed in 30 mM Tris-HCl, pH 8, containing GSH (5 mM) and substrates (from 15 to 2 mM) in a final volume of 650 μl and initiated by adding WT or mutant enzyme. The concentration of enzyme added was adjusted based on preliminary activity experiments, ranging from 0.1 and 1 μM. The reactions were stopped by adding 350 μl of ethanol, then strongly mixed by vortex, then centrifuged at 14,500 rpm during 15 min, and analyzed by HPLC using a Gemini 5-μm C18 column (150 mm long × 4.6 mm inner diameter) at 22 °C.

To visualize the deglutathionylation reactions, PAP-SG or thiodone (15 μM) was added in a 650-μl reaction mixture containing 30 mM Tris-HCl, pH 8, and 15 μM enzyme. After stopping the reaction by adding ethanol, samples were analyzed by HPLC as described above.

Size Exclusion Chromatography—Size exclusion chromatography experiments have been performed using a Superose 12HR column connected to a fast protein liquid chromatography (FPLC) system. The column was equilibrated with 30 mM Tris-HCl buffer, pH 8, containing 0.15 M NaCl, and the proteins were eluted in the same buffer at a flow rate of 0.25 ml·min^{-1}.

Crystallization and X-ray Diffraction Data Collection—Prior to crystallization, protein samples were concentrated to 40 mg·ml^{-1} PcGSTO1, in TE buffer. Crystals of the SeMet-PcGSTO1 were obtained by using the microbatch method. 1.5 μl of the PcGSTO1 solution at various concentrations and 1.5 μl of various crystallization solutions were deposited in a 72-well Microbatch plate (Hampton Research) pre-filled with paraffin oil and stored at 4 °C. Preliminary results have been obtained from two commercial screening solutions as follows: the condition B2 from the JBScreen classic kit 2 (Jena Bioscience) and the Crystal Screen solution 6 from Hampton Research. Further trials aimed to improve reproducibility and crystal quality gave the best results for PcGSTO1 solutions at 30–40 mg·ml^{-1} and crystallization solutions composed of 20–30% polyethylene glycol 4000 (PEG 4000), 0 to 0.2 M magnesium or calcium chloride, and 0.1 M Tris-HCl, pH 8.5. However, polycrystals grew frequently so that only rare portions of them were suitable for x-ray data collection. No crystal of non-selenomethionylated PcGSTO1 suitable for data collection has been obtained.

TABLE 1
Statistics of X-ray diffraction data collection and model refinement

Data collection	
Space group	*C*2
Cell dimensions	*a*, 166.8 Å, *b*, 70.3 Å, *c*, 72.6 Å; β, 98.8°
Resolution	46.66 to 2.30 Å (2.43 to 2.30 Å)[a]
R_{merge}	3.7% (18.8%)
$I/\sigma I$	19.8 (4.8)
Completeness	98.9% (96.0%)
Redundancy	4.3
Refinement	
Resolution	46.66 to 2.30 Å
No. of reflections	36,622
R_{all}/R_{free}[b]	19.2/22.8
No. of atoms	
Protein	2572 (monomer A) + 2451 (monomer B)
Ligand	1 glutathione (20 atoms) in monomer A
Water	223
B-factors	
Protein	37.9 (monomer A); 41.6 (monomer B)
Ligand	49.4
Water	38.6
Ramachandran statistics	
Residues in preferred regions	95.7%
Residues in allowed regions	4.3%
Outlier residues	0.0%
Root mean square deviations	
Bond lengths	0.011 Å
Bond angles	1.25°

[a] Values in parentheses are for highest resolution shell.
[b] R_{all} is determined from all the reflections (working set + test set), and R_{free} corresponds to a subset of 5% of reflections (test set).

Structure Solution and Quality of the Model—The PcGSTO1 structure was solved using the single wavelength anomalous dispersion protocol of Auto-Rickshaw at the EMBL-Hamburg automated crystal structure determination platform (29). The input diffraction data were prepared and converted for use in Auto-Rickshaw using programs of the CCP4 suite (28). Nine heavy atoms among the 10 requested were found using the program SHELXD (30). Initial phases were calculated after density modification using the program SHELXE (31), at 3.0 Å resolution. The initial phases were improved using density modifica-

tion and phase extension to 2.30 Å resolution using the program DM (32). 70% of the model was built by ARP/wARP (33, 34). It was progressively completed and refined by using iteratively COOT (35) and REFMAC5 (36), including diffraction data up to 2.3 Å resolution. An electron density further attributed to one GSH bound in the active site appeared in monomer A during refinement, although no GSH was added to crystallize PcGSTO1. On the contrary, the corresponding area of monomer B only displayed a few poor spherical densities that more probably corresponded to water molecules. Final model statistics are shown in Table 1.

Most residues corresponded to well defined electron density, except for the N-terminal part of both monomers that appeared too disordered to be observed. Thus, models of monomers A and B contained residues Glu^{38}–Asp^{351} and residues Phe^{54}–Asp^{351}, respectively. Furthermore, the residues Glu^{38}–Ser^{53} only observed in monomer A displayed a weaker electron density and higher *B* factors with respect to the rest of the structure. Figs. 4 and 5 were prepared by using PyMOL.

RESULTS

PcGSTO1 belongs to the first subclass of GSTs Omega displaying a CPWATR potential active site compatible with the CP(W/F)(A/T)(H/Q)R characteristic of the members of this subclass (Fig. 1, *top* and *bottom*) (9). According to the prediction softwares, PcGSTO1 possesses a signal peptide targeting to mitochondria. Different attempts to produce heterologously the full-length protein in *E. coli* resulted in a mixture of truncated proteins and suggested that this N-terminal extension contained putative cleavage sites recognized by the *E. coli* machinery or an intrinsic instability of the precursor. These data were in accordance with the presence of a signal peptide in the N-terminal part of the protein as shown previously for different other redox proteins (37, 38). To obtain a homogeneous protein preparation, a shorter form of PcGSTO1 was produced and purified removing the N-terminal extension of the protein (46 amino acids). Nevertheless, the measured enzymatic activities and specificities (full-length and without signal peptide) described in this study were similar for both forms (data not shown).

PcGSTO3 belongs to the subclass II of the GSTs Omega with an FCPFVQ active site quite similar to the FCPFAE of the HsGSTO1-1 (Fig. 1, *bottom*). A second cysteinyl residue is present at the same position into both proteins (Cys^{94}, PcGSTO3 numbering). Different attempts have been made to produce heterologously the mutated protein PcGSTO3 C94S, but they remained unsuccessful because the resulting protein was fully insoluble.

Thiol Content Determination—To investigate the putative role of the conserved cysteines in the catalytic mechanism of both proteins, site-directed mutagenesis has been used to produce the recombinant proteins in *E. coli*, PcGSTO1 C86S and PcGSTO3 C37S. The thiol content of these proteins has been determined in different conditions as follows: native oxidized corresponding to nonreducing and nondenaturing conditions; denatured oxidized corresponding to a titration in the presence of SDS; reduced and denatured corresponding to a reduction followed by a protein precipitation (see "Experimental Procedures"). A summary of these data is shown in Table 2. Nearly three and two thiols per protein were titrated for the reduced and denatured PcGSTO1 WT and PcGSTO3 WT proteins, respectively, in accordance with the expected thiol content. Under denaturing conditions and after reduction, the obtained values for PcGSTO1 C86S (around two thiols per protein) and PcGSTO3 C37S (around one thiol per protein) were in agreement with the theoretical values. Comparison of values obtained for denatured PcGSTO1 WT and C86S suggested that the sulfur of Cys^{86} was involved at least partially in a DTT-reducible link. For PcGSTO3 WT, one cysteinyl residue was probably involved in a disulfide bridge, whereas for PcGSTO3 C37S, one additional thiol group seemed to be noncovalently bound in the protein.

Fluorescence Spectrometry and Mass Analysis—PcGSTO3 contains two tryptophans adjacent to the active site. Given that proximity, we have investigated whether the intrinsic fluorescence of PcGSTO3 could change under reducing (DTT) or oxidizing (GSSG or diamide) conditions (Fig. 2). The native protein displayed an emission spectrum with a maximum at 350 nm characteristic of a fluorescence signal strongly dominated by Trp. Adding 400 μM DTT to native PcGSTO3 immediately led to a strong increase of the fluorescence signal (Fig. 2). The addition of GSSG (10 mM) on previously DTT-reduced PcGSTO3 strongly decreased the fluorescence signal, whereas addition of diamide (10 mM) did not change the reduced PcGSTO3 fluorescence spectrum (data not shown). Similar experiments have been performed with PcGSTO3 C37S; however, in this case no changes in the fluorescence spectrum have been obtained whatever the reductant and oxidant used. In accordance with thiol titrations, these data suggested that the Cys^{37} of the native PcGSTO3 WT was glutathionylated and that the tryptophan environment strongly changed after glutathionylation of this active site cysteine.

To confirm this hypothesis, PcGSTO3 WT and PcGSTO3 C37S were analyzed by mass spectrometry. A Q-TOF analysis of the DTT-reduced PcGSTO3 revealed a single protein with a molecular mass of 29,615 Da, which was consistent with a protein where the N-terminal methionine was cleaved. After treatment with GSSG, the mass of the protein increased by around 305 Da, a feature consistent with the formation of one glutathione adduct. This obtained value of 29,921 Da also corresponded to the mass of the native protein, and thus confirmed this latter also possessed a glutathione adduct on Cys^{37}. For the mutant PcGSTO3 C37S, the analysis revealed a single protein with a molecular mass of 29,599 Da, which was consistent with a protein without the first methionine and which harbored the Cys to Ser mutation. This result combined with the thiol titration was in agreement with the noncovalent binding of one reduced glutathione to PcGSTO C37S.

Similar experiments (fluorescence and mass spectrometry) have been conducted with PcGSTO1 and PcGSTO1 C86S. Even when a tryptophan residue was present near the putative catalytic cysteine, the fluorescence spectrum remained unchanged whatever the reductant (GSH/DTT) or the oxidant (GSSG/diamide) used. A Q-TOF analysis of the DTT-reduced protein revealed a single protein with a molecular mass of 40,187 Da, which was consistent with a protein where the methionine was cleaved. After treatment with GSSG, the mass of the reduced protein increased

FIGURE 1. **Alignment of PcGSTO1 (*top*) and PcGSTO3 (*bottom*) with homologs.** Amino acids involved in the catalytic site are labeled. NCBI accession numbers are as follows: PcGSTO1 (EU791894), PcGSTO3 (EU 791893), PcGSTO4 (JGI accession number 7168), *Sphingobium chlorophenolicum* PcpF (AAM 96671), *Escherichia coli* YqjG (NP_417573), and *Homo sapiens* GSTO1–1 NP_004823. For PcGSTO1, the putative signal peptide is in *italic*.

TABLE 2

Number of free thiols in WT and mutated PcGSTOs under various redox conditions

The native column is indicative of the protein thiol content measured after purification. Proteins were denatured using SDS treatment or reduced by DTT and subsequently precipitated using TCA. The thiol content per protein was quantified by 5,5-dithiobis(nitrobenzoic acid). Data are represented as mean ± S.D. (n ± 4).

	Native	Denatured	Reduced and denatured
PcGSTO1	0.46 ± 0.12	2.50 ± 0.25	3.0 ± 0.2
PcGSTO1 C86S	0.15 ± 0.03	2.0 ± 0.1	2.04 ± 0.12
PcGSTO3	0.15 ± 0.03	1.00 ± 0.03	1.80 ± 0.13
PcGSTO3 C37S	0.92 ± 0.09	1.78 ± 0.12	1.00 ± 0.04

by around 306 Da, a feature consistent with the formation of one glutathione adduct. This obtained value of 40,493 Da corresponded also to the mass of the native protein and confirmed this latter also possessed a glutathione adduct. These data combined with thiol titration results were consistent with the potential glutathionylation of Cys^{86}, because no glutathione adduct was observed on PcGSTO1 C86S.

Substrate Specificity between PcGSTO1 and PcGSTO3—Both PcGSTOs were found to be active in the two classical glutaredoxin assays as follows: the reduction of DHA and HED assays.

The activity of the PcGSTOs in these two assays increased linearly with increasing protein concentrations in the 0–750 and 0–100 nM concentration ranges, respectively. As expected, these observed "thiol transferase" activities are related to the presence of a cysteine in their catalytic site, because the mutants PcGSTO1 C86S and PcGSTO3 C37S are fully inactive in these assays (data not shown). The kinetic analyses revealed catalytic efficiency values (k_{cat}/K_m) similar between both PcGSTOs against DHA, whereas PcGSTO1 exhibited a slightly stronger activity against HED than PcGSTO3 (Table 3). Interestingly, whereas PcGSTO1 C86S remained inactive in all tested assays, PcGSTO3 C37S exhibited a classical glutathione transferase activity detected using CDNB as the substrate. Both PcGSTOs WT were fully inactive against CDNB.

The HsGSTO1.1, ortholog of PcGSTO3 has been shown to possess activity against various substrates, including dimethylarsinate and 2-bromo-4′-phenylacetophenone (12, 13). In both cases, a glutathione adduct occurred followed by a deglutathionylation reaction catalyzed by the enzyme, preventing access to the initial substrate concentrations and also to kinetic parameters. As expected, PcGSTO3 was active in these tests in the presence of glutathione, whereas PcGSTO3 C37S, PcGSTO1 WT, and PcGSTO1 C86S were fully inactive (data not shown). To determine the catalytic parameters, PAP-SG was synthesized and purified, and the activity against this substrate was followed by analytical HPLC in the presence of GSH (Table 3). In these conditions, only PcGSTO3 is active with a catalytic efficiency of around $4{\cdot}10^5$ M^{-1} s^{-1}, a value greater than that obtained for HsGSTO1-1 (10^4 M^{-1} s^{-1}) (39). The incubation of previously reduced PcGSTO3 in the presence of PAP-SG without addition of GSH led to the formation of phenacylacetophone detected by analytical HPLC (supplemental Fig. 2*A*). This deglutathionylated product is not observed either in the presence of native purified protein (*i.e.* with a glutathione adduct) or in the presence of PcGSTO3 C37S. These results are in accordance with the involvement of Cys^{37} in the deglutathionylation activity leading to the formation of a disulfide bridge with the glutathione removed from PAP-SG. This hypothesis was confirmed by mass spectrometry analysis of the resulting protein showing the presence of a glutathione adduct (data not shown).

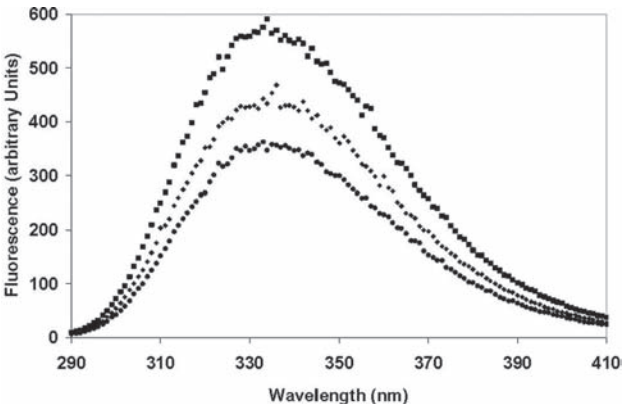

FIGURE 2. **Fluorescence spectra of PcGSTO3 under different redox states.** Emission spectra of native (♦), DTT-reduced (■), and subsequently GSSG-oxidized (●) PcGSTO3 (excitation at 290 nm) were recorded with 10 μM protein at 25 °C in TE buffer, pH 8.0,. The reduced PcGSTO3 was obtained by a treatment of 10 μM protein with 400 μM DTT. PcGSTO3 was oxidized using 10 mM GSSG after DTT reduction.

In additional experiments, we tested quinones previously incubated with reduced glutathione as substrates for PcGSTOs. In these conditions, Michael addition occurred leading to the formation of glutathione adducts corresponding to *S*-glutathionyl-*p*-hydroquinones (40, 41). As shown in Fig. 3, addition of reduced glutathione strongly modified the absorbance spectrum of 1,4-benzoquinone with the appearance of a peak maximum at 305 nm in accordance with the formation of *S*-glutathionyl-*p*-benzohydroquinone as described previously (42). Addition of PcGSTO1 led to a strong modification of the absorbance spectrum, with the disappearance of the 305-nm peak and the formation of a peak at 290 nm corresponding to *p*-benzohydroquinone. In contrast, no effect was observed in the presence of PcGSTO1 C86S and PcGSTO3 (data not shown). All these data were consistent with a deglutathionylation reaction of *S*-glutathionyl-*p*-benzohydroquinone. As expected, the activity of PcGSTO1 against *S*-glutathionyl-*p*-benzohydroquinone led to the production of GSSG as revealed by a coupled enzymatic test performed using glutathione reductase (data not shown). Similar data were obtained using menadione (2-methyl-1,4-naphthoquinone), the glutathionylated product (*S*-glutathionyl-naphthoquinone) being characterized by an absorbance peak at 425 nm (data not shown) (43). The obtained results suggested a preference of PcGSTO1 for aromatic substrates in contrast to PcGSTO3. To obtain catalytic parameters,

TABLE 3

Kinetic parameters of PcGSTO1 and PcGSTO3 in HED, DHA, and CDNB activity assays

The apparent K_m value for GSH in the DHA assay was determined using a GSH concentration range of 0.2–4 mM in the presence of 2 mM DHA for PcGSTO1 and PcGSTO3. The apparent K_m value for GSH was determined for PcGSTO3 C37S in the CDNB assay using a GSH concentration range of 0.1–2 mM in the presence of 2 mM CDNB. The apparent K_m value for DHA was determined using a concentration range of 0.1–4 mM in the presence of 3 mM GSH. The apparent turnover values (k_{cat}) for HED were determined in the presence of 4 mM GSH and 4 mM HED. The apparent K_m value for CDNB was determined using a concentration range of 0.1–2 mM in the presence of 2 mM GSH. Concerning PAP-SG and thiodione, the apparent K_m values were determined using a substrate concentration range of 0.015–4 mM. The apparent K_m and k_{cat} values were calculated by nonlinear regression using the Michaelis-Menten equation. Data are represented as mean ± S.D. (n ± 3). ND means not detected. The detection limit was estimated at 0.5 mIU for spectrophotometric tests, whereas 0.5 nmol of either menadione or phenacylacetophenone was detected using analytical HPLC.

	GSH	HED	DHA	CDNB	PAP-SG	Thiodione
K_m (mM)						
PcGSTO1	1.65 ± 0.33		1.84 ± 0.40	ND	ND	2.00 ± 0.62
PcGSTO3	1.44 ± 0.25		0.33 ± 0.07	ND	0.47 ± 0.19	ND
PcGSTO3 C37S	0.18 ± 0.04			0.97 ± 0.27	ND	ND
k_{cat} (s^{-1})						
PcGSTO1		2.67 ± 0.01	1.81 ± 0.17		ND	19.75 ± 2.31
PcGSTO3		15.75 ± 0.26	0.97 ± 0.06		19.01 ± 2.89	ND
PcGSTO3 C37S		ND	ND	18.43 ± 1.90	ND	ND

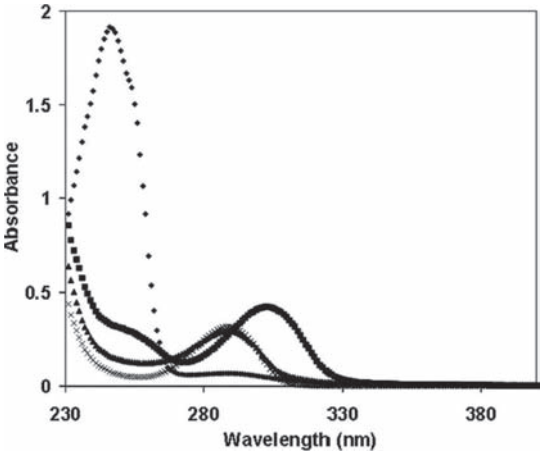

FIGURE 3. **PcGSTO1 activity against *S*-glutathionyl-*p*-benzohydroquinone.** Absorption spectra have been recorded adding successively in 1 ml at 25 °C in TE buffer, pH 8.0, 1 μM *p*-benzoquinone (♦), 5 μM reduced GSH (■), and 0.28 μM PcGSTO1 (♦). In a separate experiment, as control, absorption spectrum of 1 μM *p*-benzohydroquinone was recorded at 25 °C in TE buffer, pH 8.0.

S-glutathionyl-naphthoquinone, also called thiodione, was synthesized and purified, and the activity against this substrate was followed by analytical HPLC (Table 3). The activity of PcGSTO1 against thiodione leads to the formation of a product that elutes with the same retention time as menadione (data not shown). The k_{cat} value with this substrate is around 10-fold higher than the values obtained with HED and DHA. No activity against thiodione was detected using PcGSTO1 C86S, confirming the importance of the cysteinyl residue in the catalysis of the deglutathionylation reaction. In additional experiments, no glutathione was added in a mixture containing PcGSTO1 and thiodone, the results of the reaction being analyzed by HPLC (supplemental Fig. 2*B*). In these conditions, addition of reduced PcGSTO1 led to the formation of menadione. Analysis by mass spectrometry of the resulting protein showed the presence of a glutathione adduct (data not shown). No menadione formation was observed when PcGSTO1 C86S was used.

Dimerization—Size exclusion chromatography and dynamic light scattering were used to determine the oligomeric nature of the native forms of PcGSTO1 and PcGSTO3. The retention times of the size exclusion chromatography allowed estimation of the following native molecular masses: ~85,000 Da for PcGSTO1 and ~62,000 Da for PcGSTO3. Similar results have been obtained using dynamic light scattering suggesting that both enzymes were dimers.

Crystal Structure of PcGSTO1—The structure of PcGSTO1 has been solved to 2.3 Å resolution by single wavelength anomalous dispersion using crystals of SeMet-PcGSTO1 (Table 1). The crystals belonged to the space group *C*2, and the asymmetric unit consisted of two polypeptide chains (monomer A, residues Glu^{38}–Asp^{351}; monomer B, residues Phe^{54}–Asp^{351}; root mean square deviation, 0.348 Å for 298 superimposed Cα atoms), one GSH molecule not covalently bound and 223 water molecules. Crystal packing analysis using PISA (44) suggested that the asymmetric unit content corresponded to a dimer. The two protomers are related to each other by a noncrystallographic 2-fold symmetry axis.

PcGSTO1 adopts the canonical GST fold composed of an N-terminal thioredoxin-like domain and a C-terminal α-helix domain. It also possesses several additional features as follows: a long N-terminal coil of 77 residues; a loop that connects β2 to α2 elongated by 20 residues compared with typical GSTs; and a C-terminal extension corresponding to a ninth α-helix followed by a coil of 20 residues (Fig. 4 and supplemental Fig. 1).

This helix α9 (Phe^{319} to Ile^{333}) is the only secondary structure among these supplementary features. It adds to the α-helix domain, near the C-terminal end of helix α6, so that it also interacts with the thioredoxin-like domain via the short loop that connects the strand β1 and the α-helix α1 and via the long loop that goes from β2 to α2. Helix α9 is a structural characteristic of GST Omega (10) and is also present in GST Tau (45) and GST Delta (46). In these GSTs, the few residues that follow α9 (when they exist) go toward the thioredoxin-like domain to interact with the loops β1-α1 and β2-α2. In PcGSTO1, the long loop observed between β2 and α2 hinders this interaction, so the 20 residues that follow α9 in PcGSTO1 form a coil that runs at the opposite side of the thioredoxin-like domain, antiparallel to α9.

This C-terminal coil allows the formation of a dimer that completely differs from the usual GST dimer, where the two PcGSTO1 monomers associate exclusively via their α-helix domains (Fig. 4), with a large buried area (3100 $Å^2$) as determined by PISA (44). After α9, the 20 C-terminal residues of one monomer mainly interact with residues of the helix α5 N-terminal end (H-bonds to the side chains of Gln^{231} and Tyr^{234}), next with a residue of the α6 C-terminal end (Thr^{283}), and then with a residue of the loop that follows (Asn^{288}), in the other monomer. These direct hydrogen bonds only concern the main chain atoms of the C-terminal end and add to several hydrophobic contacts. Is this dimer a crystal artifact or a physiological entity? The large surface of contact between the protomers and the involvement of the C-terminal coil, specific to PcGSTO1 and related proteins, in the inter-monomer interactions argue for a physiological dimer, as do the dynamic light scattering and size exclusion chromatography results. Furthermore, this dimer, different from the canonical GST dimer, is similar to the dimer assigned by Cuff *et al.* (Midwest Center for Structural Genomics) in the crystal structure of a putative glutathione *S*-transferase from *Corynebacterium glutamicum* recently released by the Protein Data Dank (PDB code 3M1G).[4] The similar dimerization mode appears clearly related to a monomer structure found by DALI (47) to be the closest to the PcGSTO1 structure (root mean square deviation, 1.092 Å for 233 superimposed residues).

PcGSTO1 also possesses an N-terminal extension with respect to other GSTs, by far much longer than the 20 residues observed in GSTOs, because 77 residues precede the entrance to β1. No electron density was observed for the 37 first residues, which were thus supposed to be highly disordered. Residues Glu^{38} to Ser^{53} were only observed in monomer A (see under "Experimental Procedures"). The main chain of residues 38–53 first runs between the long loop that connects β2 to α2 and the

[4] M. E. Cuff, N. Marshall, G. Cobb, and A. Joachimiak, unpublished results.

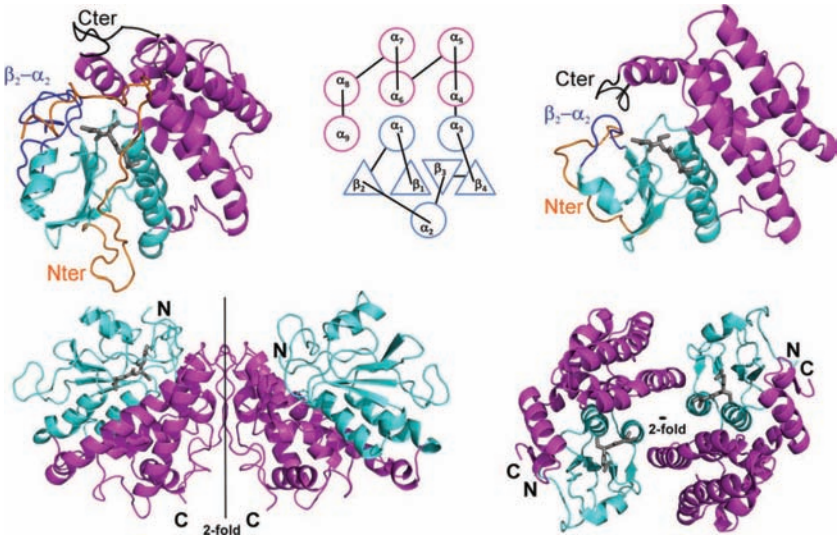

FIGURE 4. **Schematic drawing of the PcGSTO1 (*left*) and HsGSTO1-1 (10) (*right*) structures.** The monomers are displayed at the *top* separated by their common topology diagram mentioning the secondary structure numbering; the dimers are shown at the *bottom*, with orientations chosen to render their assembling at most. In each case, the thioredoxin-like domain is colored in *cyan*, and the α-helix domain is in *purple*. In monomers, the other colors emphasize the features that distinguish PcGSTO1 from HsGSTO1-1 as follows: the N-terminal coil in *orange*, the loop that connects the strand β2 to the α-helix α2 in *blue*, and the C-terminal coil in *black*. The *gray sticks* correspond to glutathione, only present in one monomer of PcGSTO1 and in the two monomers of HsGSTO1-1.

loop that goes from α2 to β3 in the N-terminal thioredoxin-like domain. Then it interacts with the short loop between α4 and α5 in the α-helix domain, where it forms a turn before it enters the region Phe^{54}–Arg^{77} observed in both monomers. This segment adopts an extended conformation to return toward the N-terminal domain. There it interacts with the loops that connect α2 to β3 and β4 to α3, before it runs all along α3, parallel to it. Then it enters β1. The conformation of the PcGSTO1 N-terminal end confers it with the original property to form part of the active site, composed of the G-site for GSH binding and the H-site, which receives the hydrophobic part of the glutathionylated substrate.

The G-site—Only monomer A displayed an electron density consistent with modeling of a GSH molecule (see under "Experimental Procedures"). However, no major structural difference between monomers A and B was observed that could account for an asymmetric binding of GSH. Nevertheless, in this structure only residues that belong to monomer A contributed to GSH binding.

No disulfide bridge exists between the GSH cysteine side chain and the catalytic residue Cys^{86} situated at the N-terminal end of helix α1 (Fig. 5). The sulfur to sulfur distance is 3.9 Å; the electron density is unambiguous, and careful analysis of the diffraction data reveals no radiation damage that could have disrupted a pre-existing disulfide bond. Superposition of the structures of PcGSTO1 and HsGSTO1-1 (where a disulfide bridge exists) shows a conserved orientation of the catalytic cysteine side chain. On the contrary, a displacement of the GSH Sγ2 atom is observed in PcGSTO1, which results from slight differences in the rotation angles around the bond between Cγ1 and Cδ1 in the GSH glutamyl residue and around the bond between Cα2 and Cβ2 in the GSH cysteinyl side chain. The latter points toward three tyrosine residues (Fig. 5), the hydroxyl groups of which form a hydrogen bond network; in particular, the GSH Sγ2 atom lies at 3.25 Å from the Tyr^{223} OH group tightly hydrogen-bonded to the Tyr^{326} OH.

In addition to the usual interactions with the main chain of a residue (Val^{158}) that precedes the conserved *cis*-proline at the entrance of the strand β3, the GSH cysteine main chain forms an additional hydrogen bond between its CO group and the Nϵ1 atom of Trp^{119}, situated in the long loop between β2 and α2 absent in most GSTs (Fig. 5). In the same way, besides the usual stabilization of the GSH glutamyl residue (main chain by residues Glu^{173} and Ser^{174} from the loop between β4 and α3 and aliphatic part of the side chain by hydrophobic contact with Trp^{88} from the N-terminal end of α1), an additional hydrogen bond was observed between the GSH glutamyl atom Oϵ1 and the Nη1 atom of Arg^{56} in the long N-terminal extension of PcGSTO1. Finally, the oxygen atoms of the carboxylic group of the GSH glycyl residue form two hydrogen bonds with the side chain of Arg^{155} situated in the loop between α2 and β3 slightly longer than in the other GSTs (Fig. 5), although usually only one bond is observed with a basic residue (48).

To summarize, some of the additional features that PcGSTO1 displays with respect to other GSTs (the long N-terminal end, the long loop between α2 and β2, and the few supplementary residues between α2 and β3) contribute to GSH binding. They squeeze GSH so much that only 45 Å^2 of its surface is accessible to solvent (Fig. 5). As a comparison, this value is 148 Å^2 in HsGSTO1.1. The active site seems so enclosed that it raises the question of its accessibility not only to the GSH part of the glutathionylated substrate but also to its hydrophobic part.

The Putative H-site—The lack of a structure of PcGSTO1 with a bound *S*-glutathionylated substrate forbids direct experimental delineation of the H-site. The PcGSTO1 surface shows no clear accessible cavity that could accommodate the hydrophobic part of the aromatic *S*-glutathionylated substrate (Fig.

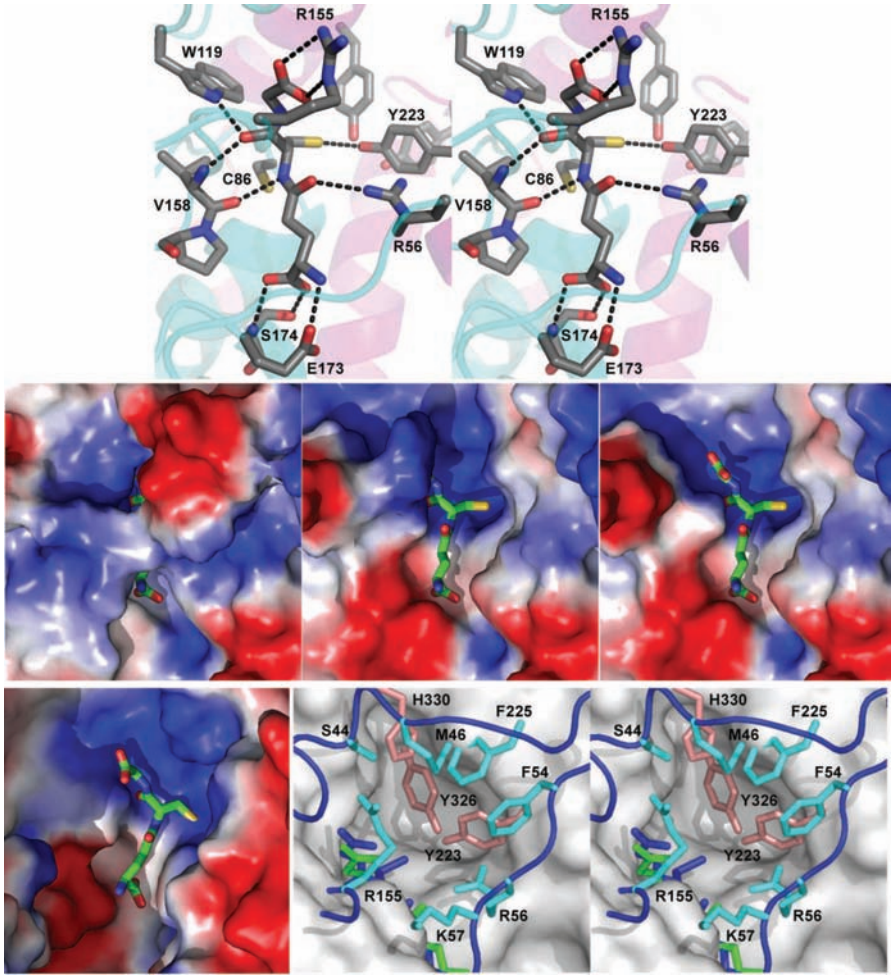

FIGURE 5. **Substrate-binding sites of PcGSTO1 and HsGSTO1-1 (10).** *Top,* stereo view of the hydrogen bond network formed by the active site residues around GSH in PcGSTO1. The schematic trace of the polypeptide main chain is suggested in *cyan* (thioredoxin-like domain) and *purple* (α-helix domain). *Middle,* PcGSTO1 electrostatic surface (*blue,* positive charge; *red,* negative charge) of PcGSTO1 in the region of GSH (shown as *sticks*). *Left panel,* calculated with all the residues observed for monomer A in the crystallographic model (Glu^{38}–Asp^{351}); *middle panel,* calculated without the N-terminal coil (*i.e.* with residues Tyr^{78}–Asp^{351}); *right panel,* same as previous but without Arg^{155}. *Bottom,* HsGSTO1-1 active site compared with PcGSTO1. *Left panel,* electrostatic surface calculated for the HsGSTO1-1dimer, in the region of GSH (shown as *sticks*). *Right panel,* stereo view of the accessible surface of HsGSTO1-1 (*gray*) in the region of GSH (*green*), superimposed to the PcGSTO1 active site containing the GSH (*blue sticks*). Residues Tyr^{223}, Tyr^{326}, and His^{330} fill the pocket defined by Board *et al.* (10) as the HsGSTO1-1 active site. Residues in *cyan* and in *orange* form a second and a third layers of residues that almost close the PcGSTO1 active site.

5). Comparison with HsGSTO1-1 emphasizes the absence of a large binding pocket; the bottom of the HsGSTO1-1 putative H-site (formed by Phe^{31}, Cys^{32}, Pro^{33}, Arg^{183}, Trp^{222}, and nearby residues (10)) is filled, in PcGSTO1, by Tyr^{223} and Tyr^{326}, which surround the GSH cysteinyl side chain, and by Arg^{56}, Cys^{86}, and His^{325} (Figs. 1 and 5). Ser^{44}, Met^{46}, Phe^{54}, Lys^{57}, Arg^{155}, Phe^{227}, and His^{330} form a narrow, deep cylindrical hole, incompatible with access and binding of a bulky substrate.

Searching in the PDB for X-ray structures of GSTs in complex with *S*-glutathionylated substrates, 1-(*S*-glutathionyl)-(2,4-dinitrobenzene) was found to be the closest analog to *S*-glutathionyl-*p*-benzohydroquinone. It has been used as a substrate by the GSTs of the classes Mu from rat (PDB code 5GST (49)) and human (PDB code 1XWK (50)), Pi from human (PDB code 18GS (51), Sigma from squid (PDB code 1GSQ (52)), and Alpha from chicken (PDB code 1VF3).[5] In each of these structures, 1-(*S*-glutathionyl)-(2,4-dinitrobenzene) leans its benzene ring against the hydrophobic side chains of the residues that belong to the α4 C-terminal end, and it often interacts with the first N-terminal residue of α1 (using PcGSTO1 numbering of secondary structures). Comparable interactions could be obtained using Trp^{88}, Tyr^{223}, and Phe^{227}, but they would require a slightly different positioning of the 1-(*S*-glutathionyl)-(2,4-dinitrobenzene) glutathionyl part with respect to the current position of GSH in PcGSTO1. Indeed, in the structure, GSH appears too shifted toward the α-helix domain, with its cysteinyl sulfur atom exactly aligned on the α1 axis. The current position of the long N-terminal loop shows that residues such as Met^{46}, Phe^{54}, or Arg^{56} could also be involved, but once again,

[5] S. C. Lin, Y. C. Lo, M. F. Tam, and Y. C. Law, unpublished results.

the main question concerns access of the substrate to its binding site, which would likely require a movement of the N-terminal loop.

DISCUSSION

Compared with other organisms, the GSTO class of enzymes is considerably larger in saprotrophic fungi and particularly in *P. chrysosporium*. From primary structure homology, this class could be divided in three different subclasses in fungi (9), one related to plant Lambda GSTs and bacterial GSTs, whereas the second and the third subclasses are related to animal isoforms. In accordance with our first phylogenetic analysis (9), Xun *et al.* (17) have recently proposed from a study of the bacterial homologs of PcpF that the proteins belonging to the Omega subclass I constitute a new class of GSTs named *S*-glutathionyl-(chloro)hydroquinone reductases.

The present x-ray structure of PcGSTO1 confirms that it should be considered as the member of a new class of GSTs. Indeed, the GST canonical fold (a thioredoxin-like domain followed by a α-helix domain) is complemented by additional specific features as follows: the long N-terminal coil (77 residues) that covers the active site and participates in glutathione binding, as does the long loop (about 30 residues) between the strand β2 and the α-helix α2, and finally the C-terminal coil (20 residues after the ninth α-helix) that allows an original dimerization mode where the monomers interact via their α-helix domain (also observed for a putative GST from *C. glutamicum*.[4] All these features clearly contrast with the HsGSTO1-1 structure (10).

As shown for members of the Omega class, the main characteristic of GHRs is the presence of a conserved cysteine in their catalytic site. From a biochemical point of view, as shown previously (10) and in this study, GHRs and GSTOs share the ability to reduce various substrates such as HED and DHA using glutathione. Concerning PcGSTO1 and PcGSTO3, we propose from fluorescence experiments (for PcGSTO3) and free thiol and mass measurements that the catalytic cysteine is involved in the deglutathionylation activity leading to the formation of a disulfide bridge with the removed glutathione from their substrate. This hypothesis is in accordance with experiments performed using thiodione or PAP-SG as substrates, because the appearance of the deglutathionylated products is observed in the absence of added glutathione, a glutathione-adduct being detected in these conditions on the resulting proteins. Furthermore, these activities require the presence of the catalytic cysteine. This common mechanism has been described at least for monothiol glutaredoxin (23), human GSTOs (10), and bacterial GHRs (16). In PcGSTO3, as shown previously for HsGSTO1-1, a change of the catalytic cysteine to a serine allows reversal of the activity of the protein allowing the mutated protein to catalyze glutathione transfer, and it also confirms the presence of a disulfide bridge between Cys-37 and glutathione in PcGSTO3 WT. For PcGSTO1, biochemical data are also in accordance with the formation of a disulfide bridge between the catalytic Cys-86 and glutathione. Similar results have been obtained for the bacterial ortholog PcpF (16). However, this disulfide bridge is not present in the crystal structure of PcGSTO1. X-rays could have disrupted the bond, but this should have taken place very rapidly at the beginning of the radiation exposure because no residual electron density corresponding to this bond was observed and the sulfur to sulfur distance is large (3.9 Å), contrary to documented cases where such a phenomenon has been described. At the moment, the reasons for the discrepancy between the PcGSTO1 x-ray structure and the results of thiol titrations concerning the presence of a disulfide bond remain to be elucidated.

As defined by Xun *et al.* (17), the main biochemical characteristic of GHRs is the ability to remove glutathione from *S*-glutathionyl-(chloro)hydroquinone substrates. In this study, we have shown that in contrast to PcGSTO3, PcGSTO1 is active against *S*-glutathionyl-*p*-hydroquinone. Binding of *S*-glutathionyl-*p*-hydroquinone can be modeled from the PcGSTO1 structure, one monomer of which was observed in complex with GSH. Glutathione adopts a conformation very close to that observed in HsGSTO1-1. However, on the contrary to the latter, GSH is more buried in PcGSTO1. It results from new interactions with residues that belong to the features characteristic of PcGSTO1 as follows: Arg^{56} in the N-terminal coil, Trp^{119} in the loop β2-α2, and Arg^{155} in the loop α2-β3. The additional coils close the cleft usually present between the Trx-like domain and the α-helix domain, near the loop β1-α1 in which is the catalytic residue Cys^{86}. Furthermore, the side chains of Tyr^{223} and Tyr^{326} here shut up the pocket present near the catalytic cysteine in HsGSTO1-1. Even if conformational adaptation occurs upon substrate binding, it would hardly concern these residues because they belong to α-helices. Hence, a substrate-binding site different from the putative pocket in HsGSTO1-1 has to be considered for the hydroquinone adduct. It could involve Trp^{88}, Tyr^{223}, and Phe^{227}. All the PcGSTO1 residues involved either in the G-site or the putative H-site are conserved in the bacterial homologs (ScPcpF and EcYqjG, see Fig. 1), although they are different or absent in other GSTs (see supplemental Fig. 1). Once again, comparison of the PcGSTO1 and HsGSTO1-1 structures in the light of their substrate-binding site emphasizes the need to consider GHRs as having a new GST structure, different from the Omega class.

In agreement with our previous phylogenetic analysis (9), the biochemical and structural data presented in this study and the recent proposition of Xun *et al.* (17), we propose to change the name of PcGSTO1 in PcGHR1 with regard to the enzymatic function and to define a new structural class of GSTs named GST xi, characterized by their canonical GST fold complemented by specific features and their new dimerization mode. Thus, this paper describes this newly defined *S*-glutathionyl-(chloro)hydroquinone reductase from *P. chrysosporium* as a member of the new structural class xi of GSTs, which would also contain the structure of the putative GST from *C. glutamicum*.

As for PcpF (16), PcGHR1 in *P. chrysosporium* could be involved in the catabolism of pentachlorophenol. In this fungus, the reductive dehalogenation of pentachlorophenol requires indeed at least two enzymes, a glutathione transferase for glutathionylation and an enzyme called glutathione conjugate reductase for deglutathionylation of the resulting *S*-glutathionyl-*p*-hydroquinones (53–55). From the biochemical data presented in this study, we postulated that PcGHR1 or the second

isoform PcGHR2 (formerly named PcGSTO2 (9)) is responsible for this latter reaction. The wide presence of GHRs in bacteria, fungi, and plants suggests an additional physiological role beyond the degradation of the recently introduced pentachlorophenol. Concerning the white rot fungi, their main physiological feature is their ability to mineralize wood components and in particular lignin. It has been suggested recently that chlorination of lignin is a phenomenon occurring through, at least in part, the activity of chloroperoxidase (56, 57). As a result, soils and decayed plant litter contain significant quantities of chlorinated aromatic polymers, which could lead after oxidation via various oxidative systems to the formation of chlorinated quinones. GHRs could play a central role in the intracellular detoxication pathways of such molecules. On the other hand, the significance of the Omega class extension in saprotrophic fungi remains unclear, and the function of the different GSTO isoforms in white rot fungi remains to be elucidated.

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Article IV

Specificities of Ure2p-like glutathione transferases from *Phanerochaete chrysosporium*

Introduction

The Ure2p class is found extended in *P. chrysosporium* in comparison with various fungi, since nine ure2p encoding genes could be found in this fungus (Morel et al., 2009a) Based on phylogenetic analysis and sequences, two subclasses could be defined. Among the subclass I, eight isoforms are related, from primary structure homology, to the single *Saccharomyces cerevisiae* Ure2p. However, this ScUre2p can be distinguished from the PcUre2ps by the presence on a prion domain in the yeast protein. ScUre2p has been shown to be involved in the nitrogen catabolite repression (NCR) (Baudin-Baillieu et al., 2003). ScUre2p is able to bind ScGLN3p retaining it in the cytoplasm. ScGLN3p is known to act as a regulator of genes involved in nitrogen metabolism. The second subclass (subclass II), contains AnGSTA (AN4905) from *Aspergillus nidulans* and one isoform from *P. chrysosporium* (PcUre2p1). AnGSTA has been shown to be involved in heavy metal and xenobiotic resistance but not in NCR contrary to the yeast isoform. This project describes the different approaches used to try to understand the role of this class of GSTs in *P. chrysosporium*.

Results

The main result of this work is the specific induction of PcUre2p4 and PcUre2p6 genes in presence of polycyclic aromatic compounds. We showed that *P. chrysosporium* is able to i) remove PAH from the culture media, especially for fluor-derivatives and ii) sequester them inside intracellular lipid vesicles. The intracellular PAH could thus create oxidative damages inside the cell and the up-regulation of PcUre2p4 and PcUre2p6 could be related to detoxication processes or oxidative stress rescue. The recombinant proteins have been produced and their enzymatic activities determined. They exhibit a classical GSH transferase activity using CDNB as substrate, while PcUre2p1, belonging to a separate subclass, rather exhibits thiol transferase activity. The *in vivo* functions of these proteins remain to be determined since no complementation in a yeast strain devoted for Ure2p gene could be obtained. We have tested the ability of the complemented strains to control NCR and to restore growth after various stresses. However, none of these conditions allowed highlighting these functions for PcUre2p1, PcUre2p4 and PcUre2p6.

Specificities of Ure2p-like glutathione transferases from *Phanerochaete chrysosporium*

Andrew A. Ngadin[1], Cécile Caupert[2], Patrick Billard[2], Jean-Pierre Jacquot[1], Eric Gelhaye[1] and Mélanie Morel[1]*.

[1]Unité Mixte de Recherches INRA UHP 1136 Interaction Arbres Microorganismes, IFR 110 Ecosystèmes Forestiers, Agroressources, Bioprocédés et Alimentation. Faculté des Sciences et Technologies, Nancy Université BP 70239, 54506 Vandoeuvre-lès-Nancy Cedex France

[2]Laboratoire des Interactions Microorganismes-Minéraux-Matière Organique dans les Sols, UMR 7137 CNRS –UHP. Faculté des Sciences et Technologies, Nancy Université BP 70239, 54506 Vandoeuvre-lès-Nancy Cedex. France

*Corresponding author: Mélanie Morel

Unité Mixte de Recherches INRA UHP 1136 Interaction Arbres Microorganismes, IFR 110 Ecosystèmes Forestiers, Agroressources, Bioprocédés et Alimentation. Faculté des Sciences et Technologies, Nancy Université BP 70239, 54506 Vandoeuvre-lès-Nancy Cedex France

Tel ++ 33 3 83 68 42 27; Mail address: mmorel@scbiol.uhp-nancy.fr

Running title: Ure2p of *P. chrysosporium*

Key words: *Phanerochaete chrysosporium*, glutathione transferase, Ure2p, polycyclic aromatic hydrocarbons

Abstract

The glutathione transferase (GST) proteins represent an extended family involved in the detoxification processes. They are divided in various classes with high diversity, depending on the organism. In *Phanerochaete chrysosporium*, the Ure2p class represents one third of the total identified GSTs and the sequences cluster into two different subclasses. Three Ure2p class members have been studied in more detail at transcriptional, biochemical and physiological levels. PcUre2p4 and PcUre2p6 of the first subclass are specifically expressed in cultures treated with polycyclic aromatic hydrocarbons and the recombinant proteins are active as typical glutathione transferases. By contrast, PcUre2p1, which belongs to the second subclass (also called cluster 2) is expressed for whatever the condition tested and is active with small molecules as substrate, such as proteins from the omega class. Physiological studies have revealed that these proteins do not have the same function as the *Saccharomyce cerevisiae* isoform, with respect to both their response to oxidative stress and its involvement in the nitrogen catabolite repression. These results suggest that fungi, especially those with saprophytic capabilities, have developed specific of Ure2p function as an adaptation to environmental constraints.

Introduction

The glutathione transferase (GST) proteins represent an extended family with high diversity depending on the organism [1,2,3]. These enzymes have such a name since they are able to transfer glutathione (GSH) onto hydrophobic molecules. Due to this activity, GSTs have been shown to be involved in biotransformation pathways, since the addition of glutathione onto molecules previously oxidized by detoxification phase I enzymes, results in the formation of compounds which are usually less toxic and more soluble. These less-toxic peptide derivatives are ready to be excreted or stored by phase III enzymes. Additionally, these proteins can exhibit other activities such as thiol transferase or peroxidase activities [4,5,6], suggesting that they could directly participate in the cellular response to oxidative stress. In a recent paper, we have studied the diversity of fungal GSTs, considering zygo-, asco- and basidiomycetes [7]. The major result of this study is the potential link which could exist between the number of GST encoding sequences in the analyzed genomes and the fungal way of life. Indeed, 46 and 27 GST-encoding genes have been found in the genomes of *Postia placenta* and *Phanerochaete chrysosporium* respectively, compared to only 10 for the pathogen *Ustilago maydis* or 11 for the yeast *Saccharomyces cerevisiae. P. placenta* and *P. chrysosporium* are ligninolytic basidiomycetes, which use unspecific oxidative reactions to degrade lignin and, by extension, recalcitrant compounds. In *P. chrysosporium*, extracellular peroxidases are involved in coordination with numerous oxidases [8]. In contrast, *P. placenta* is thought to degrade lignin by secreting various small iron-binding molecules initiating Fenton reaction [9]. Besides these extracellular systems, oxidation of recalcitrant molecules could also occur from the action of various cell-wall linked oxidases such as cytochrome p450 monooxygenases [10]. In the genome of these both fungi, a huge number of cytochromes p450 monooxigenases-related genes have been detected compared to other fungi [11,12]. Such a diversity of cytochrome p450 monooxygenases has to be related with the high

occurrence of GST encoding genes in their genome and thus to their ability to metabolize recalcitrant compounds found in their natural ecosystem.

Another interesting point is the over-representation of a particular class of GSTs called Ure2p both in the genome of *P. placenta* and *P. chrysosporium.* For these species, the Ure2p class represents one third of the total identified GSTs (9/27 for *P. chrysosporium* and 17/46 for *P. placenta*). *P. chrysosporium* Ure2p sequences cluster into two different subclasses. The first one, enclosing 8 of the 9 sequences, is related to the single Ure2p isoform of *S. cerevisiae*. In yeast this protein is known to act as a negative regulator of the nitrogen catabolite regulation (NCR) in response to primary nitrogen source by disabling Gln3p to activate transcription [13]. Moreover, the inability of a *URE2* deleted mutant strain (*Δure2p*) to grow in presence of hydrogen peroxide (H_2O_2) has been demonstrated as well as a relationship between diminishing levels of glutathione and peroxide sensitivity was established. It was suggested that the susceptibility of the *Δure2p* strain to the exogenous H_2O_2 can result from increased GSH degradation due to the deregulated localization of the gamma-glutamyl transpeptidase activating factors Gln3p/Gat1p [14]. The last *P. chrysosporium* sequence, named PcUre2p1, clusters in another group containing *GSTA* (AN4905) from *Aspergillus nidulans*. GSTA contributes to metal detoxification and contrary to the yeast isoform, it is not involved in the NCR [15].

In this study, three of the *P. chrysosporium* Ure2p proteins have been chosen for a deeper characterization based on their expected role in oxidative stress response.

Material and methods

Strains and culture conditions

The used *P. chrysosporium* strain is the homocaryon RP-78 whose genome has been sequenced [16]. The fungus was maintained on malt (4%) agar (3%) plates. The sporulation

conditions have been described previously [17]. The liquid culture medium consists of sodium acetate 5 mM pH 4.5, glucose 1%, ammonium tartrate 1.075 mM, base medium 1 % (v/v) (KH_2PO_4 20 g/l, $MgSO_4$ 5 g/l, $CaCl_2$ 1 g/l), trace medium 7% (v/v) (Nitrilotriacetate 1.5 g/l, $MgSO_4$ 3 g/l, NaCl 1 g/l, $FeSO_4$ $7H_2O$ 0.1 g/l, $CoCl_2$ 0.1 g/l, $ZnSO_4$ $7H_2O$ 0.1 g/l, $CuSO_4$ 0.1 g/l, $AlK(SO_4)$ $12H_2O$ 10 mg/l, H_3BO_3 10 mg/l, $NaMoO_4$ $2H_2O$ 10 mg/l) and $MnCl_2$ 225 µM. The fungal inoculation was made by adding 2.5 10^6 spores (OD_{650}=0.5) per flask containing 100ml of liquid medium. For the cultures on wood, the fungus was first grown for 3 days on malt agar plates and autoclaved wood chips were then placed on the fungal mat. The fungus was harvested from the wood chips 15 days later. The fungus was also grown in liquid medium containing polycyclic aromatic hydrocarbons (PAH). For this condition, the fungal pellets were first grown without PAH for 5 days to yield biomass. The fungus was then transferred into new flasks containing PAHs. To prepare these flasks, a stock solution of PAH was solubilised in hexane and added into flasks to get final quantities of 2.25 mg phenanthrene, 0.225 mg fluorene, 0.225 mg fluoranthene and 0.225 mg anthracene per flask. 100 ml of liquid culture medium, which composition is described above but without glucose, was added in each flask after complete evaporation of hexane.

Saccharomyces cerevisiae strain Y01983 (BY4741; *MAT*a; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; *ure2*::kanMX4) deleted for *URE2* (*Δure2*) was obtained from EUROSCARF collection (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/index.html). Rich yeast extract 1%, peptone 2%, glucose 2% (YPD) medium and minimal yeast nitrogen base (YNB) supplemented with amino acid drop out (Sigma), $NH_4(SO_4)$ 0.5% and galactose 2% were used. The Ure2p encoding genes of *P. chrysosporium* and *S. cerevisiae* were cloned into the pYES2 vector (Invitrogen) and their expression was achieved in yeast by induction with galactose.

For complementation tests, precultures were done in YPD medium overnight. A yeast cell suspension (OD_{600}~0.1) was then cultivated in supplemented YNB medium as described above. The cells were grown for 3 hours and treated with $CdNO_3$ 25 µM, H_2O_2 2.5 mM and 1-chloro 2,4 dinitrobenzene (CDNB) 50 µM. Growth was followed over time and cells were harvested after 20h of treatment for NAD-glutamate dehydrogenase assays.

The *Escherichia coli* strains used were DH5α for cloning experiments and BL21 (DE3) for protein production.

NAD-GDH assays

The 25 ml yeast cultures were centrifuged 20 min at 5000 rpm and the pellets were resuspended in 1 ml of 50 mM phosphate buffer pH 6.4 with 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The cells were then vortexed with glass beads for two 15 s intervals interspaced with periods of cooling in an ice bath. The slurries were then harvested and centrifuged 10 min at 14500 rpm. The supernatants were designed as the crude extracts.

NAD-GDH activity was measured using the method described by Courchesne and Magasanik [18] with some modifications. A 1ml assay contained Tris 0.1 M pH 8, 2-ketoglutarate 2.5 mM, $NH_4(SO_4)_2$ 50 mM and NADH 0.25 mM at 25°C. The A_{340} decrease was monitored. A blank in which 2-ketoglutarate was omitted was realized. The protein content was determined according to the BCA method (Pierce).

Gene expression

Gene expression was checked by semi-quantitative RT-PCR. Total RNA isolation was performed using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). RNase-free DNase treatment (Qiagen) was applied according to the manufacturer's protocol to avoid genomic DNA contamination. Reverse transcription (RT) reactions were performed with 500 ng of

total RNA using the Masterscript™ Kit (Prime) according to the manufacturer's protocol. RT products were amplified by PCR in the following conditions: DNA denaturation for 1 min at 95°C and 33 cycles at 95°C for 5s, 52°C for 45s, and 72°C for 1 min using Go Taq DNA polymerase (Promega). The suitability of the extracted RNA for RT-PCR amplification was checked by performing RT-PCR control experiments in the same amplification conditions with the ubiquitin encoding gene.

Polycyclic aromatic hydrocarbons (PAHs) quantification

PAHs from total cultures (ie. medium and biomass) were successively extracted three times with dichloromethane (DCM) (vol:vol) in separation funnels. DCM extracts were evaporated in a speedvac concentrator (Thermo Scientific RC1010), and then dried samples were dissolved in acetonitrile for HPLC analyses using a HPLC system (Dionex UltiMate 3000) with a 5 µm Agilent Eclipse PAH of 4.6 X 150 mm at 30°C of column temperature. The compounds were detected and identified through Dionex UV photodiode array detector at 254 nm. The elution buffer of 70 % Acetonitrile and 30 % H^2O was used with a flow rate of 2ml/min.

Microscopic observations

P. chrysosporium pellets grown in liquid cultures with or without PAH as indicated below were crushed and observations were undertaken with an epifluorescence microscope (Nikon E600) with an HQ-FITC-BP filter cube (Chroma) for excitation at 345 nm and emission at 485 nm (DAPI). Pictures were collected with a Nikon D60 Digital SLR camera.

Production and purification of recombinant proteins

Amplifications of GST cDNAs were performed from RT products obtained as described above, using the high proof Herculase DNA polymerase (Stratagene). The PCR products were cloned into the NcoI and BamHI sites of the pET-3d vector (Novagen) resulting in a construction devoid of a His-Tag. The recombinant plasmid was then used to transform *Escherichia coli* strain BL21 (DE3) cotransformed by the helper plasmid pSBET in order to provide the rare t-RNAs for AGG and AGA codons (Schenk et al., 1995). After induction with isopropyl β-D-1-thiogalactopyranoside (IPTG), proteins were purified using a combination of gel filtration and anion exchange chromatography as described in Rouhier et al. [19].

Enzymatic activity measurements

The activities of the recombinant proteins were assayed spectrophotometrically in 1ml reaction medium using CDNB 1 mM, β-hydroxyethyl disulphide (HED) 1 mM, dehydroascorbate (DHA) 1 mM or peroxides as substrates in Tris-HCl 30 mM pH8, EDTA 1 mM buffer [20,21,22]. For the tests using HED, DHA, H_2O_2, tert-butyl hydroperoxyde (t-BOOH) or cumene hydroperoxyde (CuOOH) as substrate, NADPH 180µM and 0.5 µl of purified glutathione reductase were added. Oxidation of NADPH and transformation of CDNB were followed at 340 nm.

The activities of the recombinant proteins using S-Phenacylglutathione as substrate were performed according to Board and Anders [23] in 1 ml reaction medium using 180 µM NADPH, 4 mM GSH, 0.5 µl of purified glutathione reductase, 0.3 mM 2-Bromo-4'-phenylacetophenone. Oxidation of NADPH was followed at 340 nm.

Bioinformatic analysis

Sequences of *P. chrysosporium* were found in the Joint Genome Institute (JGI) database (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html). The sequence alignments were performed using clustalW (http://align.genome.jp/) and predictions of subcellular localization were done using WolfpSort (http://wolfpsort.org/) and Mitoprot (http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html) softwares.

Results

Sequence analysis

The amino acid sequences of Ure2p2 to 9 are quite close showing between 25.1 and 83.2% identity [7]. The proteins exhibit the classical organization of GST *i.e.* a glutathione-binding domain (Thioredoxin domain or G-site) and a α-helical domain (GST C-domain) (Figure 1). The Trx-domain of these proteins is highly conserved, exhibiting amino acids found to be involved in the interaction with GSH in the *S. cerevisiae* isoform (ScUre2p) [24,25]. Asn124, Arg164, Glu180 and Ser181 residues are strictly conserved among the *P. chrysosporium* proteins (Figure 1A). Ala122 and His151 can be replaced by Gly and Gln respectively depending on the proteins. Moreover, PcUre2p2, 4, 6 and 7 exhibit a tyrosine in position 10 in their sequence. PcUre2p1 (Phchr 503) belongs to cluster 2 as described in a previous study [7]. Even if the global organization of PcUre2p1 remains similar to the PcUre2p described above, only Asn48 and Arg93 are conserved in GSH binding-domain (Figure 1B) and a cysteine is present at position 89. Another interesting point is the presence of a putative mitochondrial targeting peptide both in PcUre2p5 and PcUre2p1, suggesting a potential role of these proteins in the organelle.

Expression of PcUre2p genes in stress conditions

The expression of the Ure2p coding genes was monitored in two stress conditions using semi-quantitative RT-PCR (Figure 2). The first one corresponds to ligninolytic conditions, where the fungus is exposed to an oxidative stress due to the production of reactive oxygen species [26]. The second is a condition where the fungus is exposed to PAHs, which are toxic for the cell essentially due to their genotoxicity [27].

After preliminary experiments (data not shown), we focused our experiments on PcUre2p4 and 6 since they exhibited a specific gene expression profile, and on PcUre2p1 as the only member of cluster 2. No difference was highlighted concerning the gene expression in ligninolytic compared to non-ligninolytic conditions (Figure 2A): PcUre2p4 was not expressed and PcUre2p6 and PcUre2p1 were constitutively expressed in both conditions.

Concerning the second stress, *P. chrysosporium* was first grown 5 days without PAH, then collected and transferred to PAH-containing flasks. The mycelium was harvested directly after transfer (T0) and after 10 days of incubation (T10) for gene expression analysis. Whatever the culture conditions, PcUre2p1 was constitutively expressed both at T0 and T10 (Figure 2B). By contrast, PcUre2p4 and PcUre2p6 were specifically expressed after PAH treatment. PcUre2p6 gene expression was even induced just after transfer on the PAH containing medium.

Extracellular and intracellular detection of PAH

In our conditions, *P. chrysosporium* was able to dissipate between 24 to 47% of the initially added PAHs in the culture medium after 15 days of incubation (Figure 3), the highest removal occurring with cyclopentane containg PAHs (fluorene and fluoranthene). Taking advantage of the fluorescent properties of PAHs, we were able to detect their intracellular accumulation in *P. chrysosporium* in our culture conditions (Figure 4). A strong signal was detected in hyphae

treated with PAH during 10 days compared to the control. This accumulation occurred mainly in hyphae, few in spores, and increased with incubation time with PAHs and the signal seemed to be localized in small vesicles.

Enzymatic activities of PcUre2p4, PcUre2p6 and PcUre2p1

The recombinant PcUre2p4, PcUre2p6 and PcUre2p1 proteins have been produced in *Escherichia coli* and purified. Their activities have been tested *in vitro* using various substrates.

PcUre2p4 and PcUre2p6 exhibited activity against CDNB, suggesting a classical GSH transferase activity (Table 1). Both proteins have been found to be inactive as thiol transferase and dehydrascorbate reductase. Contrary to ScUre2p, PcUre2p4 and PcUre2p6 had no glutathione peroxidase activity whatever the peroxide used (hydrogene peroxide, t-butyl peroxide or cumene peroxide). In comparison with PcUre2p4 and 6, the PcUre2p1 primary sequence is slightly modified (Figure 1) and this had a consequence on the catalytic properties of the protein. The purified recombinant protein is fully inactive against CDNB and peroxides. However, PcUre2p1, which belongs to cluster 2, was able to reduce HED and DHA, thus exhibiting a thioltransferase activity (Table 1). This glutaredoxin-like activity has been usually restricted to cysteine-containing GSTs in particular to omega GSTs. A detailed analysis of the primary and tertiary structures of PcUre2p1 shows the presence of one cysteinyl residue in the G-site (Cys 89), precisely in helix 3 of the thioredoxin fold. For comparison purpose, previous results obtained on the human omega GSTs (GTOs) are reported in table 1 [28, 29]. Changing the Cys32 into an Ala in HsGTO1 results in a lost of thiol transferase activity and in an increased activity with CDNB. This is presumably because the inability to form a disulfide allows the bound GSH to form a stable thiolate that readily participates in glutathione conjugation reactions [22]. Surprisingly, the mutated C89S

PcUre2p1 remains fully active against HED and DHA and does not exhibit any activity against CDNB (data not shown). Despite this difference, PcUre2p1 seems to be functionally related to omega GSTs since it is active using HED and DHA. We have then tested other substrates known to be modified by omega proteins. HsGTOs are able to reduce *S*-Phenacylglutathione [23]. We found that PcUre2p4 and 6 are not active against this substrate, while PcUre2p1 is able to reduce it (Figure 4).

All these results suggest that PcUre2p4 and 6 are able to glutathionylate aromatic substrates with rather high molecular weight, while PcUre2p1 acts on smallest molecules by deglutathionylating them. Another interesting point is that the activity of PcUre2p1 is not mediated by its cysteinyl residue, since the C89S mutant remains fully active whatever the condition tested.

Functional analysis of PcUre2p

In *S. cerevisiae*, Ure2p is involved both in the regulation of nitrogen assimilation and oxidative stress response. A mutant deficient in Ure2p (*Δure2*) is sensitive to oxidative stress [14] and allows the transcription of genes involved in nitrogen metabolism whatever the nitrogen source due to the constitutive activity of Gln3p, which is a positive transcriptional regulator. We have used this strain to define the function of PcUre2p4, PcUre2p6 and PcUre2p1. We first tested the role of PcUre2p in the control of the nitrogen metabolism by measuring the activity of the NAD-glutamate dehydrogenase. The gene encoding this enzyme is only expressed when Gln3p is translocated to the nucleus. When Ure2p is induced, it binds to Gln3p, preventing its migration inside the nucleus and consequently the transcription of the nitrogen-regulated genes [18]. The mutant strain exhibited a maximum of NAD-GDH activity since no Ure2p can bind Gln3p (Figure 5). By contrast, no activity was detected in the control corresponding to the mutant strain transformed with the endogenous gene ScUre2p. ScUre2p

was thus able to bind Gln3 as expected, preventing the expression and thus the activity of NAD-GDH. A maximum of activity was detected in the mutant strain complemented with PcUre2p4, PcUre2p6 and PcUre2p1. Besides this, we checked that all PcUre2p encoding genes were expressed in yeast by RT-PCR (data not shown). Thus these results suggest that PcUre2p proteins are not able to bind Gln3p and to control nitrogen-related gene expression contrary to the yeast isoform.

We also tested the involvement of PcUre2p proteins in various stress conditions (H_2O_2 2.5 mM, $CdNO_3$ 25 µM and CDNB 50 µM) by comparing growth of the *Δure2p*, *Δure2p*-Scure2p, *Δure2p*-PcUre2p4, *Δure2p*-PcUre2p6, *Δure2p*-PcUre2p1 strains (Figure 6). In a control condition, the mutant and the complemented strains exhibited a lower growth than the control (*Δure2p*-ScUre2p), suggesting that the lack of Ure2p affects growth even without any stress (Figure 6A). For the CDNB treatment, an increased latency period was observed for the mutant and the complemented strains, and then the growth rate was the same as the control strain (Figure 6B). We observed quite the same profile after H_2O_2 treatment (Figure 6C). In these stress conditions, no difference was observed between the mutant and the mutant complemented with PcUre2p. These results suggest that the *P. chrysosporium* Ure2p don't have the same role than ScUre2p in protecting the cell against oxidative stress. We additionally showed that for a Cd treatment, the strains expressing PcUre2p, especially PcUre2p4, were more sensitive than mutant one (Figure 6D).

Discussion

The single Ure2p isoform of *S. cerevisiae* has been well studied for its prion properties and its involvement in the nitrogen catabolite repression (NCR). More recently its role in the oxidative stress response has been demonstrated [14]. However, nothing was really known about this particular class of GST in other fungi. We showed in a previous paper that this

class exists in many asco- and basidiomycetes but not in zygomycetes [7]. The major difference between the yeast isoform and PcUre2p is the presence of the prion domain in ScUre2p [30]. This Gln/Asn rich sequence at the N-terminal end is required for aggregation properties of the protein. However, deletion of this N-terminal region has no effect on the stability or folding of the protein *in vitro* [31]. Among the fungi analysed, only the *Candida albicans* protein exhibits the N-terminal prion domain, suggesting that the other proteins have evolved separately. In particular, the isoforms exhibiting a Tyr instead of a Phe in position 105 in the *S. cerevisiae* sequence appeared more recently [32], suggesting that the prion domain has been lost during evolution. PcUre2p 2, 4, 6 and 7 exhibit a tyrosine in position 10 in their sequence. This tyrosine hydroxyl group is though to act as a hydrogen bond donor to the sulphur of GSH, lowering its pK_a to stabilize a nucleophilic thiolate [33]. The presence of this residue at the end of the first β-sheet defines a subgroup of GSTs called Y-GST type [32]. Based on their taxonomic distribution, it has been suggested that this type has evolved more recently compared to the so-called S/C-GST type. PcUre2p1 belongs to this S/C-type exhibiting a cysteine in position 89. Among all the fungi considered in our previous study [7], only two *P. chrysosporium* sequences (PcUre2p7 and PcUre2p1) and one *Sclerotinia sclerotiorum* sequence exhibit such a residue, almost all the other sequences –even belonging to cluster 2- having a Asn. Interestingly, bacterial homologous sequences exhibit a Ser at this position (data not shown). The hydroxyl group of serine is used to activate the bound GSH, while a Cys is able to form a disulfide bond with GSH. These differences suggest that the proteins do not show the same catalytic properties.

P. chrysoporium and *P. placenta* are two ligninolytic fungi with two different strategies of wood degradation. Interestingly, both possess a high number of Ure2p proteins [7]. Using *P. chrysosporium* as a model, we tried to understand the role and the significance of such an abundance of Ure2p isoforms in this fungus.

The three proteins we have characterized do not have the same specificities. None of them was able to complement the ΔUre2p mutant for its function in the NCR. However, a homologue of the yeast Gln3p is present in the genome of *P. chrysosporium* (Phchr 43861), suggesting that the NCR could exist in this fungus. Because *URE2* sequence is not completely annotated, it is difficult to discriminate between these following hypotheses: (i) *P. chrysosporium* Ure2p sequences do not have the amino acids required for the interaction with the yeast Gln3, but could interact with the *P. chrysosporium* Gln3p protein, or (ii) the proteins we have chosen among the PcUre2p isoforms are not those interacting with Gln3p. The second noticeable phenotype of the ΔUre2p mutant is its sensitivity to H_2O_2 [14]. Our results of growth kinetics corroborate these previous data and show a delay of the ΔUre2p growth in the control condition and the CDNB and H_2O_2 treatments at the beginning of the kinetics. However, in a second step, the yeast cells exhibited the same growth rate to finally reach almost the same OD after 27 hours. It has been previously suggested that the sensitivity of this mutant to H_2O_2 was due to impairment in the amount of intracellular glutathione [14]. The fact that the strain was able to rescue a growth rate similar to the control in a second step suggests that other detoxification systems, independent of GSH such as thioredoxins or thiol peroxidases [34], were induced at that time. It was not the case for the Cd treatment since the only way to detoxify this metal was shown to be strictly dependant on GSH. Indeed, Cd detoxification occurs via the conjugation of GSH on the metal for its further vacuolar sequestration [35].

In our conditions, none of the three tested PcUre2p is able to rescue the observed phenotypes. Moreover, an increased sensibility of the ΔUre2p strain complemented with PcUre2p4 was observed for the Cd treatment. Overexpressing PcUre2p4 in yeast could emphasize the lack of free GSH in the cell because of a putative requirement of GSH by PcUre2p4 to glutathionylate hydrophobic molecules. This could thus increase the sensibility of the cells to

Cd, which toxicity has been well demonstrated as causing oxidative damage [36]. Surprisingly it is not really the case for PcUre2p6 even if we show that it exhibits the same specificities than PcUre2p4. This may be explained by a lower $Km_{(GSH)}$ for PcUre2p4 (4.8 mM) compared to PcUre2p6 (28 mM).

The main conclusion is that PcUre2p1, 4 and 6 don't have the same activity than ScUre2p in protecting cells against oxidative, heavy metal or aromatic compounds stress. We can hypothesize that PcUre2p are not directly involved in rescuing oxidative stress such as GSTs exhibiting peroxidase activities, but rather act to detoxify specific substrates; small compounds for PcUre2p1 and more hydrophobic ones for PcUre2p4 and 6. The specific expression of PcUre2p4 and PcUre2p6 genes after PAH treatment is in accordance with these enzymatic data. PAHs are aromatic molecules that are degraded at least by three mechanisms in fungi: one uses the cytochrome P-450 system which is composed of a superfamily of monooxygenases, one uses the Fenton reaction [37] and the other uses the soluble extracellular enzymes of lignin catabolism, including lignin peroxidase (LiP), manganese peroxidase (MnP) and laccases which are nonspecific and oxidize a wide variety of organic compounds [38,39]. However, in our conditions, no LiP or MnP activity was detected in the culture medium during the kinetic, we rather observed an intracellular storage of PAH in lipid vesicles as it has been previously shown in *Fusarium solani* [40]. Thus, a high proportion of PAH removal could be due to their internalization inside the hyphae rather than their extracellular degradation and we can hypothesize a role of PcUre2p4 and 6 in this process, perhaps by glutathionylating PAHs.

PcUre2p1 was able to reduce HED and *S*-Phenacylglutathione such as proteins from the omega class. In *A. nidulans*, the homologue GSTA contributes to metal and xenobiotic detoxification, as evidenced by the sensitivity to selenium, silver, nickel, sulphanilamide and pyrrolnitrin of strains lacking a functional copy of *GSTA* [15]. The analysis of the soluble

proteome of *P. chrysosporium* has revealed the presence of PcUre2p1 in a standard culture condition with a relative abundance of 0.12 [41]. Moreover, the authors have shown a 3 fold-upregulation of the protein after copper treatment. Another high scale study revealed an induction of PcUre2p1 gene in response to nonylphenol [42]. In our experiment we showed that the gene is constitutively expressed both in ligninolytic and PAH conditions. Because of its putative mitochondrial localization, the protein may have a role in reducing small toxic molecules in this organelle.

Nothing was really known about the role of fungal Ure2p-like enzymes, most of the studies focusing on the yeast isoform. We showed in this study that the homologues in *P. chrysosporium* do not have the same role than ScUre2p and that *S. cerevisiae* cannot be systematically used as a model to extrapolate data on other fungi. The repartition into 2 distinct subclasses in many fungi suggests that these proteins have evolved differently inside the Ure2p class, adapting their functional specificities to environmental constraints. The putative link between the saprophytic properties of the fungi and the function of the Ure2p proteins remains to be elucidated.

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Table 1: Enzymatic activities of recombinant *P. chrysosporium* and human proteins, using 1-chloro-2,4-dinitrobenzene (CDNB), β-hydroxyethyl disulphide (HED) and dehydroascorbate (DHA). The specific activities are expressed in µmol/min/ mg protein.

	CDNB	HED	DHA
PcUre2p4	0.07(0.01)	ND	ND
PcUre2p6	3.03 (1.78)	ND	ND
PcUre2p1	ND		
PcUre2p1C89S	ND	2.00 (0.21) (Schmuck et al., 2005)	0.13 (0.005) (Schmuck et al., 2005)
HsGTO1	0.18 (0.006) (Board et al., 2000)	2.92 (0.12) (Board et al., 2000)	0.16 (0.005) (Board et al., 2000)
HsGTO2	ND	1.5 (0.62) (Schmuck et al., 2005)	13.8 (0.29) (Schmuck et al., 2005)

ND: not detected

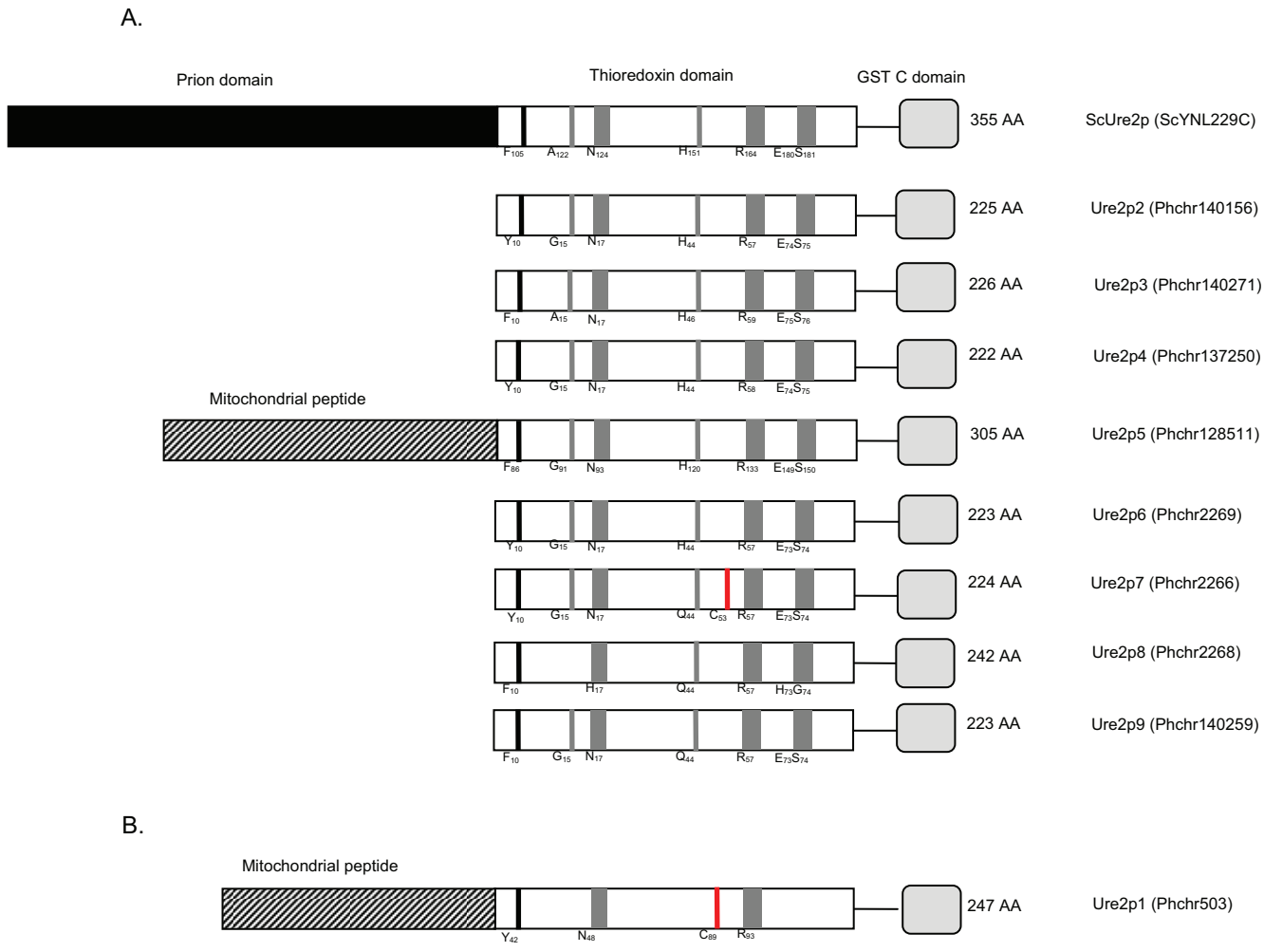

Fig. 1: Comparative analysis of PcUre2p sequences from *Saccharomyces cerevisiae* and *Phanerochaete chrysosporium*. Only the amino acids of the N-terminal domain known to be involved in the activity of the proteins have been reported.

A: Comparison between sequences for the first subclass including the yeast isoform.

B: Organisation of the sequence of PcUre2p1, which belongs to the second subclass (cluster 2).

The proteins ID are given in brackets.

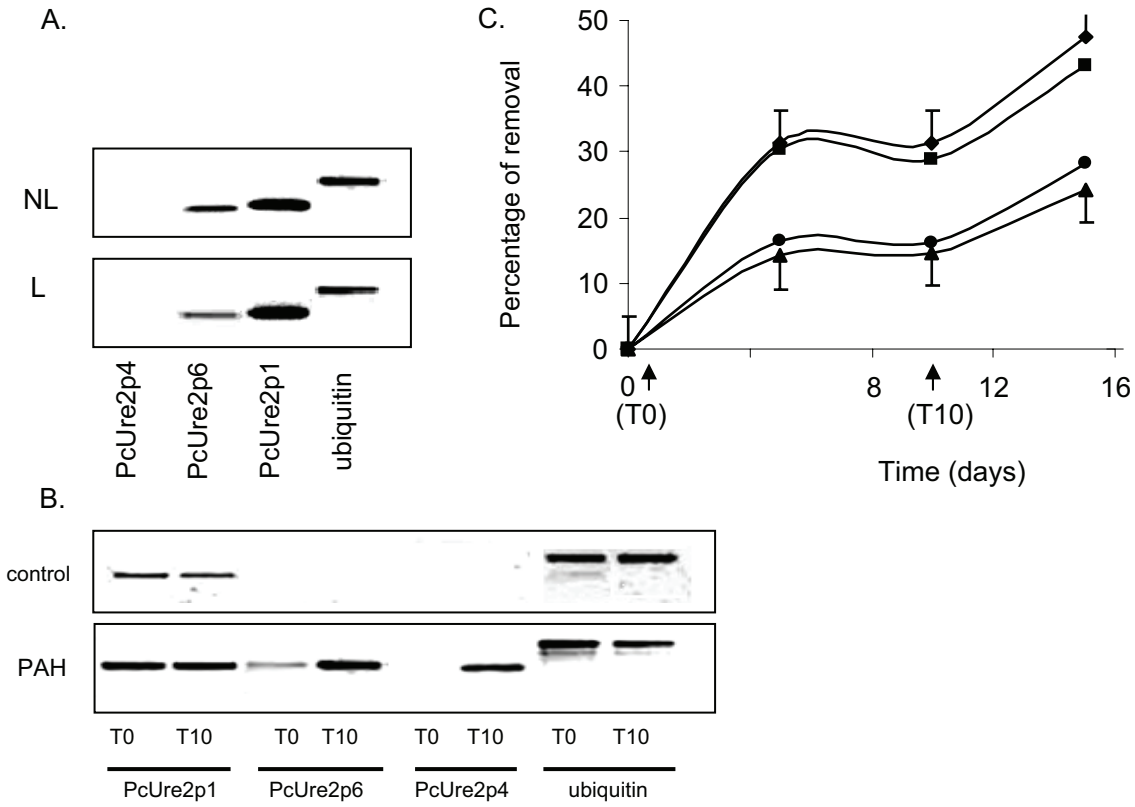

Fig. 2: Expression of PcUre2p coding genes using RT-PCR.

A: PcUre2p gene expression in ligninolytic (L) compared to non-ligninolytic conditions (NL) after 15 days of culture on solid media.

B: PcUre2p gene expression immediately after transfer on PAH (To) and after 10 days of PAH treatments on liquid media.

The culture conditions are described in the material and methods. The suitability of the samples was confirmed with similar amplifications of the ubiquitin coding gene (data not shown).

C: Percentage of PAH removal by the fungus according to time, determined by HPLC quantification. The arrow indicates the condition used for the gene expression experiment shown in figure 2B. (T0 and T10). Fluorine (◆), phenanthrene (●), anthracene (▲) fluoranthene (■).

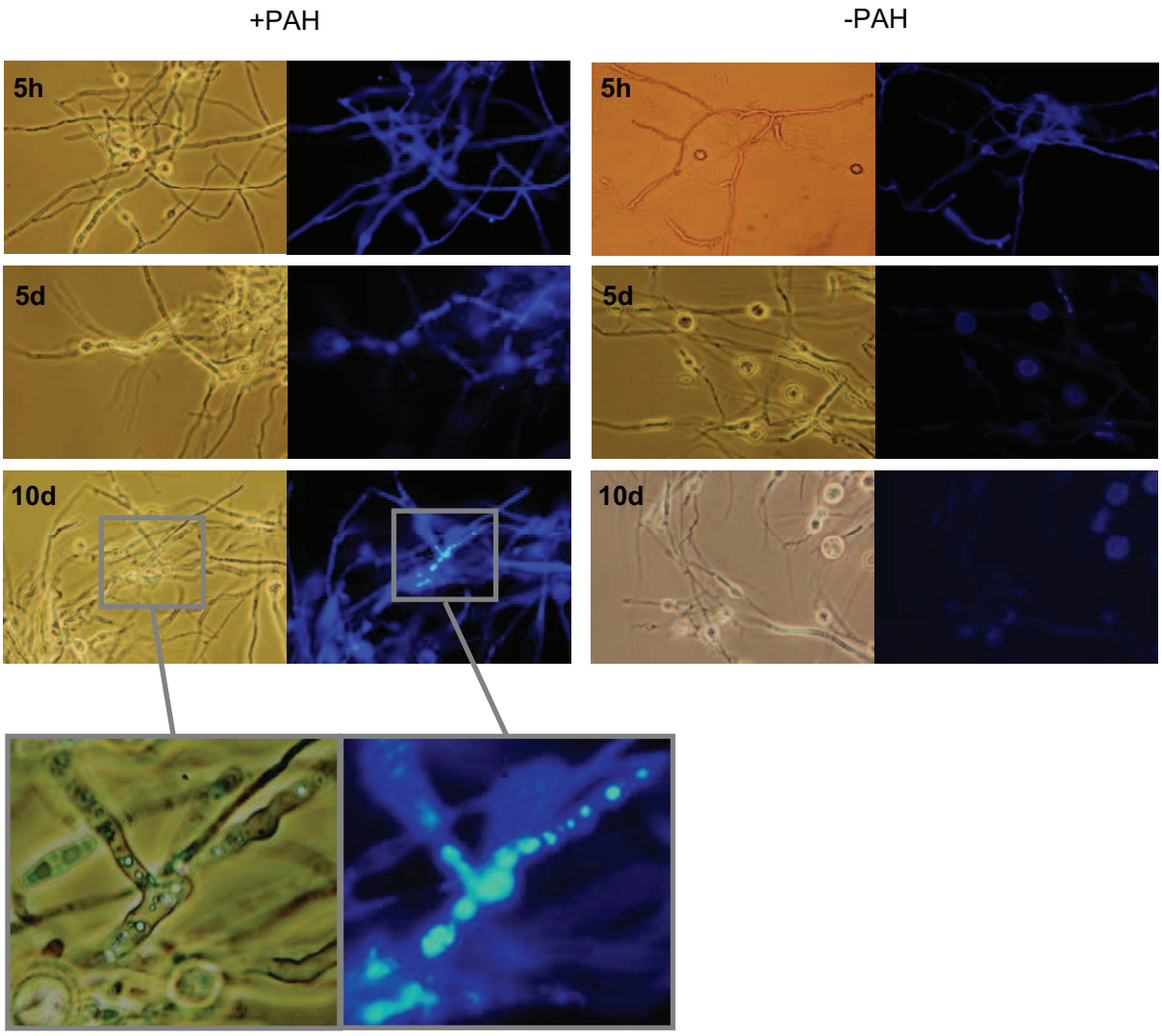

Fig. 3: Microscopic observations of *P. chrysosporium* hyphae and spores after PAH treatment for 5 hours, 5 days and 10 days compared to control cultures without PAH. The fluorescence of PAH was revealed using the DAPI filter as described in the Material and Methods.

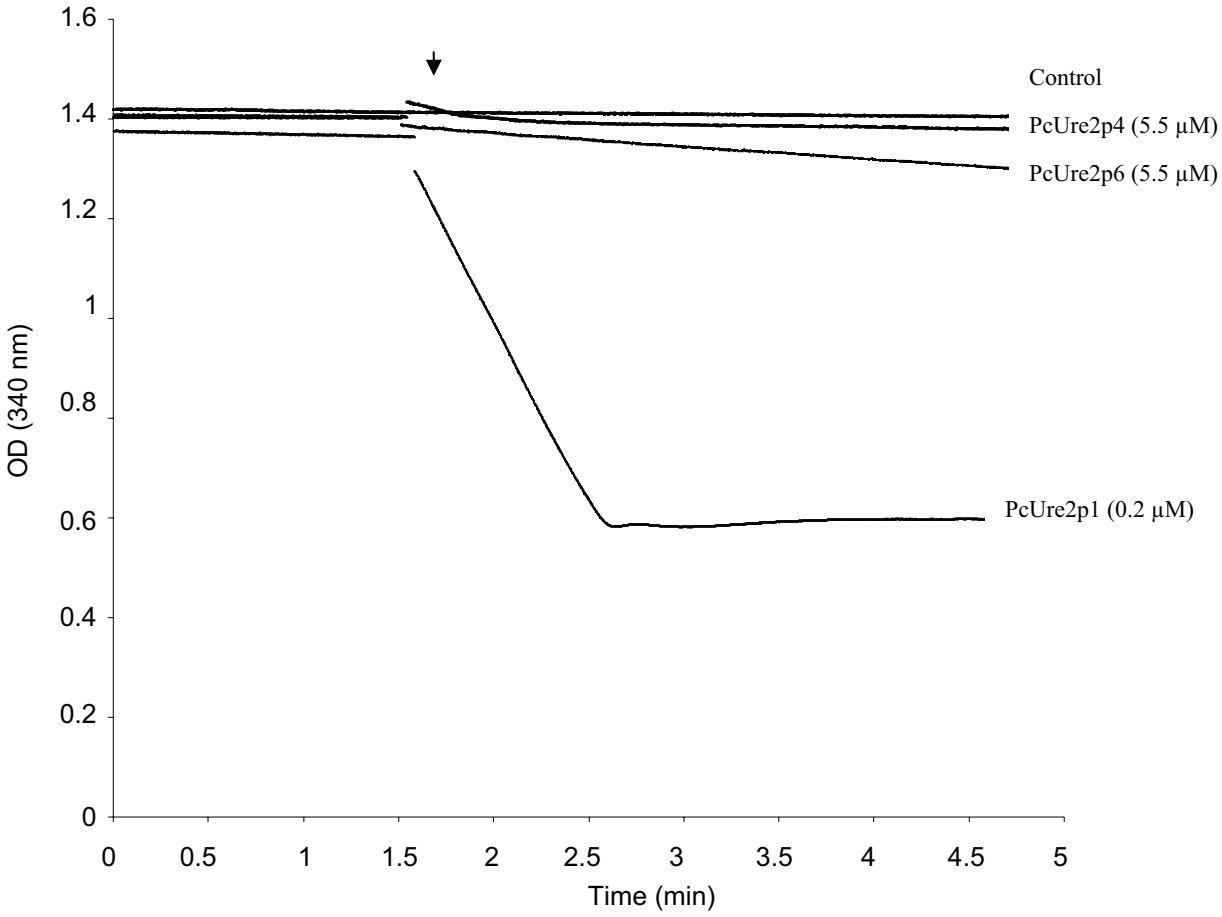

Fig. 4: Reduction activity measurements of recombinant PcUre2p proteins using S-Phenacylglutathione as substrate. The absorbance was followed at 340 nm according to time. The arrow indicates the enzyme addition (5.5µM for PcUre2p4 and 6 and 0.2µM for PcUre2p1). The control corresponds to the enzymatic mix without any enzyme.

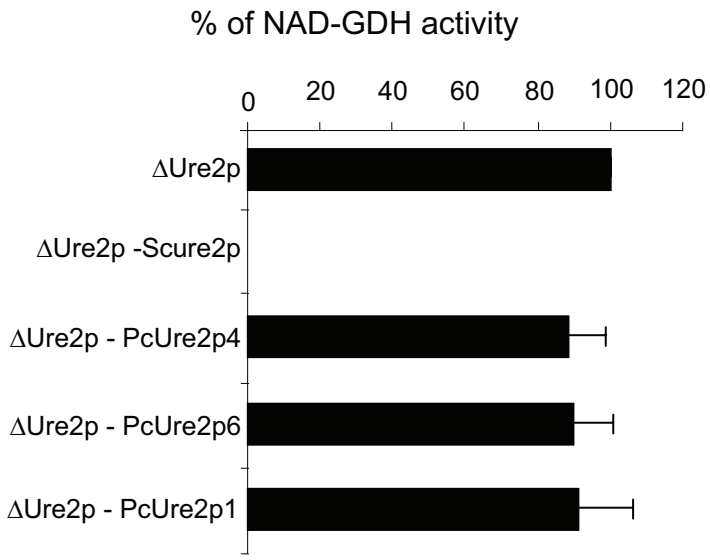

Fig. 5: NAD-GDH activity in the transformed yeast strains. ΔUre2p is the mutant strain transformed with the empty pYes2 vector. The other strains correspond to the mutant transformed with the pYes2 vector harbouring either the endogenic ScUre2p or the eterologous PcUre2p4, 6 and 1. The results are expressed as percentages of the higher activity found for the mutant ΔUre2p.

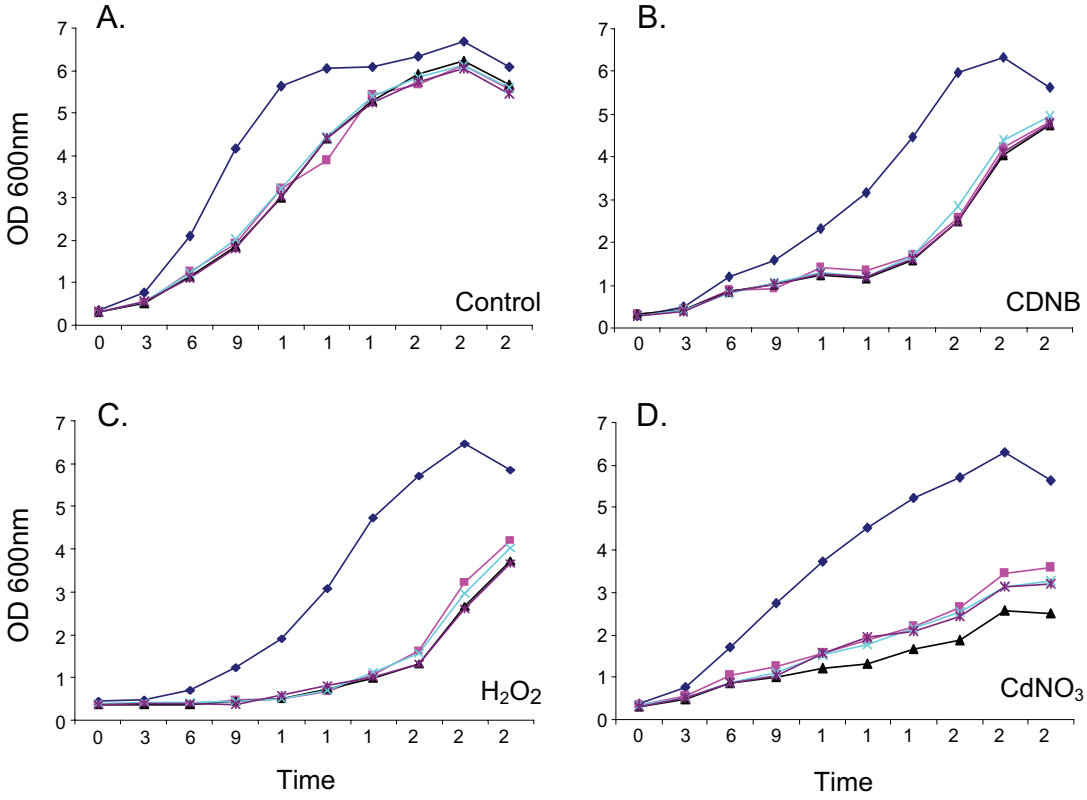

Fig. 6: Growth kinetics of the yeast strains in various stress conditions. The yeast cells were precultured in YPD medium overnight, transferred to a selective medium for 3 hours and various compounds were added. The time (0h) shows the OD at 600nm after 3 hours of preculture on selective medium, and then OD measurements were performed every 3 hours.

The control condition (A) was performed using a selective medium without any toxic compound. CDNB (50µM) (B), H_2O_2 2.5mM (C) and $CdNO_3$ (25µM) were added to the culture after 3 hours of preculture on selective medium.

ΔUre2p-ScUre2p (◆), ΔUre2p (■), ΔUre2p-PcUre2p4 (▲), ΔUre2p-PcUre2p6 (×), ΔUre2p-PcUre2p1 (✳).

The data are the mean of 2 independent biological repetitions.

Additional Results

Transcriptomic analysis of *P. chrysosporium* genes under PAH treatments

Introduction

A global transcriptomic approach has been carried out to have an overview of gene expression in *P. chrysosporium* in condition of PAH treatment. The aim was to analyze expression of genes coding for all *P. chrysosporium* Ure2p, for the other members of the GST superfamily and the global antioxidant system, and to identify candidates involved in PAH degradation and stress response. This analysis was carried out to complete the previous expression data obtained by RT-PCR for PcUre2p4, PcUre2p6 and PcUre2p1 (Ngadin et al., unpublished).

Experimental procedures

Strains and culture conditions

The used strain and the culture conditions are those described previously (Martinez et al., 2004; Tien and Kirk, 1988). The fungal pellets were first grown without PAH for 5 days to yield biomass. The fungus was then transferred into new flasks containing PAHs. To prepare these flasks, a stock solution of PAH was solubilised in hexane and added into flasks to get final quantities of 2.25 mg phenanthrene, 0.225 mg fluorene, 0.225 mg fluoranthene and 0.225 mg anthracene per flask. 100 ml of liquid culture medium, which composition is described above (Verdin et al., 2005) but without glucose, was added in each flask after complete evaporation of hexane. The fungus was harvested at T0 corresponding to the beginning of the kinetic (5 days of culture) and 10 days after PAH treatment. A control was made without adding any PAH (Figure 1).

Expression microarrays

From a data set of 9998 unique *P. chrysosporium* gene predictions, each Roche NimbleGen (Madison, WI) array featured 6 unique probes per gene, all in duplicate. For each condition, total RNA was purified from triplicate cultures using the RNeasy Plant mini Kit (Qiagen) according to the manufacturer using DNase treatment. RNA was converted to double-strand cDNA using the Smarter^{TM} PCR cDNA Synthesis Kit (Clontech) according to the manufacturer protocol and purified using the Qiaquick PCR Purification Kit (Qiagen). The complete protocol for labelling and hybridization is available at the Nimblegen website: http://www.nimblegen.com/products/exp/index.html.

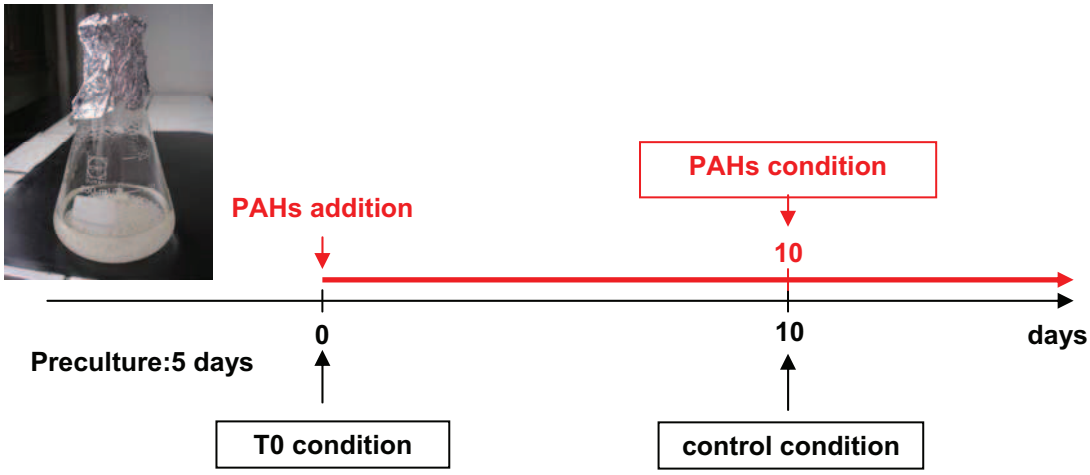

Figure 1: Description of the conditions used for the transcriptomic analysis. The fungus was precultured for 5 days before PAH treatment for 10 days. The fungus was harvested at T0 and 10 days later for the control and the PAH conditions.

Results and discussion

The statistical analyses of the data were not conclusive using Array Star program. No gene was found significantly regulated. Since Array Star is a very restrictive program, we also analysed the results using CyberT Bayesian test. The Bayesian *t* test allows statistical inference to be made even when experiments are replicated only at nominal levels. It assumes that genes with similar expression levels have similar measurement errors (Long et al., 2001). This analysis revealed 43 genes significantly upregulated after PAH treatment compared to the control with fold>5, *p*-value<0.05 and PPDE>0.85 (PPDE, a posteriori probability of differential gene expression; significant when close to 1). However, if we look at the values more in detail, we did not consider these results as reliable since several problems have been highlighted:

1- All the experiments have not been performed at the same time since Nimblegen failed the hybridization step of some samples. Some new samples have thus been prepared and sent separatly.
2- The principal component analysis (PCA) revealed huge variations between biological samples (Figure 2). The culture system used could generate high variability according to the development of the pellets, the oxygenation state in relation with the size of the pellets and the proportion of spores since the fungus is able to sporulate in this condition.
3- Globally, the signals of the 6 probes for a same gene are very variable (see Table 1 for PcUre2p4, PcUrep6 and PcUre2p1 as examples). This could be explained by problems of amplification, cDNA degradation or array design. The variability observed between biological replicates could thus also arise from this heterogeneity.

Perspectives

Nimblegen technical services are currently working on the data to better understand where the problem of hybridization and probe design is. To be able to bring this project to a successful conclusion, we plan to make new experiments using static fungal cultures and more biological repetitions.

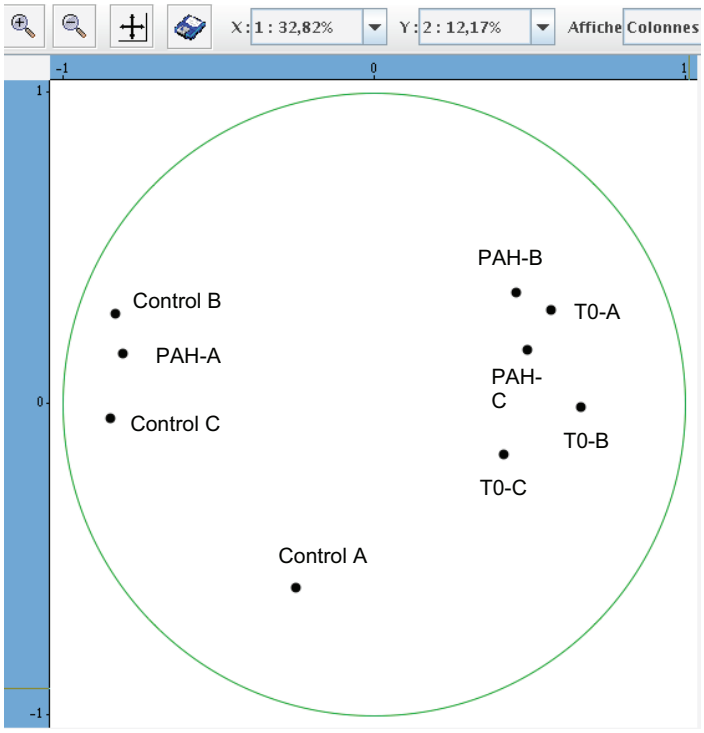

Figure 2: Principal component analysis (PCA) of total normalized expression data between the control conditions, the PAH treatments and at the begining of the kinetic (T0) as described in the materials and methods part.
A, B and C are biological repetitions. The analysis has been perfomed using the ArrayStar program.

Table 1: Normalized expression data for PcUre2p4 (Seq ID 137250), PcUre2p6 (Seq ID 2269) and PcUre2p1 (Seq ID 503) under T0, control and PAH treatments. Data from the 6 probes are reported for each gene.
A, B and C are biological repetitions.

		T			Contr			PAH		
SED I	Prob	A	B	C	A	B	C	A	B	C
PHCHR1 137	PchrP00044	60.1	727.7	55.9	203.6	1094.	649.1	3668.	225.6	44.2
PHCHR1 137	PchrP00044	46.5	647.0	29.8	163.0	1286.	706.0	4052.	122.0	39.8
PHCHR1 137	PchrP00044	52.8	880.5	86.6	132.7	1175.	664.3	4151.	335.0	47.3
PHCHR1 137	PchrP00044	173.3	2052.	131.9	294.0	1686.	819.8	6137.	181.2	79.8
PHCHR1 137	PchrP00044	136.2	112.5	50.8	96.1	1007.	499.0	5036.	131.9	88.1
PHCHR1 137	PchrP00044	1486.	3033.	548.0	344.0	357.6	284.5	1534.	116.2	1552.
PHCHR1 2	PchrP00068	70.5	18.7	25.1	21.3	42.8	75.8	302.2	31.5	25.2
PHCHR1 2	PchrP00068	27.8	25.9	21.8	15.3	31.9	20.6	142.5	205.1	27.6
PHCHR1 2	PchrP00068	73.8	80.0	40.7	28.4	85.6	49.7	331.0	294.8	67.4
PHCHR1 2	PchrP00068	144.8	128.8	93.0	96.1	120.4	125.7	408.4	1110.	347.0
PHCHR1 2	PchrP00068	19.5	18.7	32.6	20.4	55.0	57.0	731.4	56.0	31.5
PHCHR1 2	PchrP00068	42.8	47.4	105.9	172.7	280.0	257.4	4013.	326.0	102.6
PHCHR1 5	PchrP00128	1057.	2187.	496.2	308.5	235.9	146.4	199.5	1276.	1132.
PHCHR1 5	PchrP00128	31.0	28.9	25.9	52.6	25.9	23.9	26.0	41.2	47.3
PHCHR1 5	PchrP00128	39.4	39.8	40.7	31.5	23.3	25.0	36.4	101.6	67.4
PHCHR1 5	PchrP00128	119.2	72.4	129.3	51.2	44.5	49.7	112.4	320.0	65.1
PHCHR1 5	PchrP00128	62.5	42.8	129.3	29.1	27.5	47.8	68.3	415.2	77.3
PHCHR1 5	PchrP00128	178.5	104.6	156.9	43.5	32.7	35.3	65.5	534.1	160.4

Reference

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DISCUSSION, CONCLUSION ET PERSPECTIVES

DISCUSSION, CONCLUSION ET PERSPECTIVES

1 The Glutathione -S-Transferases functions

Explosive advances in genomic have provided researchers with a complete inventory of the predicted proteins produced by eukaryotic and prokaryotic organisms. This has allowed the description of many protein superfamilies. Among them, Glutathione-S-Transferases (GSTs) constitute a complex and widespread superfamily classified as enzymes of secondary metabolism (Dixon et al., 2010). Usually, they are involved in xenobiotic detoxification by catalyzing the conjugation of electrophilic xenobiotic substrates with the tripeptide glutathione in response to biotic and abiotic stress (Reddy and Gold, 2001; Edwards and Dixon, 2005). However, the characterization of a large diversity of cytosolic GSTs in mammalian, insects, plants, bacteria and fungi has highlighted multiple other functions in the cell (Buetler and Eaton, 1992; Sheehan et al., 2001; Wagner et al., 2002; Morel et al., 2009a). In medicine, GSTs have been explored as molecular targets for the design of new anticancer drugs (Chronopoulou and Labrou, 2009; Sau et al., 2010) and as recombinant allergens for diagnosis, immunotherapy, study of hypoallergenic variants and epitope mapping (Lorenz et al., 2001; Hiller et al., 2002). In plants, overexpression of GST genes enhances the potential for phytoremediation of herbicides (Kawahigashi, 2009). These enzymes have thus been explored for the development of biosensors for such toxic compounds (Kapoli et al., 2008). Expression of GST encoded genes was found to be induced by dehydration and exposure to ozone, hydrogen peroxide, lipid peroxidation products, UVB, different pathogens, herbicides, salicylic acid and in lesion mimic mutants that develop hypersensitive response-like symptoms (Wagner et al., 2002; Lan et al., 2009), suggesting various roles in a biotic and biotic stress responses. Some GSTs are also thought to be involved in plant secondary metabolism as transporters (Dixon et al., 2010). In fungi, GSTs are notably involved in essential processes as plant-pathogen interactions (Mehta et al., 2008), mycorrhizal interactions, lignocellulose degradation (Morel et al., 2009a), fungicide degradation (Reddy and Gold, 2001) and xenobiotics biodegradation (Fragoeiro and Magan, 2008; Minami et al., 2009). Moreover, a GST of *Pochonia chlamydosporia* is involved in the synthesis of hypothemycin, a fungal secondary metabolite. This compound is an attractive candidate as an anticancer agent (Reeves et al., 2008). Fungal GSTs have been poorly studied in comparison

with their animal and plant counterparts. The few available data, concerning mainly yeast, indicate that they are potentially involved in protecting cells against damages resulting from oxidative stress, heavy metals and antifungal compounds (Veal et al., 2002; Rai et al., 2003; Barreto et al., 2006). This prompted us to investigate the GST network in the wood-decaying fungus *P. chrysosporium*.

2 Diversity of fungal GSTs

The phylogenetic analysis of fungal GSTs reported in this manuscript has revealed seven classes of fungal GSTs, named GTT1, GTT2, Ure2p, Omega, EFBγ, MAK16, and GTE, four of them being fungal specific: Ure2p, GTT1, GTT2 and GTE (Morel et al., 2009a). Another interesting point revealed by this phylogenetic analysis is the high diversity of the number of fungal GST isoforms. It is attempting to connect this GSTs number with the way of life since this latter is mainly increased in saprophytic species. The main extended classes are omega, Ure2p and GTE, these extensions depending of the considered species. The extension of these classes raises the question of the function(s) of these enzymes and of their potential redundancy. Genomes are in constant evolution, due to multiple events as mutations and rearrangements of DNA sequences. A limited number of point mutations in a gene can be generated by chemical mutagens, radiation, or error-prone DNA replication. More extensive structural alterations can be accomplished by homologous recombinations, and major changes can be obtained by fusion of genes or other large coding sequences. Nevertheless, only events conducting to functional advantages will be selected and conserved. Concerning evolution of proteins, our current understanding of enzyme evolution supports the notion that redesign of existing structures, rather than *de novo* design, is a major route to enzymes and other proteins with novel functions (Mannervik et al., 2009). The human GSTs have been extensively studied showing that the different isoforms share similar overall topologies. All members are functional dimeric enzymes possessing two different domains, one more or less conserved involved in the glutathione binding (G-site), the second being more variable and involved in the recognition of electrophilic substrates (H-site). Inside different classes, several members are quite similar from a point of view of primary structure. For instance, in the alpha class, HsGSTA1-1 and HsGSTA1-4 are highly homologous; however these isoforms are functionally different. While GSTA1-1 is regarded as a highly promiscuous enzyme with catalytic activity toward structurally diverse substrates, GSTA4-4 is recognized for its specificity toward alkenals such as 4-hydroxynonenal (Balogh et al., 2010). Another example

could be found in the GTO class, where a single mutation changes totally the activity of the enzyme. As shown in the paper III presented in this manuscript for PcGTO3, the replacement of the catalytic cysteine of HsGTO1-1 by a serine leads to a drastic change of the catalytic properties. The WT protein is able to perform deglutathionylation reactions, whereas the mutated protein exhibits a classical transferase activity.

All these examples suggest that the extension of the fungal GST classes could result from an adaptation of the saprophytic fungi to their natural environment, minor structural changes in the catalytic site leading to new functions. Such extension is observed in particular in wood-decaying fungi as *P. chrysosporium*. Wood is composed of many compounds often recalcitrant to biodegradation as extractives or of course lignin. The oxidative mechanisms mediated by wood-rot fungi during wood degradation lead to the production of potentially toxic molecules. The high diversity of such oxidative by-products could explain the high number of GSTs found in these organisms. This hypothesis has been also proposed to explain the huge number of cytochrome P450 monooxygenases found in these organisms. Another hypothesis, which has been proposed to explain the diversity of plant GSTs, could be their involvement in secondary metabolism and in particularly in metabolites transport (Dixon and Edwards, 2010).

Another interesting point is that the diversity of GSTs, at least in wood-decaying fungi, is mainly due to the extension of some class or even subclasses. For instance, *in P. chrysosporium*, the ure2p subclass 2 is highly extended (this latter contains 8 isoforms) whereas the ure2p subclass 1 contains only one isoform (PcUre2p1). In *P. chrysosporium*, the extension of the omega class concerns essentially isoforms related to mammals GSTs. We demonstrated in this study in accordance with the work of Xun et al (Xun et al., 2010) that the omega subclass 1 represents indeed a distinct functional and structural class named now GHR for glutathionyl Hydroquinone Reductase, related to bacterial GSTs. In these examples (PcGHR and PcUre2p1), both enzymes could result from a horizontal gene transfer as shown by the high homology found with bacterial GSTs. In another hand, both enzymes could be located in mitochondria as suggested by the presence of a target peptide, which could explain their bacterial origin. In contrast, the extended subclasses or classes (omega, ure2p and etherase-like) are either related to higher eukaryotic organisms (omega) or specific to fungi. Further investigations are required to understand the mechanisms linked to the extension of these specific classes.

	Prot ID	Gene expression/induction conditions	Recombinant protein	Activity	Substrate	3D Model	Comment
PcGHR1	EU791894	expressed	OK	deglutathionylation	HED, DHA, hydroquinone	XRD	Crystal structure solved by our group[4]
PcGTO2	126388	*	*	*	*	HM	65 % seq. identity with PcGTO1[4]
PcGTO3	EU791893	expressed	OK	deglutathionylation	HED, DHA, phenacylglutathione,methylarseniate	HM	31 % seq. identity with Human GSTO1-1[5]
PcGTO4	7168	expressed	OK	deglutathionylation	HED, DHA, phenacylglutathione,methylarseniate	HM	28 % seq. identity with Human GSTO1-1[5]
PcGTO5	7169	expressed	*	*	*	HM	28 % seq. identity with Glycine max GSTT[6]
PcGTO6	3911	expressed	*	*	*	HM	45 % seq. identity with PcGTO7
PcGTO7	6880	expressed	OK	GSH transferase	CDNB, MBBr	XRD	
PcGTO8	6881	*	*	*	*	HM	60 % seq. identity with PcGTO7
PcGTT2.1	6766	induced (wood, oxidative stress)	OK	peroxidase	t-BOOH, Cu-OOH	XRD	
PcGTT2.2	6683	induced (oxidative stress)	*	*	*	XRD	
PcUre2p1	503	induced (copper[1])	OK	deglutathionylation?	HED, DHA, phenacylglutathione	HM	43 % seq. identity with E. coli YfcG[7]
PcUre2p2	140156	expressed	*	*	*	HM	37 % seq. identity with yeast Ure2p[8]
PcUre2p3	140271	expressed	*	*	*	HM	38 % seq. identity with yeast Ure2p[8]
PcUre2p4	137250	induced (PAH)	OK	GSH transferase	CDNB	HM	40 % seq. identity with yeast Ure2p[8]
PcUre2p5	128511	expressed	OK	GSH transferase	CDNB	XRD	
PcUre2p6	2269	induced (PAH)	OK	GSH transferase	CDNB	HM	40 % seq. identity with yeast Ure2p[8]
PcUre2p7	2266	*	*	*	*	HM	39 % seq. identity with yeast Ure2p[8]
PcUre2p8	2268	*	*	*	*	HM	32 % seq. identity with yeast Ure2p[8]
PcUre2p9	140259	induced (oxidative stress, wood[2])	*	*	*	HM	40 % seq. identity with yeast Ure2p[8]
PcPhl	7971	induced (benzoic acid[3])	*	*	*	HM	46 % seq. identity with Zea mays GSTT[9]

Table 6: Available data for *Phanerochaete chrysosporium* GSTs.
PAH: polycyclic aromatic compounds, GSH: glutathione, t-BOOH: ter-butyl hydroperoxide, Cu-OOH: cumene hydroperoxide, HED: hydroxyethyl disulfide, DHA: dehydroascorbate, CDNB: chloro-dinitrobenzene, MBBr: Monobromobimane, XRD: X-ray Diffraction, HM: Homology modeling.
1 Ozcan et al., 2007; 2 Sato et al., 2009; 3 Matsuzaki et al., 2008; 4 Meux et al., 2011; 5 Board et al., 2000; 6 Axarli et al., 2009; 7 Wadington et al., 2009; 8 Umland et al., 2001; 9 Neuefeind et al., 1997.

3 How to describe the GST functions?

To understand the functions of members of a protein superfamily, different approaches are required, in particular when the studied enzymes exhibit similar activities with artificial substrates. An overview of the obtained results in our research team on PcGSTs is given Table 6. We will describe in this part the strategy retained in this work and propose further experiments to pursue the investigation of fungal GSTs, such of them being under the way in our research team.

3.1 Regulation of gene expression

Genes encoding GSTs are often tightly regulated and GST transcripts are routinely identified as being strongly upregulated in stress studies, although these correlations have generally not led to any major new insights about GSTs function. In *P. chrysosporium*, various transcriptomic studies have been performed in conditions linked to the ligninolytic ability or to the bioremediation potential of this fungus revealing in some cases specific up-regulation of different GST genes (Minami et al., 2007; Minami et al., 2009; Sato et al., 2009; Vanden Wymelenberg et al., 2009; Vanden Wymelenberg et al., 2010; Kim et al., 2010).

In the experiments performed during this thesis, we have demonstrated the specific expression of Pcure2p4 and Pcure2p6 during polycyclic aromatic hydrocarbons degradation. Based on the previous studied, at least three different systems could be involved in PAH degradation by fungi. These include cytochrome P450 monooxygenase, Fenton reaction (Veignie et al., 2009) and extracellular enzymes involved in lignin oxidation which are nonspecific and oxidize a wide variety of organic compounds (Tortella et al., 2005; Wang et al., 2009). It is known that *P. chrysosporium* P450 monooxygenase can catalyse oxidation of xenobiotic compounds including toluene, phenanthrene and benzo[a]pyrene (Bezalel et al. 1997; Luykx et al. 2003; Maspahy et al. 1999).

To pursue the investigation of the GSTs functions in PAH degradation, we planned DNA arrays experiments. Even if these experiments have been unsuccessful due probably to the pellet heterogeneity in the used culture conditions, this strategy should indeed give insight on the GSTs function. This indirect method could be powerful to look for the co-ordinated expression of family members with genes encoding proteins with known enzyme activities.

Transcriptional co-regulation is often observed in primary metabolism, and to a more limited extent in the pathways leading to secondary products (Wei et al., 2006). This strategy has been used for plant GSTs and has given new insights about the putative functions of plant GSTs (Dixon et al., 2010). Nevertheless, in our case, the culture conditions need to be optimized (probably using static cultures) before to try again.

3.2 GST biochemistry

From a biochemical point of view, GSTs share the ability to bind and to react with various substrates suggesting a broad substrate acceptance and then a lack of specificity. The recent advances made in various organisms have in fact highlighted strong compound specificity for plant, bacterial and fungal GSTs (Dixon and Edwards, 2010; Masai et al., 2007, Meux et al.,2011), suggesting that the major limitations is to find the physiological substrates of these isoforms. From the results presented in this work and also complementary data obtained in our research team, it appears that the *P. chrysosporium* GSTs could be divided into two functional classes according to their catalytic properties. The first catalyzes glutathionylation, i.e. glutathione transfer to electrophilic compounds, the second one catalyzing deglutathionylation reaction called also thiol transferase activity.

3.2.1 Enzymes involved in deglutathionylation

From the results obtained from biochemical characterization, we have shown that PcGSTs catalyzing deglutathionylation experiments are members of the omega class with the exception of Pcure2p1.

PcGHR1, PcGTO3 and PcGTO4 possess a catalytic cysteinyl residue, which is essential in the activity. The retained strategy has shown a difference of specificity between these studied GSTs. PcGTO3 and PcGTO4 are active against phenacylglutathione and dimethylarseniate as their human ortholog HsGTO1.1 (Board et al., 2008; Meux et al., 2011), whereas PcGHR1 is active against substrates possessing a quinone motif. We proposed that PcGHR1 is probably involved in the well-used fungicide pentachlorophenol degradation pathway (Reddy and Gold, 2001). Concerning PcGTO3 and 4, recent advances made in our team showed that both enzymes are able to react with a commercial fluorescent substrate,

CMFDA (5-chloromethylfluorescein diacetate), leading to a covalent link between the substrate and the enzymes. The binding occurs through a thioester linkage suggesting that these enzymes possess an esterase activity (Meux et al., unpublished data). This activity has been confirmed using substrates as p-nitrophenolbutyrate. These biochemical data suggest that these enzymes could react with alkyl substrates. It could be very interesting to pursue these investigations in order to study the potential interactions of these isoforms with such potential substrates. It has been indeed shown in mammals, that some GSTs are involved in the response against lipid peroxidation (Catala, 2009; Awasthi et al., 2004). Due to oxidative stress, lipid peroxidation lead to the formation of various by-products in particular alkenals as 4-hydroxynonenal (HNE) which can cause apoptosis and differentiation, modulate cell growth and various signal transduction pathways (Kreuzer et al., 1998). HNE has been shown to be mainly metabolized in human via its conjugation to glutathione, this conjugate being further metabolized through different pathways (Hartley et al., 1995). The putative involvement of PcGTOs in such processes requires further investigations.

Through the different GSTs biochemically investigated during this thesis, we have found that PcUre2p1 exhibits also a deglutathionylation activity similar to that one observed with PcGTO3 and 4. However, no cysteine is present in the putative active site and mutational studies have shown that no cysteine is involved in the catalysis (Morel and Gelhaye, unpublished), remaining the catalytic mechanism unknown. Recently, the structures of two *E. coli* GSTs related to PcUre2p1 have been resolved (Wadington et al., 2009; Stourman et al., 2011). Both enzymes display disulfide-bond oxidoreductase activity as PcUre2p1 and are active against glutathionylspermidine, the predominant redox-active thiol in the stationary phase in this bacterium. It has been proposed that these both enzymes are members of a new subfamily of GSH transferases called nu-class, due in particular to the presence of two GSH binding sites. The structure of PcUre2p1 is underway in collaboration with the team of C. Didierjean (Crm2, UHP-Nancy), the first results demonstrating also the presence of two glutathione bound in the structure (unpublished data). An investigation of *P. chrysosporium* genome revealed the absence of putative enzymes involves in the synthesis of glutathionylspermidine, suggesting another function in the fungus. Further investigations are required to understand the function of this particular GST.

3.2.2 Enzymes involved in glutathionylation

The main activity usually found in GSTs concerns glutathione transferase reactions. To detect such activity, different substrates have been described depending on protein nature. Basically, GSTs are able to perform various chemical transformations as alkylation, arylation, and addition to isothiocyanates, transacylation, hydroperoxide reduction, transnitrosylation, and steroid isomerisation (Kurtovic et al., 2008). For the proteins studied during this work, we have tested different compounds and showed that Pcure2p4 and Pcure2p6 are able to catalyze the conjugation of glutathione on aromatic molecules as CDNB, but both enzymes are not able to reduce peroxides. To confirm the potential role of these both enzymes in the PAH degradation, other tools are required to identify potential substrates. It is clearly a limitation of this biochemical approach using artificial substrates in particularly could these enzymes accepting a broad range of substrates.

3.3 Natural substrates fishing/ Activity based profiling

As stated above, to unravel the function of the different GST isoforms, additional tools are required to identify potential substrates. Such approaches could be conducted using activity-based profiling screening or natural substrates fishing. We propose such strategies to pursue the investigations of fungal GSTs.

3.3.1 Activity-based protein profiling (ABPP)

During the last years, the (ABPP) has mainly been developed in the medical field to access the protein activities in the proteome. ABPP is based on the use of labelled small reactive molecules that react with active site residues of proteins in an activity-dependent manner. The resulting labelled proteins can be detected in protein gels or blots and purified and identified by mass spectrometry. This technique can be used to highlight enzyme-substrate interactions. The principle is to use a fluorescent probe, which is able to interact with the protein. The variation of fluorescence will reflect the capacity of the probe to compete with various substrates for interacting with GST. ABPP coupled to a detection using fluorescence polarization (FP) has been recently demonstrated to be efficient to detect potential inhibitors/substrates of HsGTO1 (Weerapana et al., 2010). Despites the similarity of the reactions catalyzed by the various classes of GSTs, it has been shown a clear specificity of

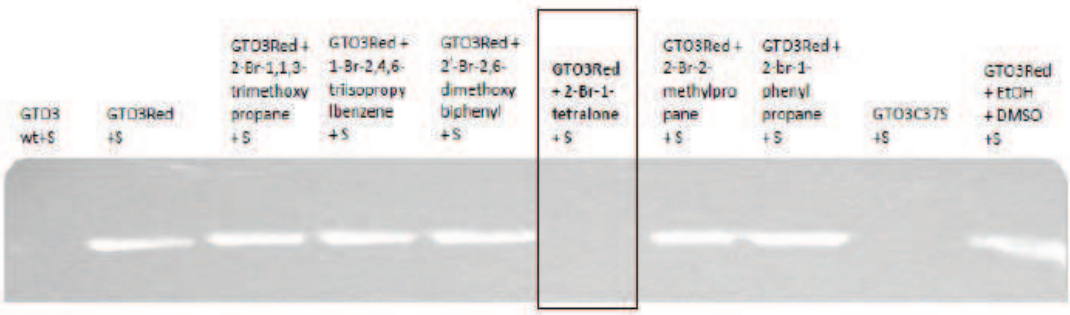

Figure 16: SDS-PAGE gel of PcGTO3 incubated with various substrates and the fluorogenic CMFDA probe

S: fluorescent CMFDA
Red: reduced
GTO3C37S: the catalytic cysteine in position 37 has been replaced by a serine

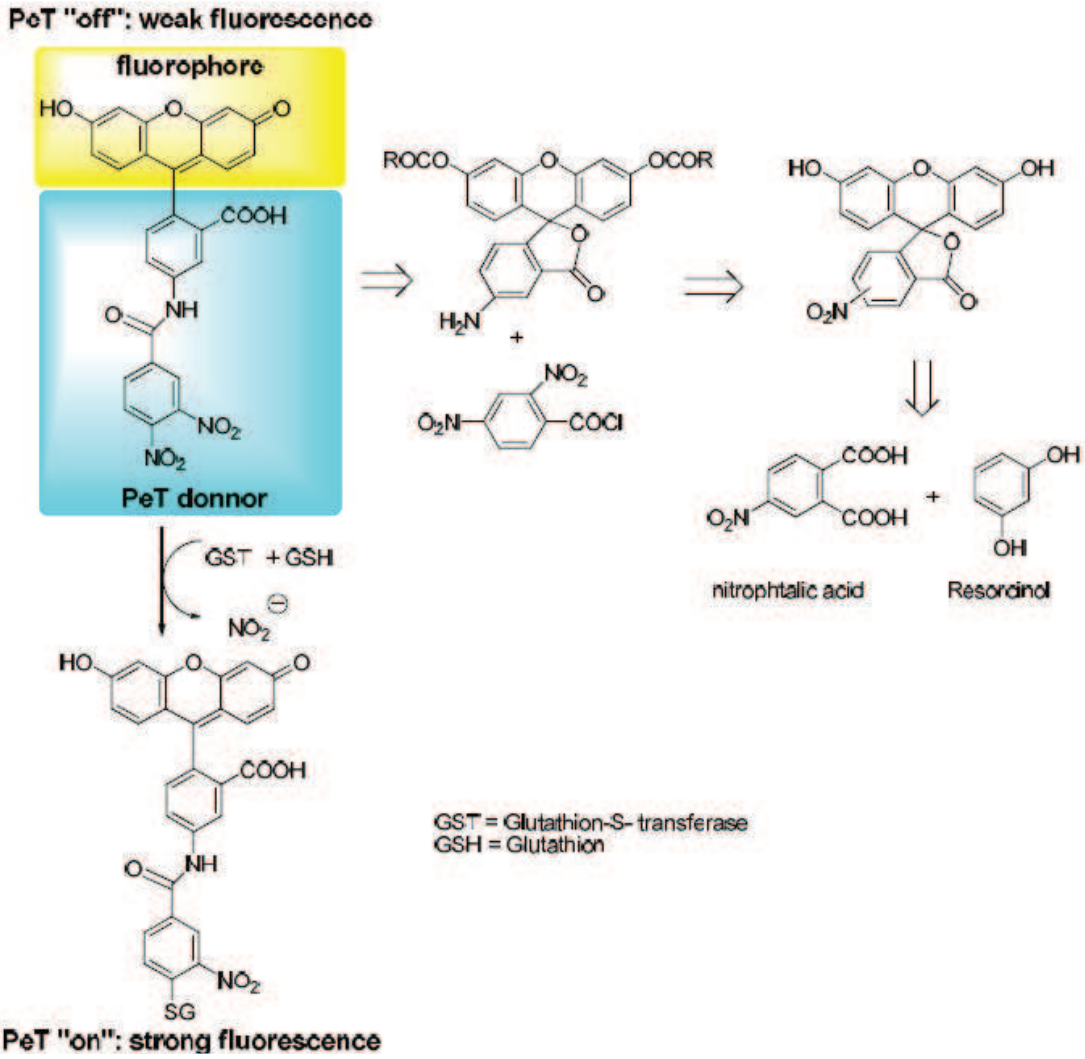

Figure 17: Synthesis and mode of action of GST fluorogenic probes (Fujikawa et al., 2008)

various isoforms using advanced physiological tools (Dixon and Edwards, 2010). We propose an ABPP approach to take profit of this characteristic. Indeed, the lack of activity specificity allows the use of fluorogenic probe according to their activity-type (deglutathionylation or GSH transferase) to highlight their high substrate specificity. Design of probes for classes or subclasses of cysteine-containing GSTs has been recently made for the human ortholog HsGTO1.1 (Bachovchin et al., 2009; Son et al., 2010) allowing the development of specific inhibitors (Bachovchin et al., 2009). Specific probes could be used according to the activity-type of GSTs.

GSTs with deglutathionylation activities exhibit a catalytic cysteine. CMFDA (5-chloromethylfluorescein diacetate) is an example of fluorescent compound that is able to specifically bind cysteines. Our preliminary experiments have shown that PcGTO3 and PcGTO4 could be labelled through their catalytic cysteine using such fluorogenic compounds. Moreover, first experiments using this method have shown that PcGTO3 could react with substrates as 2-Br-1-tetralone (unpublished data, Figure 16).

For PcUre2p1, which does not possess any catalytic cysteine and no esterase activity, other substrates, such as BADAN (6-bromoacetyl-2-dimethylaminonaphtalene) could be used (Svensson et al., 2002).

Concerning proteins possessing transferase activity, different substrates have been recently described and could be used in such approaches to screen chemical libraries. This approach should lead to the identification of inhibitors taking into account the high diversity found in C-terminal part (H-site) of these enzymes, responsible for the substrate binding (Fujikawa et al., 2008). For instance, the structure of such a probe is shown in Figure 17.

3.3.2 Natural substrate fishing

To unravel the putative functions of GSTs, it is clear that we need to accede to their physiological substrates. The ABPP method described above could be a way to screen chemical libraries in order to identify potential substrates for studied GSTs. However, it could be useful also to use this methodology to screen physiological metabolites. As alternative, recent advances made in plant GSTs have shown that the identification of physiological substrates could be performed using a strategy of “ligand fishing”. Using this approach, the

plant GSTs lambda have been shown to be involved in the flavonoid metabolism (Dixon and Edwards, 2010). Briefly, proteins of interest will be immobilized on a static matrix (strep-Tag) and exposed to a mixture of potential ligands. The complex proteins-ligands will then be eluted and analyzed either by GC-MS or LC-MS for identification of the ligands.

3.4 Genetic tools

Besides the biochemical approaches based on recombinant proteins use and *in vitro* experiments, additional *in vivo* experiments are required to access to the physiological function of GSTs. During this work, we tried to complement the yeast strain deficient for the Scure2p gene. No functional complementation was observed whatever the retained screening test (oxidative stress or nitrogen metabolism), suggesting that *P. chrysosporium* Ure2p4, Ure2p6 and Ure2p1 do not exhibit the same functions than the yeast counterpart. As discussed above, the saprophytic basidiomycete *P. chrysosporium* possesses a GST network strongly different that one of *S. cerevisiae*, this latter being clearly not the best choice as host for complementation experiments.

In these conditions, genetic tools are required to investigate the functions of GSTs in *P. chrysosporium*. Several studies have described the genetic manipulation of *P. chrysosporium* (Alic et al., 1991; Randall and Reddy, 1991; Ma et al., 2001; Matityahu et al., 2008). Thanks to collaboration with Gary Foster (UK), who has developed a “toolkit” for basidiomycete transformation, our research team can access to optimized plasmids to overexpress or inhibit gene expression in *P.chrysosporium*. Preliminary tests showed that protoplast transformation was efficient to overexpress GFP and RFP in the fungus using hygromycin as a selective marker. Plasmids constructions are underway to perform RNAi experiments focusing in particular on Pcure2p4 and 6. We will try to identify potential phenotypes in the transformed strains during PAH degradation. The subcellular localization of these proteins, such as their gene expression induction could be followed in fungal cells using GFP fusions.

In addition, a tagged protein of interest could be expressed in the fungus and complexes protein-ligands could then be trapped using affinity chromatography. This method has been recently used successfully to identified physiological substrates of various plant

GSTs using the biotin-streptavidin system (Dixon and Edwards, 2009; Dixon and Edwards, 2010), demonstrating the feasibility of such *in vivo* approaches.

4 Conclusion

The major aim of this work was to investigate the superfamily of GSTs in a wood-decaying fungus *P. chrysosporium*. This work has been based on different complementary approaches: phylogenetic analysis, biochemical characterization, *in vivo* experiments, which are required to understand the function of these enzymes since they belong to a complex superfamily involved in many detoxication processes as well as in other endogenous pathways.

Recent advances on the evolution of this superfamily in poplar have suggested that the C-terminal domain has been subject to relaxed functional constraints or divergent directional selection, which may have allowed rapid changes in substrate specificity, affinity, and activity (Lan et al., 2009). Thus, assigning *in vivo* functions of GSTs remains an ambitious challenge and the relevance of generalization of data obtained from a model fungus such as *P. chrysosporium* to other fungi is still an open question.

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Résumé: *Phanerochaete chrysosporium* est un champignon ligninolytique largement étudié pour ses capacités à dégrader la lignine et certains xénobiotiques grâce à un important système d'enzymes extracellulaires. Son génome est entièrement séquencé et constitue un inventaire de séquences protéiques prédites qui a permis la description de nombreuses superfamilles de protéines. Parmi elles, les Glutathion-S-Transférases sont essentiellement impliquées dans le métabolisme secondaire du champignon. Cependant, malgré les nombreux travaux montrant l'implication de ces enzymes dans la réponse aux stress, le développement cellulaire et plus globalement dans certaines fonctions métaboliques, leurs réelles fonctions restent inconnues à cause de leur grande diversité et le manque de données concernant leurs spécificités catalytiques. *P. chrysosporium* possède 27 isoformes de GSTs qui se regroupent en 7 classes. Parmi elles, 3 sont étendues chez les champignons saprophytes : les classes Omega, Ure2p et ethérases. Deux membres de la classe Omega ont été caractérisés au niveau biochimique et montrent des spécificités de substrat. En effet, PcGTO1 fait partie d'une nouvelle classe appelée S-glutathionyl-p-hydroquinone reductase, alors que PcGTO3 est plutôt active avec le phenylacetophenone. La structure tridimensionnelle de PcGTO1 suggère que l'enzyme appartient également à une nouvelle classe structurale que nous avons appelée xi. La deuxième classe majoritaire que nous avons étudiée est la classe des Ure2p qui est composée de 9 isoformes et se regroupent en 2 sous-classes. Trois isoformes ont été étudiées au niveau transcriptionnel, biochimique et physiologique. PcUre2p4 et PcUre2p6 appartenant à la première sous-classe sont spécifiquement exprimés dans des cultures fongiques en présence d'hydrocarbures aromatiques polycycliques et l'activité des protéines recombinantes correspondantes est classique des GSTs à savoir le transfert de glutathion sur un substrat hydrophobe. A l'inverse, PcUre2p1 qui appartient à la deuxième sous-classe est exprimé de manière constitutive au niveau transcriptionnel et la protéine présente une activité thiol transférase comparable aux protéines de la classe Omega. Les analyses physiologiques menées grâce à la complémentation de souche déficience de *Saccharomyces cerevisiae* ont montré que PcUre2p1, PcUre2p4 et PcUre2p6 n'avaient pas la même fonction que l'isoforme de la levure puisqu'aucune complémentation n'a été détectée en ce qui concerne la résistance au stress ou la régulation du métabolisme azoté. Ces résultats suggèrent que les champignons, en particulier ceux qui présentent des propriétés saprophytes ont développé des spécificités de fonction de leur GSTs probablement en réponse à des contraintes environnementales.

Mots-clés: *Phanerochaete chrysosporium,* Glutathion S-Transferases, Omega, Ure2p, xenobiotiques, hydrocarbures aromatiques polycycliques.

Abstract: *Phanerochaete chrysosporium* is a ligninolytic fungus widely studied because of its capacities to degrade wood and xenobiotics through an extracellular enzymatic system. Its genome has been sequenced and has provided researchers with a complete inventory of the predicted proteins produced by this organism. This has allowed the description of many protein superfamilies. Among them, Glutathione-S-Transferases (GSTs) constitute a complex and widespread superfamily classified as enzymes of secondary metabolism. However, despite the numerous associations of GSTs with stress responses, cell development and metabolism in various organisms, the functions of these enzymes remain usually evasive mainly due to their high diversity and also to the lack of knowledge about their catalytic specificities. In *P. chrysosporium* 27 GST isoforms have been highlighted and clustered into 7 classes. Among them 3 are extended in saprophytic fungi: the Omega, the Ure2p and the etherase classes. Two members of the Omega class have been characterized at the biochemical level showing difference in substrate specificities. Indeed, PcGTO1 is member of a new class of S-glutathionyl-p-hydroquinone reductase, while PcGTO3 is rather active with phenylacetophenone. The three-dimensional structure of PcGTO1 confirms the hypothesis not only of a new biological class, but also of a new structural class that we propose to name GST xi. The second extended class we have studied is the Ure2p one. It is composed of 9 isoforms in *P. chrysosporium* and clusters into 2 subclasses. Three Ure2p class members have been studied in more details at transcriptional, biochemical and physiological levels. PcUre2p4 and PcUre2p6 of the first subclass are specifically expressed in cultures treated with polycyclic aromatic hydrocarbons and the recombinant proteins are active as typical glutathione transferases. By contrast, PcUre2p1, which belongs to the second subclass is constitutively expressed whatever the condition tested and is active with small molecules as substrate, such as proteins from the Omega class. Physiological studies have revealed that these proteins do not have the same function than the *Saccharomyce cerevisiae* isoform, concerning both the response to oxidative stress and its involvement in the nitrogen catabolite repression. These results suggest that fungi, especially those with saprophytic capabilities, have developed specificities of GST function as an adaptation to environmental constraints.

Keywords: *Phanerochaete chrysosporium,* Glutathione S-Transferases, Omega class, Ure2p class, xenobiotics, polycyclic aromatic compounds.