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par **Filipe GAMA**

Les glutarédoxines : de la réduction des peroxyrédoxines de type II aux systèmes d'assemblage des centres fer-soufre

Soutenue le 10/11/2010

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« En science, la phrase la plus excitante que l'on peut entendre, celle qui annonce des nouvelles découvertes, ce n'est pas "Eureka" mais "c'est drôle". »

Isaac Asimov

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Abréviations

ADN : acide déoxyribonucléique
Aft : activator ferrous transport
AOX: alternative oxydase
APS : ammonium persulfate
Apx : ascorbate peroxydase
ATP : adénosine triphosphate
Bet : bromure d'éthidium
BSA : bovine serum albumin
CDSP : chloroplastic drought-induced stress protein
COOH : cumene hydroperoxyde
DAH: déhydroascorbate
DEAE : diéthyl amino éthyle
DHLA: acide dihydrolipoïque
Dsb: Disulfide bond formation protein
DTT : dithiothréitol
EDTA : ethylene diamine tetra acetic acid
EOR : espèce oxygénées réactives
ENR : espèce nitrées réactives
EST : expressed sequence tag
FBPase : fructose, 1-6, biphosphatase
FNR : fumarate nitrate réductase
Fra : fe repressor of activation
FTR : ferrédoxine thiorédoxine réductase
G6PDH : glucose-6-phosphate déshydrogénase
GAPDH : glycéraldéhyde-3-phosphate déshydrogénase
GFP : green fluorescent protein
GR : glutathion réductase
Grx : glutarédoxine
GSH : glutathion
GSSG : glutathion oxydé
Gst : glutathion-S-transferase
Gpx : glutathion peroxydase
H_2O_2 : peroxyde d'hydrogène
IPTG : isopropyl-ß-D thiogalactoside
Isc : iron sulfur cluster
kcat : efficacité catalytique
kDa : kilo dalton
Km : constante d'affinité de Michaelis-Menten
LA : acide lipoïque
LB : Luria-Bertani
MDAH : monodéhydroascorbate
Msr : méthionine sulfoxyde réductase
mV : millivolt
NADP-MDH : NADP malate déshydrogénase
NADPH : nicotinamide adénine dinucléotide phosphate
NOS : NO synthase
NTR : NADPH thiorédoxine réductase

Abréviations

Nif : nitrogen fixation
pET : plasmide d'expression à polymérase de phage T7
PAGE : polyacrylamide gel electrophoresis
PCR : polymerase chain reaction
PDI : protéine disulfure isomérase
PICOT : Protein kinase C cousin of thioredoxin
Prx : peroxyrédoxine
PSI : photosystème I
PSII : phosystème II
Rli : RNase L inhibiteur
RMN : résonance magnétique nucléaire
rpm : rotations per minute
SDS : sodium dodécyl sulfate
SOD : superoxyde dismutase
Suf : sulphur mobilization
tBOOH : tert-butyl hydroperoxyde
TDX: thiorédoxine contanant un domaine tétratricopeptide
TEMED : N,N,N',N'-Tetramethylethylenediamine
Tpx : thiol peroxydase
Trx : thiorédoxine

INTRODUCTION

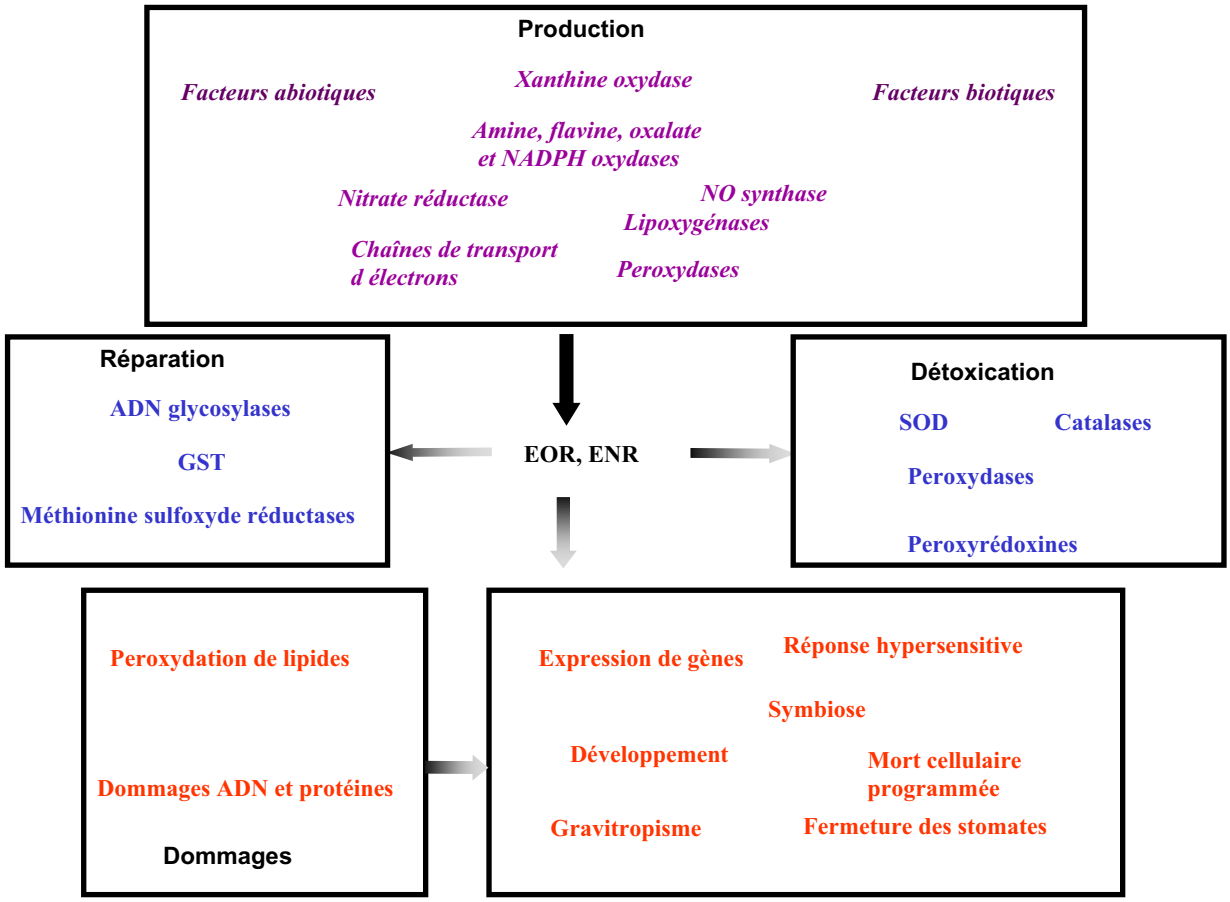


Figure 1 : Les causes et conséquences des EORs et ENRs. Ces composés peuvent être d'origine biotique (bactéries, virus, animaux) ou abiotique (sécheresse, inondation, présence de métaux), mais dans tous les cas le métabolisme cellulaire sera modifié jusqu'à engendrer un état de stress oxydant. Ces composés oxydants formés vont oxyder des molécules cellulaires, ces dégâts étant soit prévenus soit réparés à l'aide de systèmes complexes dont font partie les SODs, les catalases, les peroxydases ou encore les méthionines sulfoxyde réductases. Les dommages contre lesquels ces mécanismes luttent peuvent toucher bon nombre de processus physiologiques et peuvent aboutir à la mort des cellules pour les cas les plus sévères.

Introduction

I Le stress oxydant chez les plantes :

A Les causes et effets du stress oxydant

Les variations de l'environnement auxquelles sont sujets les végétaux les contraignent à posséder une forte capacité d'adaptation. Lorsque ces changements sont trop brutaux, ils peuvent provoquer l'apparition d'un stress oxydant caractérisé par la formation d'espèces oxygénées réactives (EOR) et nitrées réactives (ENR). Ce stress peut ainsi provenir de facteurs abiotiques, par exemple dans le cas d'un stress lumineux, d'une sécheresse, d'une exposition au froid, à la chaleur ou aux UV, ou de conditions d'hypoxie (Mittler, 2002). Il peut également être de nature biotique comme l'attaque par des insectes et des animaux, ou par des micro-organismes pathogènes tels des virus, des bactéries ou des champignons. Ces facteurs étrangers à la plante vont bouleverser son métabolisme, conduisant à la formation de composés réactifs qui peuvent induire différentes réactions de la plante (Noctor and Foyer, 2000). La figure 1 résume les différents facteurs provoquant la production d'EOR et ENR, quelques enzymes impliquées dans leur détoxication et certains composés ou voies métaboliques affectées.

Parmi les EOR, les principaux composés formés sont le peroxyde d'hydrogène (H_2O_2), le radical hydroxyle ($OH^{\bullet}$), l'anion superoxyde ($O_2^{\cdot-}$) et l'oxygène singulet ($^1O_2$). Ces EOR possèdent un fort pouvoir oxydant et vont réagir avec la plupart des molécules biologiques, entraînant d'importantes modifications de leurs propriétés physico-chimiques aux conséquences néfastes pour l'intégrité de la cellule. De plus, certaines molécules non chargées sont également capables de diffuser à travers les parois végétales et pourront ainsi causer de multiples dégâts comme l'oxydation des bases de l'ADN, ou encore l'oxydation de protéines notamment au niveau des cystéines et des méthionines. En effet, ce sont les deux acides aminés les plus sensibles et puisqu'ils sont assez souvent impliqués dans la fixation de métaux ou dans les propriétés

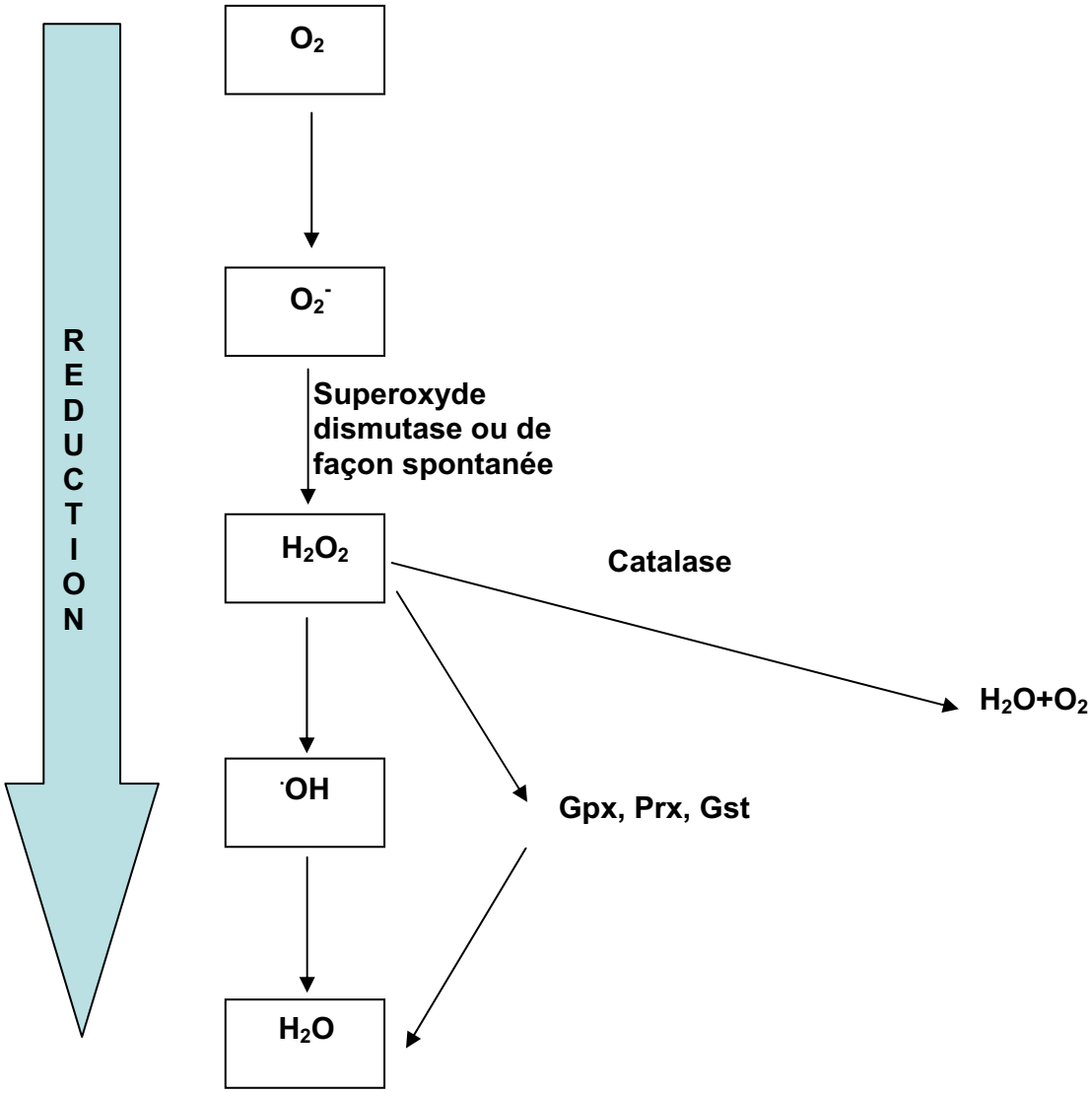


Figure 2: Différentes espèces réactives de l'oxygène (EOR) selon une chaîne de réduction. La molécule de dioxygène est la plus oxydante, par gain successif d'électron, un composé de moins en moins oxydant est formé en passant par l'anion superoxyde, le peroxyde d'hydrogène, le radical hydroxyle et enfin l'eau.
Est aussi présenté le processus de dégradation de l'anion superoxyde en deux étapes. Tout d'abord les superoxyde dismutases dégradent l'anion superoxyde en peroxyde d'hydrogène qui sera alors pris en charge par les Gpxs, les Prxs ou les Gsts ou les catalases pour donner de l'eau.

catalytiques de nombreuses enzymes et protéines, leur oxydation peut entraîner une perte d'activité de ces protéines. Les lipides membranaires (phosphatidylcholine, acides gras, esters de cholestérol...) sont également sujets à modification, le plus souvent à une peroxydation, ce qui provoquera éventuellement des modifications de la fluidité membranaire.

B Formation des EOR

Au cours du métabolisme basal, ces composés oxydants sont produits à faible concentration. Ils sont le plus souvent issus de la réduction partielle de l'oxygène se déroulant à la suite de fuite d'électrons au niveau des chaînes de transport d'électrons, chloroplastique ou mitochondriale. De plus, la photorespiration dans le peroxysome, le catabolisme des lipides ou le fonctionnement de diverses oxydases complètent la liste des processus majeurs responsables de la production des EOR (figures 1 & 2).

Dans les mitochondries, les EOR et plus particulièrement $O_2^{\cdot-}$ sont générés au niveau des complexes I et III en raison d'une fuite d'électrons de flavoprotéines réduites vers l'oxygène moléculaire (Imlay, 2003). Chez les mammifères, les EOR sont ainsi formés principalement dans la mitochondrie, alors que chez les organismes photosynthétiques, ce compartiment n'est responsable que d'une quantité relativement faible d'EOR à la lumière masi en revanche beaucoup plus importante à l'obscurité. Cependant, il semblerait que la mitochondrie soit un facteur clé de la régulation redox et de la signalisation dans les cellules végétales (Noctor *et al*., 2007). La faible quantité d'EOR produite dans cet organite peut être au moins partiellement expliquée par la présence de l'alternative oxydase (AOX) qui constitue une voie alternative pour la réduction de l'O_2 ne faisant pas intervenir le complexe III. En effet, l'AOX est surproduite en présence d'H_2O_2 et des mutants dans lesquels le gène codant cette enzyme a été interrompu, accumulent 5 fois plus d'EOR (Maxwell *et al*., 1999; Wagner, 1995). Ainsi, 2 à 3 % de l'oxygène utilisé par la

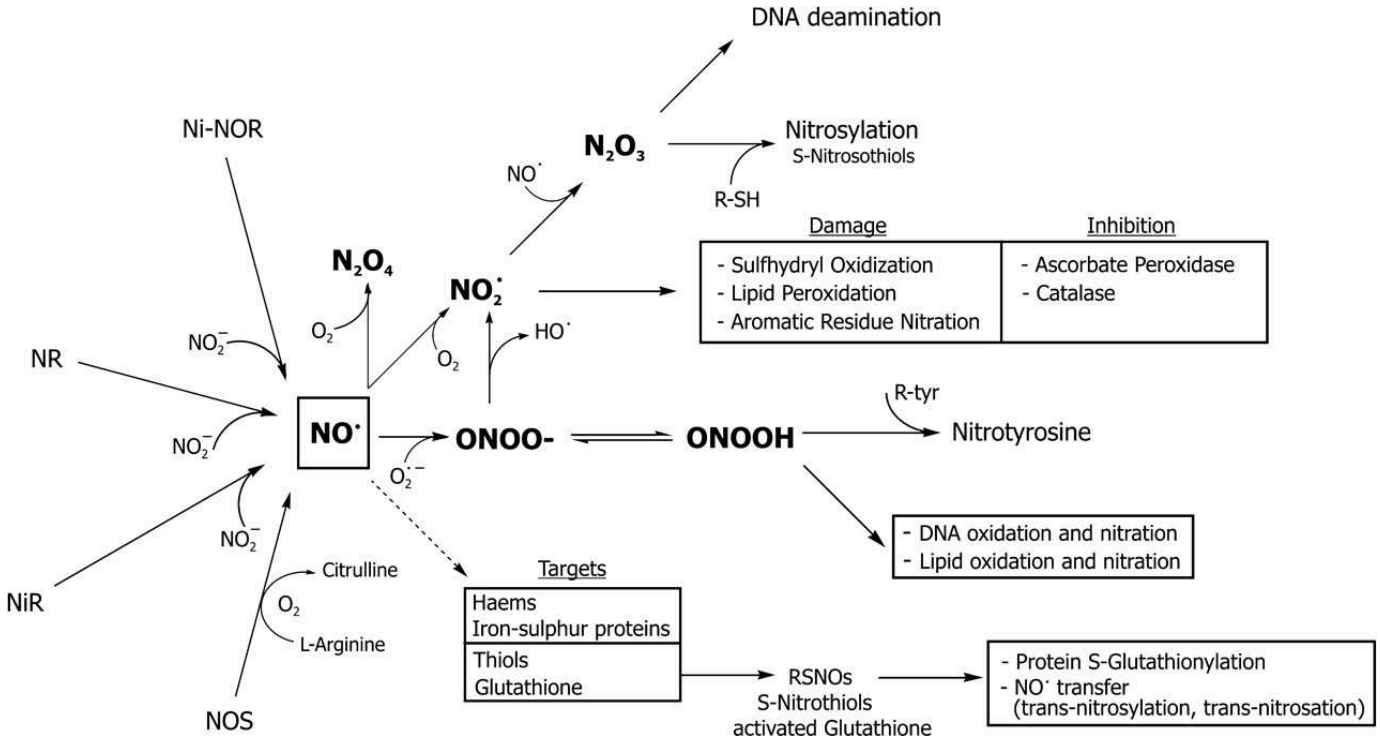


Figure 3 : Production des ENR et les effets sur le métabolisme.
Le $NO^{\bullet}$ peut réagir avec l'anion superoxyde pour former du peroxynitrite ($ONOO^-$) et de l'acide peroxynitrique (ONOOH). Le $NO^{\bullet}$ peut également réagir avec du O_2 formant ainsi du dioxyde d'azote ($NO_2^{\cdot}$), composé hautement réactif, et du tetraoxyde diazote (N_2O_4). Les ENR vont causer des dommages sur les groupements thiols, les lipides, les résidus aromatiques, l'ADN et toutes les protéines qui contiennent des hèmes, des centres fer-soufre ou des thiols (Rinalducci *et al*., 2008).
Ni-NOR, nitrite oxyde nitrique réductase; NiR, nitrite réductase; NOS, oxyde nitrique synthase; NR, nitrate réductase; RSNOs, *S*-nitrosothiols.
.

mitochondrie chez les plantes sont convertis en anion superoxyde puis en peroxyde d'hydrogène au niveau de la matrice (del Rio *et al*., 2002; Oracz *et al*., 2007). Dans le chloroplaste, il existe plusieurs sites de production continue des EOR générées par la lumière. En effet, au cours du transport d'électrons, l'O_2 dégagé peut être éliminé physiologiquement du chloroplaste par réduction ou par assimilation (Apel and Hirt, 2004). Il existe trois systèmes de consommation de l'O_2 produit : la photorespiration qui dérive de la réaction d'oxygénation du ribulose 1-5 biphosphate via la fonction oxygénase de la Rubisco ; la réduction directe par le photosystème I (PSI) et la ferrédoxine (réaction de Mehler) ; et la respiration du chloroplaste (Apel et Hirt, 2004). Au niveau du PSI, la photoréduction de l'O_2 produit l'anion superoxyde qui sera réduit en peroxyde d'hydrogène (Asada, 2006). De l'oxygène singulet est également produit par le photosystème II (PSII), principalement par réaction entre la chlorophylle excitée dans un état triplet et l'oxygène. Cette production a d'abord été décrite au niveau du PSII isolé puis ensuite démontrée *in vivo* (Fryer *et al*., 2002). Un stress provoqué par un excès de lumière va engendrer une surproduction d'oxygène singulet provoquant l'inactivation du centre réactionnel du PSII, processus appelé photoinhibition. Ce phénomène est en particulier marqué par une diminution/dégradation d'une des protéines majeurs de ce complexe, la protéine D1 (Hideg *et al*., 2002).

Concernant le peroxysome, lors du cycle photorespiratoire suite à l'action de la glycolate oxydase, on observe une formation de peroxyde d'hydrogène. L'anion superoxyde est également formé dans cet organite lors de la formation d'acide urique à partir de xanthine, réaction catalysée par la xanthine oxydase (del Rio *et al*., 2006).

Enfin, la formation des radicaux hydroxyles se produit essentiellement par la réaction entre H_2O_2 et des métaux actifs d'un point de vue redox, tels que le Fe^{2+} et Cu^{2+}, réaction appelée réaction de Fenton (réaction 1) (Rachmilovich-Calis *et al*., 2009). Le Fe^{3+} ainsi formé peut à son tour réagir avec $O_2^{\cdot-}$ (réaction d'Haber-Weiss, réaction 2) pour reformer du Fe^{2+} et entretenir le

cycle (Rachmilovich-Calis *et al*., 2009). Il est donc aisément compréhensible que la cellule doive réguler très finement à la fois les concentrations en EOR mais aussi la proportion de métaux libres dans la cellule. Voici ci-dessous les deux réactions présentées :

$Fe^{3+} + O_2^{\cdot -} \longrightarrow Fe^{2+} + O_2$ (Réaction 1)

$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$ (Réaction 2)

C Formation des ENR

Les ENR sont également produites constitutivement dans la cellule. La formation de ces ENR et leurs effets sur les composants cellulaires sont présentés en figure 3. L'élément central est l'oxyde nitrique ($NO^{\bullet}$). C'est un radical gazeux formé en présence ou non d'enzymes, qui est produit dans les plantes de façon constitutive. Cependant, cette production augmente considérablement lorsque la plante est soumise à différentes conditions de stress comme l'accumulation de métaux lourds, l'hypoxie, l'attaque par un pathogène ou lors de certains processus développementaux (le développement racinaire, la fermeture des stomates, la germination ou la sénescence) (Besson-Bard *et al*., 2009; Delledonne, 2005; Lamattina *et al*., 2003). Une production d'oxyde nitrique a été observée dans le peroxysome de nombreuses plantes (*Olea europeae, Helianthus annuus* et *Arabidopsis thaliana*) suite à l'oxydation de L-arginine (Alderton *et al*., 2001; Barroso *et al*., 1999; Guo and Crawford, 2005). Plusieurs voies de production ont été proposées, la production enzymatique du $NO^{\bullet}$ peut provenir soit du nitrite grâce à l'activité de nitrate réductases et de nitrite-oxyde nitrique réductases, soit de l'arginine grâce à une NO synthase (NOS) (Rinalducci *et al*., 2008). Une fois produit, le $NO^{\bullet}$ est un composé très réactif qui peut être suroxydé en d'autres oxydes d'azote. Il peut notamment former du

peroxynitrite ($ONOO^-$) en présence d'anion superoxyde, en équilibre avec de l'acide peroxynitrique. Le premier, va se décomposer rapidement et former ainsi des radicaux hydroxyls ($OH^\bullet$) et dioxyde d'azote ($NO_2^\bullet$). Il peut aussi être éliminé par les thiol-peroxydases (voir le paragraphe concerné).

Outre leur rôle dans la signalisation cellulaire, ces ENR vont engendrer de nombreuses modifications. Ainsi, elles peuvent initier la peroxydation des lipides, nitrosyler les groupements thiols des protéines pour donner des groupements *S*-nitrosothiols mais aussi engendrer la nitration des résidus aromatiques des protéines, en particulier les tyrosines. Le $NO^\bullet$ sera également très réactif vis-à-vis des hèmes et des centres fer-soufre ce qui conduit éventuellement à l'inactivation des protéines qui les contiennent (Requena *et al*., 2001).

D Rôles des EOR dans la signalisation

Les EOR sont produites constamment au niveau cellulaire et sont impliquées dans différents mécanismes de la vie cellulaire comme le déroulement de son cycle de division ou l'entrée en apoptose, ainsi que dans la régulation de la transcription des gènes ou de l'activité de certaines protéines (Scandalios, 2002). On sait que la production d'EOR dans les mitochondries et les chloroplastes ont comme résultat des changements au niveau du transcriptome nucléaire, sans connaître pour autant l'identité précise des signaux dits rétrogrades entre ces organites et le noyau. Il est notamment possible que les EOR changent l'activité des facteurs de transcription en modifiant leur état redox.

Le peroxyde d'hydrogène, l'anion superoxyde, le NO et l'$^1O_2$ sont *a priori* les composés oxydants ayant les rôles les plus importants au niveau de la signalisation cellulaire (Foyer and Noctor, 2005; Foyer and Noctor, 2009). Chez les procaryotes, H_2O_2 et $O_2^{\bullet-}$ régulent un grand nombre de gènes par l'intermédiaire du facteur de transcription du type OxyR ou du système

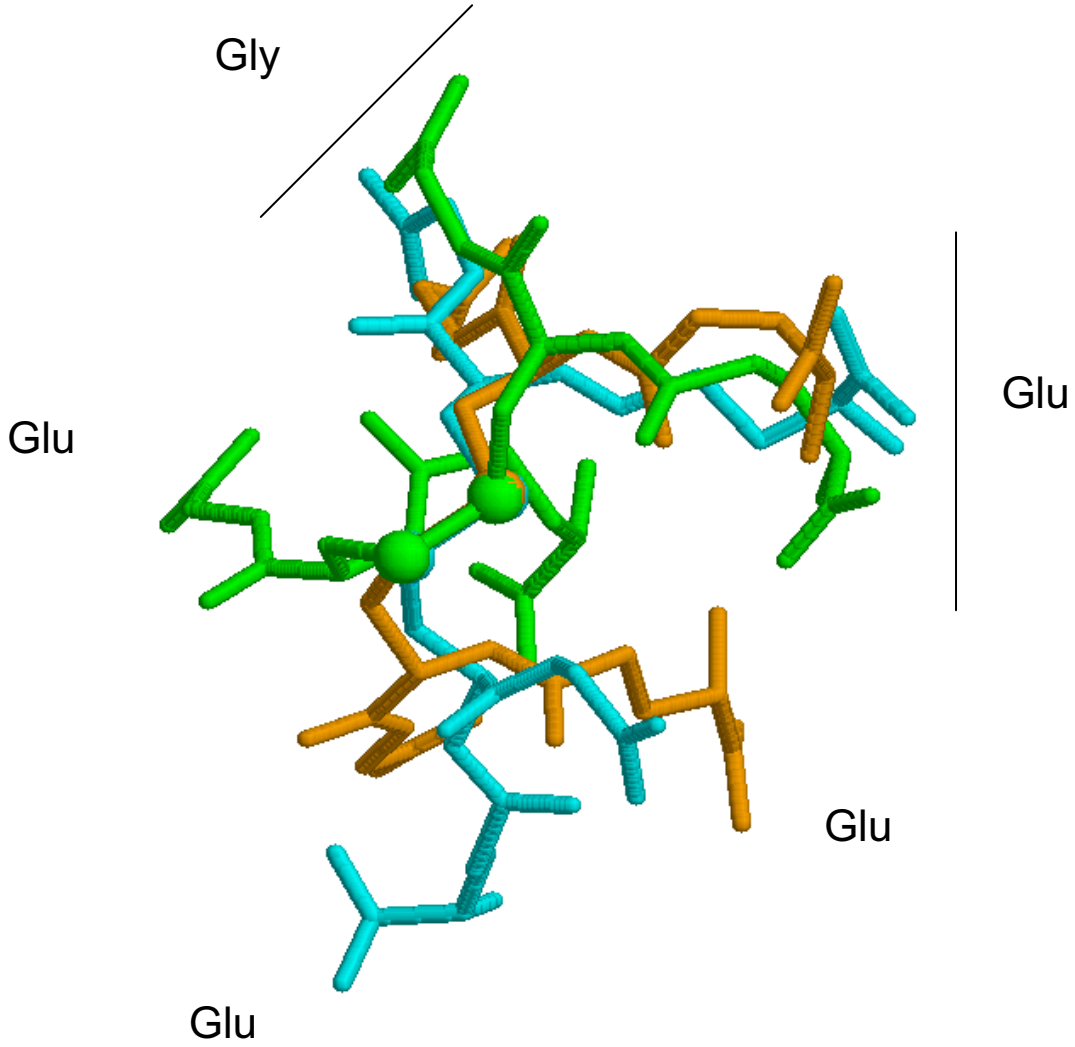


Figure 4: Molécule de glutathion oxydée.
Orientation du glutathion oxydé (GSSG) dans les structures de trois protéines différentes (2GRT : human glutathione reductase R37W, cyan, 1GRA human glutathione reductase, orange, 1YKC : human glutathione S transferase, vert). Le GSH est un tripeptide, le γ-L-Glutamyl-L-cystéinylglycine. Sous sa forme oxydée, un pont disulfure se forme entre deux molécules de GSH pour donner le GSSG. Le pont disulfure est représenté par la liaison présente entre les deux boules représentant les atomes de soufre. La superposition des trois molécules de glutathion oxydé au niveau du disulfure révèle la grande flexibilité de la molécule. En conséquence il n'existe pas de site « universel » de liaison du glutathion dans les protéines glutathion-dépendantes.

SoxR/S (Monje-Casas *et al*., 2001). Chez les plantes supérieures, les EOR jouent notamment un rôle primordial dans la germination, en effet, ils agissent comme signal positif capable de lever la dormance des semences, particulièrement le peroxyde d'hydrogène, cela en interagissant avec la signalisation hormonale, ce qui conduit à des changements dans l'expression des gènes ou de l'état redox cellulaire (Bailly *et al*., 2008). Il existe également un modèle très complet chez *Arabidopsis thaliana* montrant que le peroxyde d'hydrogène et le NO jouent un rôle majeur dans le processus de fermeture des stomates, au travers de la modification de l'expression de nombreux gènes, la plupart codant des protéines entrant dans la régulation redox, mais certains codant pour des protéines ayant des fonctions de signalisation (Bright *et al*., 2006; Desikan *et al*., 2001). De plus, à travers l'inhibition d'enzymes telles que les catalases et ascorbate peroxydases, deux enzymes importantes dans les systèmes de détoxication vont, selon les variations de leur concentration, activer ou réprimer certaines voies de signalisation ou voies métaboliques (Durzan and Pedroso, 2002).

II Les systèmes enzymatiques de dégradation des EOR

Avant d'introduire les systèmes enzymatiques, il est important de noter qu'il existe également des systèmes non-enzymatiques de dégradation des EOR. Il s'agit le plus souvent du glutathion, de l'ascorbate, de l'α-tocophérol, de l'acide lipoïque et des caroténoïdes.

Le glutathion (GSH) est un tripeptide (γ-L-glutamyl-cystéinylglycine) constituant la plus grande réserve de thiols non protéiques, que l'on retrouve dans tous les compartiments cellulaires soit sous forme réduite (GSH) soit sous forme oxydée (forme disulfure (GSSG), nitrée (GSNO) ou oxygénée (GSOH)). La figure 4 nous montre une molécule de glutathion sous forme disulfure. Il est considéré comme le tampon redox cellulaire et peut réduire directement des substrats oxydés.

Il est connu pour réduire notamment les glutarédoxines, certaines peroxyrédoxines et glutathion peroxydases. Il peut se fixer de manière covalente sur des groupements thiols libres, processus appelé glutathionylation. Ce phénomène serait responsable de la protection de ces thiols en condition oxydante et pourrait également jouer un rôle régulateur pour de nombreuses protéines participant à diverses voies métaboliques et à la signalisation cellulaire (Bedhomme *et al*., 2009; Gao *et al*., 2009).

L'ascorbate est une molécule présente dans tous les compartiments cellulaires mais particulièrement abondante dans le chloroplaste. Il est connu pour régénérer les ascorbate peroxydases, après quoi il se retrouve oxydé sous forme de monodéhydroascorbate (MDHA) ou déhydroascorbate (DHA). Ces deux composés sont ensuite principalement réduits respectivement par des MDHA et DHA réductases, participant au cycle ascorbate-glutathion (Sanmartin *et al*., 2007).

L'acide lipoïque (LA), sous forme libre, pourrait aussi constituer un antioxydant en réagissant avec certains EOR comme notamment $O_2^{\cdot -}$ et $OH^{\cdot}$, ou en chélatant certains métaux (Petersen Shay *et al*., 2008). Toutefois, sa fonction première semble être de servir de cofacteur à plusieurs enzymes mitochondriales (Taylor *et al*., 2004). Sa forme réduite est l'acide dihydrolipoïque (DHLA) alors que sa forme oxydée est le LA.

Les caroténoïdes sont des pigments dont font partie les carotènes et les xantophylles. On les retrouve donc dans tous les organes colorés d'une plante (fleur, feuille, fruit, racine…etc). Ils sont notamment capables de réagir avec l'oxygène singulet produit au niveau du chloroplaste protégeant ainsi la chaîne de transport d'électrons photosynthétique (Havaux *et al*., 2007).

Introduction

A Les superoxyde dismutases

Les superoxyde dismutases (SODs) font partie d'un groupe de métalloprotéines essentielles pour la protection des cellules en présence de dioxygène. Ces enzymes ont comme fonction la dismutation d'$O_2^{\cdot -}$ en H_2O_2 qui sera réduit à son tour par différents types de peroxydases. Les SODs ont été isolées et caractérisées chez de nombreux organismes. Les principaux types connus sont mentionnés ci-dessous. Une classe de SOD possède du Cu(II) et du Zn(II) au niveau du site actif (Cu/Zn SOD), une autre classe présente du Mn(III) (Mn SOD), une autre du Fe(III) (Fe SOD), et enfin une quatrième classe avec du Ni(III) (Ni SOD). Ces métaux changent de valence lors de la réaction par la perte d'électron associée à l'étape de réduction, cependant ils seront chargés de façon identique en fin de catalyse, qui se décompose en deux étapes, une perte d'électron suivie d'un gain d'électron pour l'enzyme. On retrouve généralement les Cu/Zn SODs dans le cytosol et le chloroplaste des cellules eucaryotes et chez certains procaryotes. En revanche, les Mn SODs sont présentes chez les procaryotes et dans les mitochondries, les Fe SODs sont retrouvées chez les procaryotes, les algues et dans les chloroplastes de certaines plantes supérieures, et enfin la présence de Ni SODs a été démontrée chez *Streptomyces coelicolor* (Scandalios, 2005).

A l'inverse d'autres organismes qui ne contiennent qu'un type de SOD par compartiment cellulaire, les plantes possèdent tous les différents types de SOD sauf celles à nickel, et un nombre de gènes plus important, confirmant la plus grande complexité des systèmes de détoxication des EOR. La surexpression de SODs de tabac n'améliore pas la tolérance au stress oxydant ce qui tend à prouver qu'une autre voie de régulation serait limitante, cependant, l'expression de SODs de pois chez le tabac augmente la protection des membranes endommagées par un traitement au méthyl-viologène (Allen *et al*., 1995).

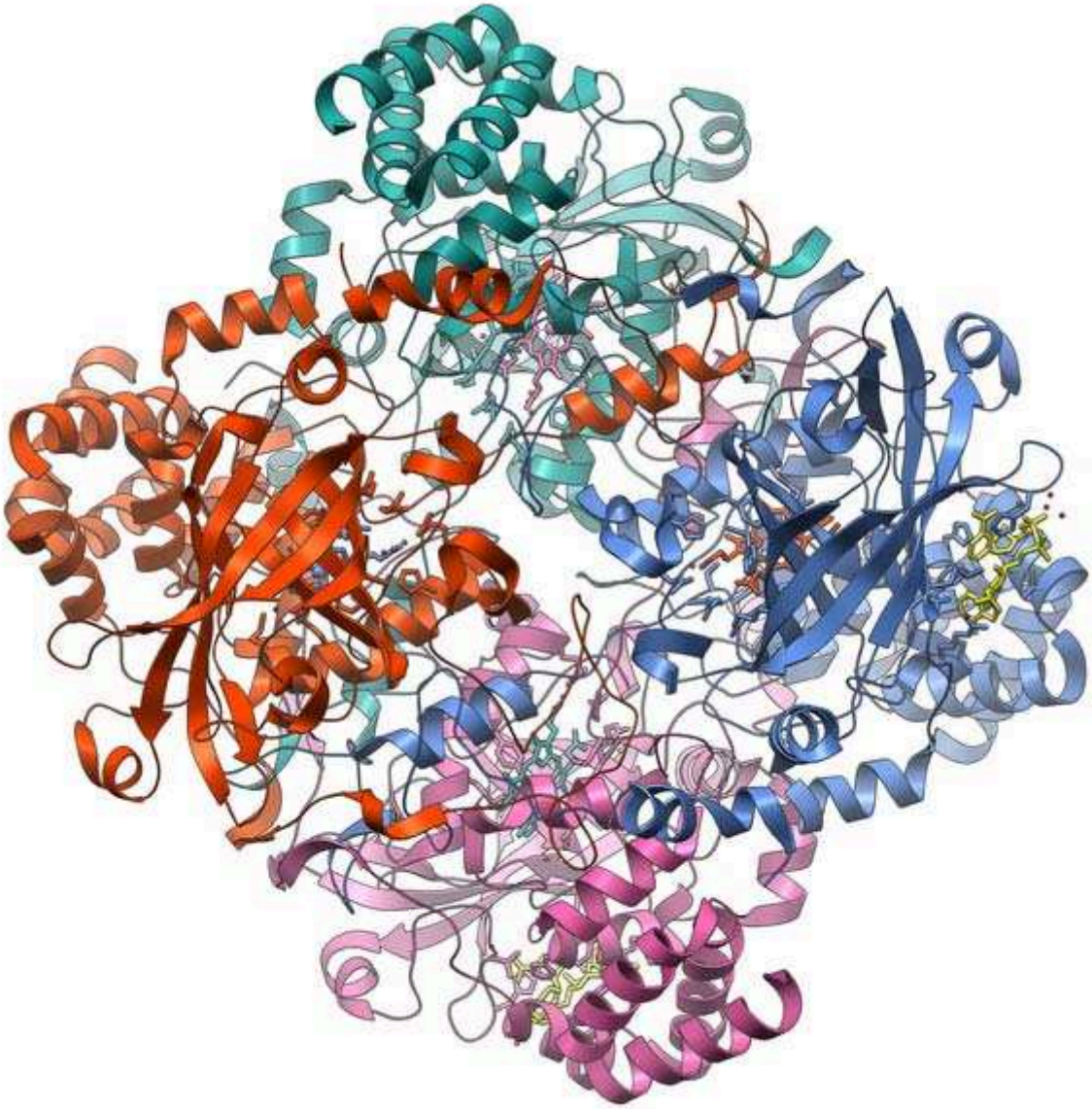


Figure 5 : Structure tridimensionnelle d'une catalase organisée en homotétramère. Chaque monomère possède une structure caractérisée par la présence de feuillets β entourés par des hélices α et à l'intérieur de laquelle on retrouve un hème.
Catalase de *Thermus thermophilus* (PDB : 2V8U).

Les Cu/Zn SODs sont susceptibles d'être attaquées par le peroxynitrite, formant ainsi un intermédiaire qui peut attaquer les résidus tyrosine selon la réaction suivante (Beckman and Crow, 1993) :

$SOD\text{-}Cu_2^{+} + {}^{-}OONO \dashrightarrow SOD\text{-}CuO...NO_2$

$SOD\text{-}CuO...NO_2^{+} + H\text{-}Tyr \dashrightarrow SOD\text{-}Cu_2^{+} + HO^{-} + NO_2^{-}Tyr$

Ces réactions modifient la valence de l'atome de cuivre de la superoxyde dismutase qui ne peut plus alors effectuer son action sur $O_2^{\cdot -}$. Cependant ces modifications sont réversibles.

B Les catalases

Les quantités d'H_2O_2 formées par le métabolisme, pouvant devenir préjudiciables pour la cellule, doivent absolument être régulées par des enzymes de détoxication. Parmi celles-ci, les catalases ont été découvertes il y a plus d'un siècle, le terme catalase ayant été utilisé pour la première fois par Loew en 1900. Ce sont des enzymes dont le rôle est de catalyser la décomposition du peroxyde d'hydrogène en eau et O_2 ($2H_2O_2 \rightarrow O_2 + 2H_2O$) (Antoniuk *et al*., 2000). Ces enzymes sont réputées comme étant parmi les plus actives avec un turn-over exceptionnellement élevé (autour de 20 000 s^{-1}). Il existe trois classes de catalases. Les deux premières contiennent un hème : les catalases typiques et les catalase-peroxydases. Ces catalases typiques présentent une structure généralement tétramérique (figure 5), possèdent une masse moléculaire comprise entre 200 et 400 kDa et contiennent un hème par monomère. Elles sont retrouvées chez les eubactéries, archaebactéries, protistes, champignons, plantes et animaux et forment le groupe le plus important (Zamocky and Koller, 1999). Il est possible quelquefois de retrouver du NADPH lié à chaque sous-unité, ce produit ne fonctionne pas comme cofacteur dans

ce cas mais plutôt comme protecteur du site catalytique de l'enzyme (Kirkman *et al*., 1987). Les catalase-peroxydases, elles, ne sont pas présentes chez les plantes ni les animaux. Ces deux groupes ont une activité catalytique et peroxydatique. Le troisième groupe, appelé dimanganèse-catalases, est constitué de protéines de bactéries uniquement (par exemple *Thermus thermophilus*), contenant un site actif présentant un dimanganèse. Bien qu'elles catalysent la même réaction, ces trois protéines diffèrent significativement au niveau de l'architecture de leur site actif et également en ce qui concerne leur mécanisme d'action. La dimanganèse-catalase est composée de six sous-unités, le centre actif étant composé de deux atomes de manganèse profondément ancrés dans un monomère entre quatre hélices α (Antoniuk *et al*., 2000).

C Les ascorbate peroxydases (Apx)

Ce sont des peroxydases à hème également, qui réduisent majoritairement H_2O_2 *(Raven et al.*, 2004). Comme leur nom l'indique, ces enzymes utilisent l'ascorbate pour leur régénération. Elles dépendent donc principalement du cycle ascorbate-glutathion. Ces enzymes sont relativement bien connues, notamment leur mécanisme d'action. Selon l'état d'oxydation du fer au niveau de l'hème, la structure du site actif est modifiée, régulant ainsi l'activité de cette peroxydase (Badyal *et al*., 2008). La première étape est la formation d'un intermédiaire oxydé par 2 électrons qui sera ensuite réduit par un substrat de nature organique généralement. Cette première étape est sujette à un mécanisme acido-basique, une histidine conservée au niveau du site actif étant le catalyseur acido-basique du clivage de la molécule d'H_2O_2 (Hiner *et al*., 2002). Les plantes possèdent plusieurs isoformes d'Apx réparties dans différents compartiments subcellulaires, notamment le chloroplaste et le cytosol. Dans le chloroplaste, on trouve des isoformes dans le stroma et d'autres accrochées aux thylacoïdes. Bien que très similaires, ces deux

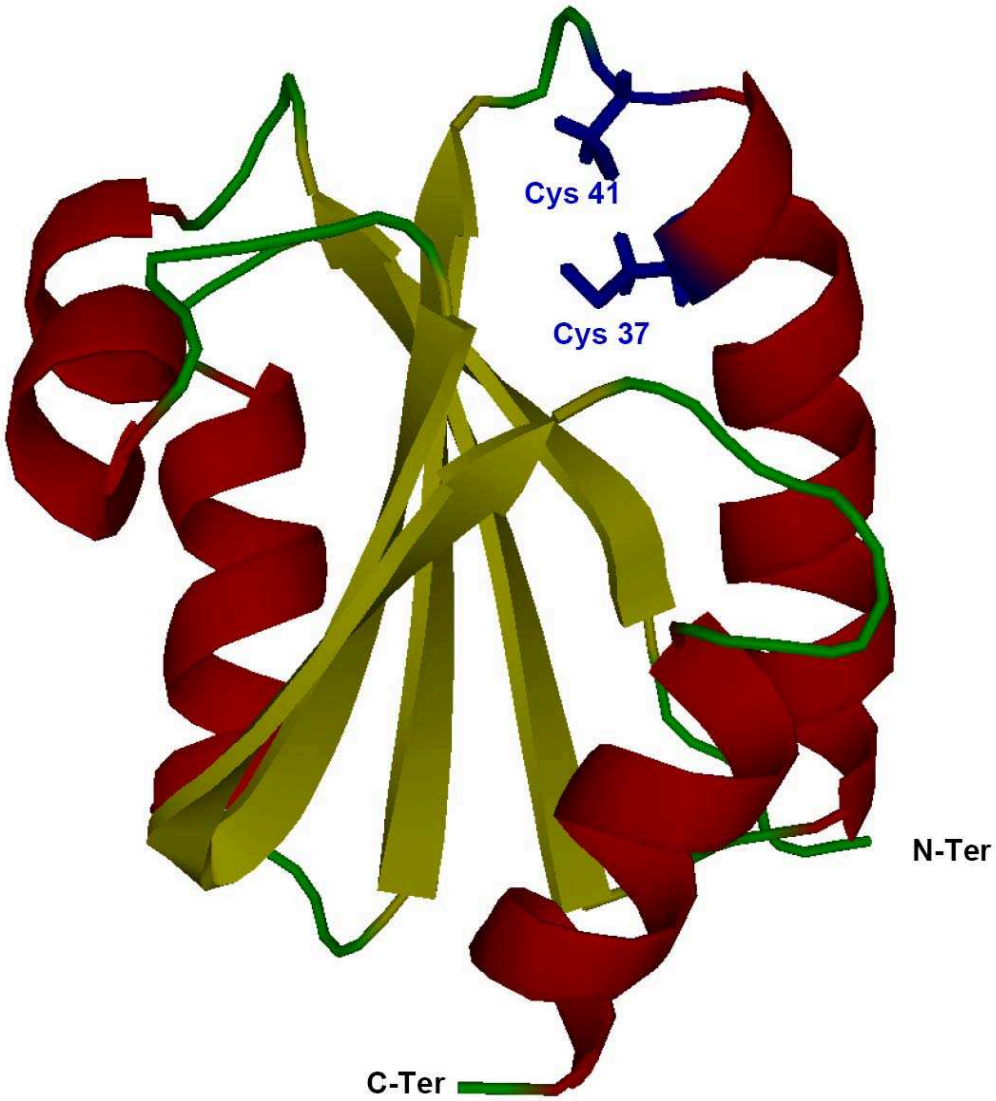


Figure 6 : Structure tridimensionnelle de la Trxh1 de peuplier.
PDB: 1TI3. On observe les 4 feuillets β au centre de 4 hélices α caractéristiques de la structure "Trx-fold". Les cystéines du site actif sont représentés en bleu.

types d'Apx diffèrent par la présence d'une extension C-terminale pour l'ancrage dans les membranes thylacoïdales.

D Les thiol peroxydases (Tpx): peroxyrédoxines (Prx) et glutathion peroxydases (Gpx)

a Classification

On dénombre 15 thiol peroxydases chez *Populus trichocarpa* (9 Prxs et 6 Gpxs) et 17 chez *Arabidopsis thaliana* (9 Prxs et 8 Gpxs) (Rouhier and Jacquot, 2005). Le nombre d'isoformes est généralement plus important chez les organismes photosynthétiques que chez les organismes non-photosynthétiques. Une des explications réside dans la présence d'un site de formation supplémentaire d'EOR, le chloroplaste. De plus, peut être aussi parce qu'il est moins étudié, il semble que le phénomène d'épissage alternatif soit moins fréquent chez les végétaux. En effet, il n'est pas rare chez les mammifères notamment qu'un seul gène puisse donner plusieurs isoformes (2 ou 3 voire plus) distribuées dans différents compartiments subcellulaires, compensant de la sorte un nombre de gènes moins élévés.

Les Tpxs sont des petites protéines dont la masse moléculaire d'un monomère varie généralement de 10 à 20 kDa. Chaque monomère possède une structure 3D comportant un motif de type thiorédoxine (figure 6). Ces enzymes, tout du moins celles qui contiennent des cystéines et non pas des sélénocystéines comme résidus catalytiques, ont en général une efficacité catalytique plus faible (autour de 10^4-10^5 M^{-1} s^{-1}) comparée aux autres peroxydases, catalases et Apxs. Toutefois, elles ont pour particularité de réduire une gamme plus large et plus complexe de substrats tels des hydroperoxydes, des lipides peroxydés mais également pour certaines le peroxynitrite (Dietz *et al*., 2006).

Les Tpx d'organismes photosynthétiques se regroupent en 5 sous-groupes selon le nombre, la position des cystéines impliquées dans la catalyse et aussi selon leur état d'oligomérisation

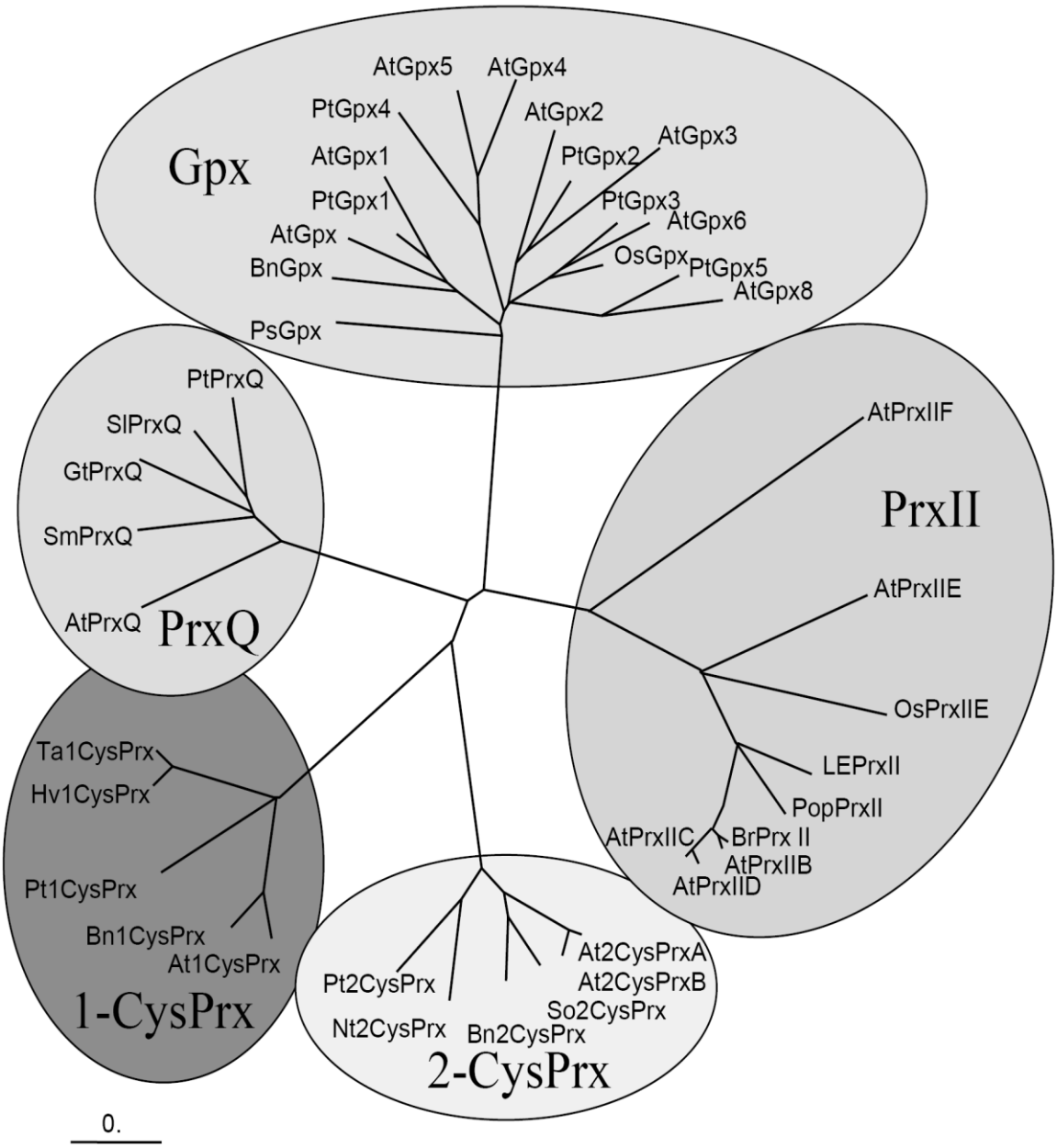


Figure 7 : Arbre phylogénétique des thiol peroxydases de plantes. On retrouve 5 groupes, les Prxs 1-Cys, les Prxs 2-Cys, les Prxs Q, les Prxs de type II et enfin les glutathion peroxydases.
At: *Arabidopsis thaliana*, Pt: *Populus trichocarpa*, Os: *Oryza sativa*, Bn: *Brassica napus*, Br: *Brassica rapa*, Ps: *Pisum sativum*, Le: *Lycopersicon esculentum*, So: *Spinacia oleracea*, Nt: *Nicotiana tabacum*, Hv: *Hordeum vulgare*, Ta: *Triticum aestivum*, Gt: *Gentiana triflora*, Sl: *Sedum lineare*, Sm: *Suaeda maritime*, Ss: *Suaeda salsa*.

(figure 7) (Rouhier and Jacquot, 2005), 1 sous-groupe Gpx et 4 sous-groupes de Prx (2-Cys Prx, 1-Cys Prx, Prx II et Prx Q).

Les Prxs et Gpxs possèdent toutes une cystéine conservée du côté N-terminal, appelée cystéine catalytique ou peroxydatique, mais l'on peut également retrouver une deuxième cystéine appelé cystéine de recyclage qui servira à la régénération de l'enzyme (Rouhier and Jacquot, 2005; Wood *et al*., 2003). Pour les Prx dicystéiniques (Prx 2-Cys, Prx Q et Prx II), l'intervalle entre les deux cystéines conservées varie : il est de 112 acides aminés pour les Prx 2-Cys, de 5 pour les Prxs Q et de 25 pour les Prxs de type II (Dietz, 2003; Rouhier *et al*., 2002). Il est toutefois important de noter que, bien que les Prx II possèdent la plupart du temps 2 cystéines conservées (la Prx IIC d'*Oryza sativa* ne possède pas la seconde cystéine par exemple), ces protéines fonctionnent comme des Prxs à 1 cystéine (voir plus loin) (figure 8). Concernant les Prxs Q, un certain nombre d'isoformes chez les algues et les cyanobactéries ne possèdent pas non plus la deuxième cystéine (Latifi *et al*., 2007). Initialement, les Prxs étaient supposées être réduites par les thiorédoxines (Trxs), ce qu'il leur a valu le nom de thiorédoxine peroxydases, cependant il est apparu que certaines pouvaient également être réduites par le GSH et les glutarédoxines (Grxs).

b Mécanismes d'action

Le mécanisme d'action des Tpxs se décompose globalement en trois étapes : (i) l'attaque nucléophile de la cystéine catalytique sur le substrat provoquant sa réduction et la fomation d'un acide sulfénique (SOH), c'est l'étape dite de réduction, (ii) la formation d'un pont disulfure suite à l'attaque de l'acide sulfénique par une cystéine de recyclage ou un thiol externe et (iii) la régénération de l'état initial par réduction du pont disulfure par une oxydo-réductase, principalement les thiorédoxines et glutarédoxines (Declercq *et al.*, 2001; Dietz, 2003; Hirotsu *et al*., 1999; Hofmann *et al*., 2002; Rouhier and Jacquot, 2002). A l'heure actuelle, les Prxs de type

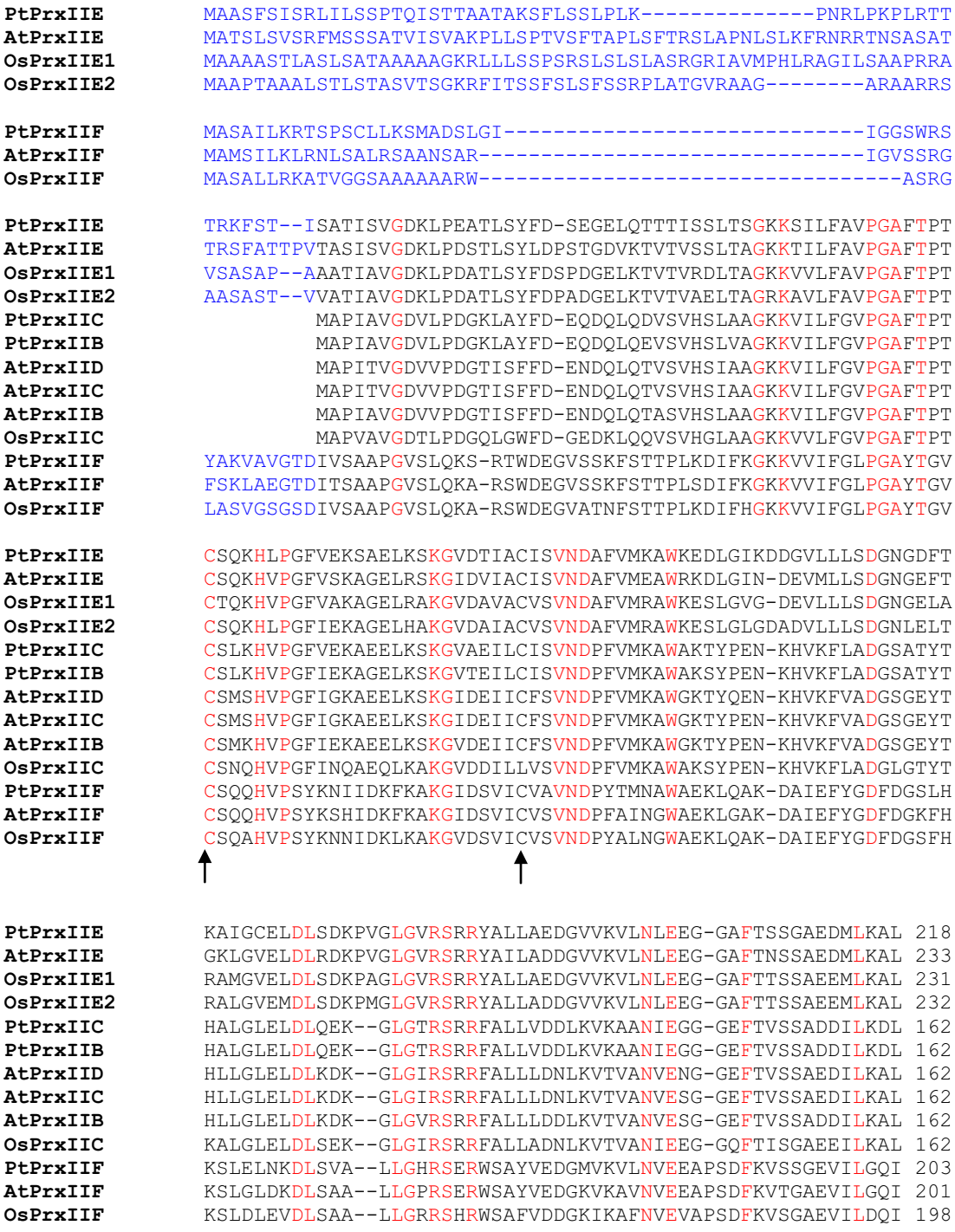


Figure 8 : Alignement des séquences en acides aminés des Prxs IIB, IIC, IID, IIE et IIF d'*Arabidopsis thaliana*, *Populus trichocarpa*, et *Oryza sativa*. En rouge sont présentés les acides aminés strictement identiques et en bleu les séquences d'adressage présumées en position N-terminale. On s'aperçoit que les deux cystéines (flèchées) sont éloignées de 25 acides aminés, sauf dans le cas de la PrxIIC d'*Oryza sativa* qui ne possède ni la cystéine de régénération, ni aucune autre cystéine susceptible de jouer un rôle similaire.

II sont à peu près les seules Prxs à être réduites par le système GSH/glutarédoxine tout en conservant pour certaines la possibilité d'utiliser le système thiorédoxine (Brehelin *et al*., 2003; Finkemeier *et al*., 2005; Rouhier *et al*., 2001). Dans quelques rares cas, il a été montré que l'activité Tpx pouvait être alimentée soit uniquement par le glutathion (GSH) soit par les cyclophilines (Finkemeier *et al*., 2005; Laxa *et al*., 2007). La réduction de l'acide sulfénique constitue une étape importante puisqu'il peut se produire une suroxydation aboutissant à la formation d'acides sulfinique (SO_2H) ou sulfonique (SO_3H), inactivant la protéine, après réaction avec une deuxième ou une troisième molécule de peroxyde (Wood *et al*., 2003). Les sulfirédoxines sont spécialisées dans la réduction de l'acide sulfinique en acide sulfonique participant ainsi à la régulation de l'activité des Prxs. Cette régulation est importante puisqu'elle aboutit à une modification de la signalisation cellulaire via la dégradation des EORs par les Prxs (Iglesias-Baena *et al*., 2010). Selon les Prxs, le potentiel redox du pont disulfure formé varie de -325mV à -288mV à pH7 (Konig *et al*., 2002; Rouhier *et al*., 2004a). Les différents mécanismes d'action propres à chaque type de peroxyrédoxines sont représentés dans la figure 9.

Au niveau structural et conformationnel, les Prxs diffèrent énormément. Les Prxs 2-Cys, les premières caractérisées structuralement, forment un homodimère de type "tête-à-queue" lors de leur oxydation. De plus, elles présentent une forte propension à s'oligomériser, allant jusqu'à former des décamères (Wood *et al*., 2003). La forme décamère est stable à des concentrations égales ou supérieures à 1.5µmol/L (Barranco-Medina *et al*., 2008). Les Prxs de type II et les Prxs 1-Cys forment également des dimères mais non covalents (Li *et al*., 2005; Noguera-Mazon *et al*., 2006). Elles peuvent également se retrouver sous forme dimérique, montrant une orientation perpendiculaire des feuillets β. La Prx Q est vraisemblablement présente sous forme monomérique contenant un pont disulfure intramoléculaire en guise d'intermédiaire réactionnel. Des études structurales et mécanistiques assez poussées dans le laboratoire ont permis d'aboutir à un modèle assez précis décrivant le fonctionnement de la Prx IIB de peuplier et sans doute des autres Prx II.

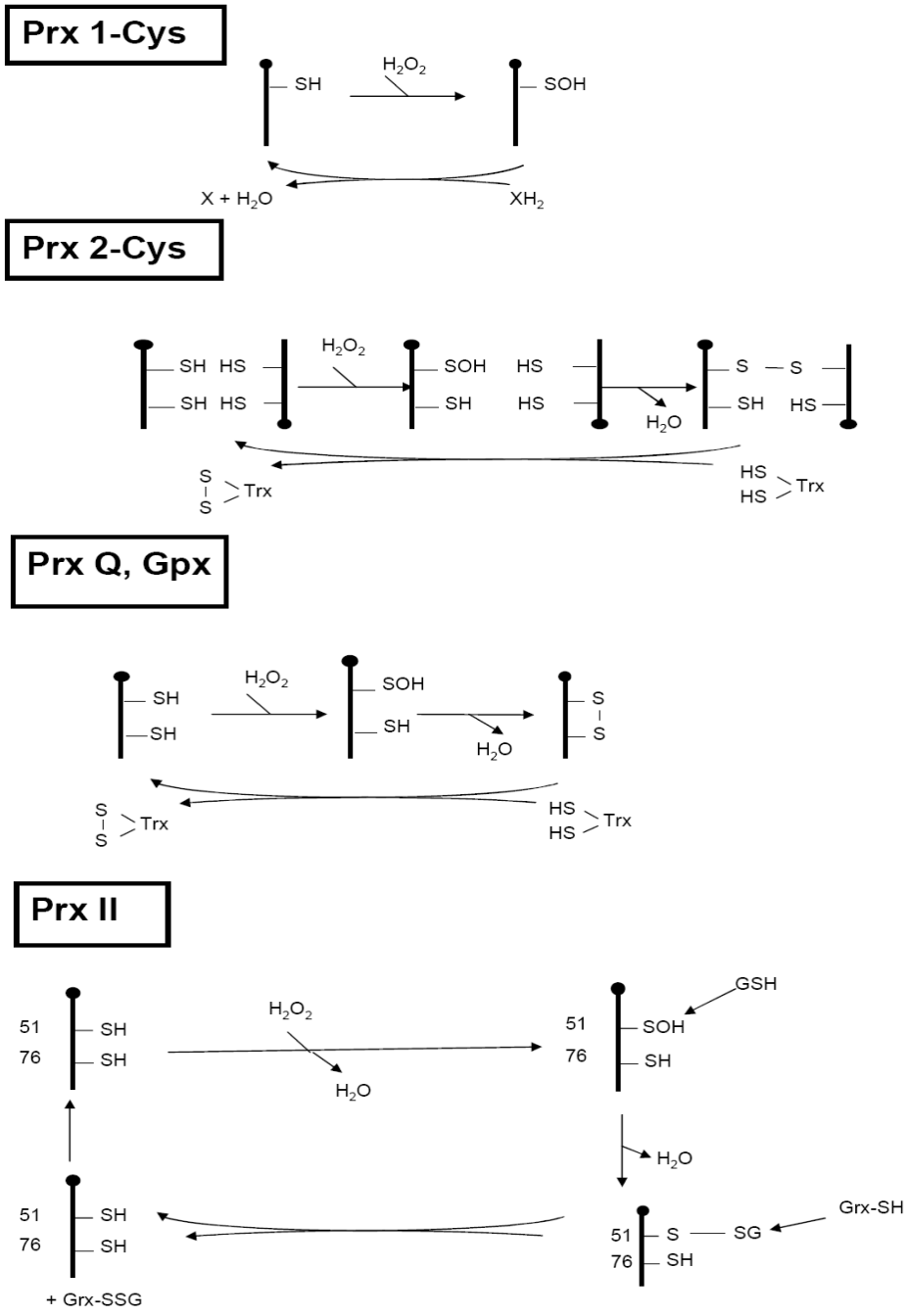


Figure 9 : Mécanismes d'action des différents types de peroxyrédoxines.
D'après Rouhier et Jacquot, 2002 et réactualisé.
Le mécanisme réactionnel des Prxs à une cystéine est simple : après action de l'enzyme, celle-ci présente un acide sulfénique qui sera réduit par une molécule dithiol comme une thiorédoxine. Les mécanismes des Prxs dithiols sont plus complexes. Tout d'abord il peut soit se former un pont disulfure intramoléculaire, soit intermoléculaire aboutissant à un homodimère. Ces deux sortes de ponts seront ensuite réduites par une thiorédoxine. Les Prxs de type II peuvent être réduites par le système Trx ou Grx. Dans ce cas, une molécule de GSH va attaquer le thiol oxydé de la Prx formant une protéine glutathionylée. Celle-ci sera alors déglutathionylée par une Grx qui elle-même sera régénérée par l'attaque d'une nouvelle molécule de GSH.

En effet, au niveau tridimensionnel, la cystéine de recyclage, semble *a priori* trop éloignée et présente dans une partie trop rigide pour pouvoir, même avec un changement conformationnel très important, se retrouver en position de former un pont disulfure avec la cystéine peroxydatique. Cette observation, couplée au fait (i) que certaines Prxs ne possèdent pas cette cystéine de recyclage, (ii) que le mutant pour cette cystéine conserve partiellement son activité et (iii) que le système GSH/Grx soit le système utilisé préférentiellement, suggèrent que l'acide sulfénique est attaqué directement par le réducteur. Le modèle proposé pour cette classe de Prx, en tous les cas pour une régénération par le système GSH/Grx implique la glutathionylation de la cystéine peroxydatique puis la réduction de cet adduit par les Grxs. Ce mécanisme est similaire à celui utilisé par les MsrB à 1 cystéine (Tarrago *et al*., 2009). Pour quelques Prxs II, incluant la Prx IIF, la réduction de l'adduit glutathion peut être effectué par le GSH seul, en dehors de la présence de Grxs mais cette réaction est moins efficace (Finkemeier *et al*., 2005; Gama *et al*., 2007; Gama *et al.*, 2008). Il est à noter que la glutathionylation pourrait faire passer la protéine d'un état dimérique à un état monomérique comme le suggèrent des analyses effectuées par résonance magnétique nucléaire (RMN) (Noguera *et al*., 2005). Pour la régénération par le système Trx l'hypothèse est que l'acide sulfénique est réduit directement par les Trxs comme suggéré dans le cas de la Prx BCP d'*E. coli* et montré pour le couple MsrB1 et CDSP32 (Tarrago *et al*.), 2010) .

c Rôles physiologiques, expression tissulaire et subcellulaire dans les plantes

Au travers de la réduction de différents substrats (H_2O_2, ROOH, ONOO-), les thiol peroxydases vont jouer de très nombreux rôles dans les cellules, d'autant plus qu'on les retrouve dans tous les compartiments de la cellule.

Les Prx 1-Cys, sont retrouvées à la fois dans le cytosol et dans le noyau. Elles sont principalement exprimées dans les graines mais il a récemment été montré qu'elles étaient également localisées dans les tissus végétatifs au niveau des feuilles, des tiges, des pétioles et des racines où elles interviendraient dans la résistance au stress oxydant (Haslekas *et al*., 2003; Mowla *et al*., 2002; Requejo and Tena, 2006). Dans les graines, les Prx 1-Cys jouent un rôle dans le maintien de dormance et la protection des tissus de la graine contre les dommages causés par une trop forte oxydation (Haslekas *et al*., 1998; Lee *et al*., 2000). De plus, des graines surexprimant cette Prx et soumises à un stress oxydant (NaCl, mannitol, méthyl viologène), ont des difficultés à germer (Haslekas *et al*., 2003). On peut ainsi penser que cette protéine entrerait dans la régulation de la germination notamment en stoppant la germination lorsque les conditions sont défavorables. Les organismes photosynthétiques ne possèdent généralement qu'une isoforme mais le peuplier par exemple en possède deux, appelées Prx 1-CysA et 1-CysB, qui proviennent donc vraisemblablement d'un événement de duplication. Récemment, alors que planent toujours des interrogations quant au réducteur physiologique de ces protéines, il a été proposé que, dans des cellules de blé soumises à un stress oxydant, la protéine se retrouve dans le noyau, et pouvait être réduite par une NTR (Pulido *et al*., 2009). Les autres réducteurs proposés suite à des études sur des Prx 1-Cys provenant de divers organismes sont le GSH, les Trx, les glutathion transférases (Gst) et l'ascorbate (Kang *et al*., 1998; Monteiro *et al*., 2007; Pedrajas *et al*., 2000; Pedrajas *et al*., 2010; Ralat *et al*., 2006). Aucun de ces réducteurs ne fonctionne avec la Prx 1-Cys de peuplier.

Les Prx 2-Cys, premières Prxs à avoir été décrites chez les bactéries et les levures (Kim and Rhee, 1988; Storz *et al*., 1989), sont localisées au niveau des chloroplastes chez les plantes supérieures, et plus particulièrement au niveau des thylacoïdes (Konig *et al*., 2002). Il semble que *Chlamydomonas reinhardtii* possède une isoforme chloroplastique homologue des Prx 2-Cys, la Prx1, qui jouerait un rôle similaire au niveau de son unique chloroplaste (Dayer *et al*., 2008). Elle est ainsi plutôt exprimée dans tous les tissus précoces et verts de la plante (Baier and Dietz, 1999;

Broin *et al*., 2002; Cheong *et al*., 1999). A l'aide de plants d'*A. thaliana* antisens, les fonctions physiologiques de cette protéine ont pu être étudiées (Baier and Dietz, 1999; Baier *et al*., 2000). Chez ces mutants, certaines protéines chloroplastiques avaient perdu leur activité et la photosynthèse était significativement ralentie. Ces résultats suggèrent fortement une fonction de protection de la machinerie photosynthétique par la Prx 2-Cys. Un autre rôle pour cette protéine a été défini avec des mutants de pomme de terre surexprimant la protéine CDSP32 (Broin and Rey, 2003). Il a été démontré que la sécheresse ou un traitement au méthyl viologène provoquaient la peroxydation des lipides présents dans les thylacoïdes et également une suroxydation de la Prx 2-Cys. La protéine CDSP32 empêcherait donc l'oxydation de la Prx 2-Cys, maintenant ainsi son action contre l'oxydation des lipides des membranes des thylakoïdes en réponse à des situations de stress oxydant. Chez lez animaux, sa suroxydation et sa régéneration lente, dépendante de l'ATP, jouent un rôle dans la signalisation cellulaire (Rhee *et al*., 2005).

A l'instar des Prx 2-Cys, la Prx Q, la dernière à avoir été étudiée chez les plantes *(Kong et al*., 2000), est également adressée dans les chloroplastes à proximité du photosystème II et peut-être dans le lumen des thylakoides. Ceci explique pourquoi on la trouve principalement dans les organes verts tels les feuilles (Lamkemeyer *et al*., 2006; Petersson *et al*., 2006; Rouhier *et al*., 2004b). La Prx Q du peuplier semble être uniquement exprimée au niveau des feuilles (Rouhier *et al*., 2004b). Soumise à une infection par *Melampsora larici populina*, un pathogène causant la rouille du peuplier, la plante présente des variations de l'expression de cette protéine semblables à celles de la Prx IIB, aussi bien pour une réaction compatible qu'incompatible, indiquant que ces Prx, dans leur compartiment sub-cellulaire respectif, sont associées aux systèmes de défense du peuplier, vraisemblablement au travers de leur capacité à réduire des peroxydes (Rouhier *et al*., 2004b). Comme tous les composants du chloroplaste, l'abondance de cette enzyme s'effondre pendant la sénescence. On retrouve aussi une diminution des quantités de transcrit sous l'effet d'un stress salin, d'un traitement à l'ascorbate ou d'une baisse subite de l'intensité lumineuse

(Dietz *et al*., 2002; Horling *et al*., 2003). En revanche, on dénote une surexpression après une augmentation de l'intensité lumineuse, et en réponse à un stress oxydant causé par H_2O_2, le ter-butyl hydroperoxyde (t-BOOH) ou le diamide (Horling *et al*., 2003).

Chez toutes les plantes, le sous- groupe des Prx II est composé de plusieurs membres qui ont des localisations diverses, les Prx IIB, C et D sont cytosoliques, les Prx IIE sont chloroplastiques alors que les Prx IIF sont mitochondriales (Brehelin *et al*., 2003; Finkemeier *et al*., 2005; Rouhier and Jacquot., 2005).

Les Prxs II sont généralement exprimées dans la plupart des organes des plantes. Seules les Prx IIC and D, en tout cas celles d'*A. thaliana*, semblent avoir une expression très localisée, au niveau du pollen (Brehelin *et al*., 2003). L'expression de la Prx IID varie très peu, mais elle n'a pas été analysée en détail (Brehelin *et al*., 2003). Chez *A. thaliana*, l'expression des transcrits de la Prx IIB augmente suite à un traitement au NaCl et au t-BOOH, cependant aucune modification n'apparait lorsque la plante est traitée avec de l'ascorbate (incubation de morceaux de feuilles avec différentes concentrations d'ascorbate et de déhydroascorbate) ou avec des variations d'intensité de lumière (Horling *et al*., 2002; Horling *et al*., 2003). La Prx IIB du peuplier présente des variations de quantité de protéines lors de l'interaction avec *Melampsora larici populina* (Rouhier *et al*., 2004a). On observe une augmentation de l'abondance de cette enzyme suite à une réaction incompatible et inversement, une baisse suite à une réaction compatible.

L'expression de la Prx IIC d'*A. thaliana* varie considérablement sous l'effet d'un stress salin, d'un apport d'ascorbate ou de conditions oxydantes causées par du H_2O_2, du t-BOOH ou du diamide, et également lorsque la plante est privée de phosphore (Dietz *et al*., 2002; Horling *et al*., 2003).

Les Prx IIE et IIF ont été l'objet de mon travail puisque très peu de données étaient disponibles au début de ma thèse. Chez *A. thaliana*, la Prx IIE est plutôt exprimée dans les tissus

reproducteurs et présente des modifications d'expression semblables à celles d'autres Prx chloroplastiques telles les Prxs 2-Cys et Q. On note une augmentation de l'expression en conditions de forte intensité lumineuse, et une baisse en réponse à une intensité lumineuse. De plus, des traitements à l'ascorbate et au NaCl provoquent également une baisse de la quantité de transcrit alors qu'en conditions oxydantes aucune variation n'est observée (Dietz *et al*., 2002; Horling *et al*., 2003). La Prx IIF mitochondriale est exprimée constitutivement dans tous les tissus sans variation, ou très peu et quel que soit le traitement appliqué. Il semblerait donc que cette enzyme joue un rôle de maintien d'activité basale dans la mitochondrie (Baier *et al*., 2000; Brehelin *et al*., 2003; Dietz *et al*., 2000; Horling *et al*., 2003).

Le dernier groupe de thiol-peroxydases est constitué par les Gpxs. La plupart des Gpxs d'animaux sont des enzymes contenant une sélénocystéine au sein de leur site actif, ce qui en fait un système antioxydant très efficace chez les animaux (Maiorino *et al*., 1990). On retrouve ces sélénoprotéines également chez des organismes photosynthétiques unicellulaires tels que l'algue verte *Chlamydomonas reinhardtii* (Fu *et al*., 2002; Novoselov *et al*., 2002). Cependant les protéines des plantes supérieures (terrestres) possèdent une cystéine à la place de la sélénocysteine (Eshdat *et al*., 1997). Ainsi, ces Gpxs de plante supérieures et de levure et sans doute bactériennes réduisent les peroxydes, de manière plus efficace ou quelquefois exclusivement en utilisant le système thiorédoxine plutôt que le GSH comme source de régénération (Herbette *et al*., 2002; Jung *et al*., 2002; Tanaka *et al*., 2005). Bien que présentant une séquence primaire plus proche des Gpxs animales glutathion dépendantes que des Prxs et possédant une structure également caractéristique des Gpxs, ces Gpxs à cystéine forment un groupe de peroxydases dépendantes des thiorédoxines (Koh *et al*., 2007; Navrot *et al*., 2006).

8 isoformes existent chez *Arabidopsis thaliana* prédites pour être localisées aussi bien dans le cytosol, le chloroplaste, la mitochondrie, que le réticulum endoplasmique. Chez le peuplier,

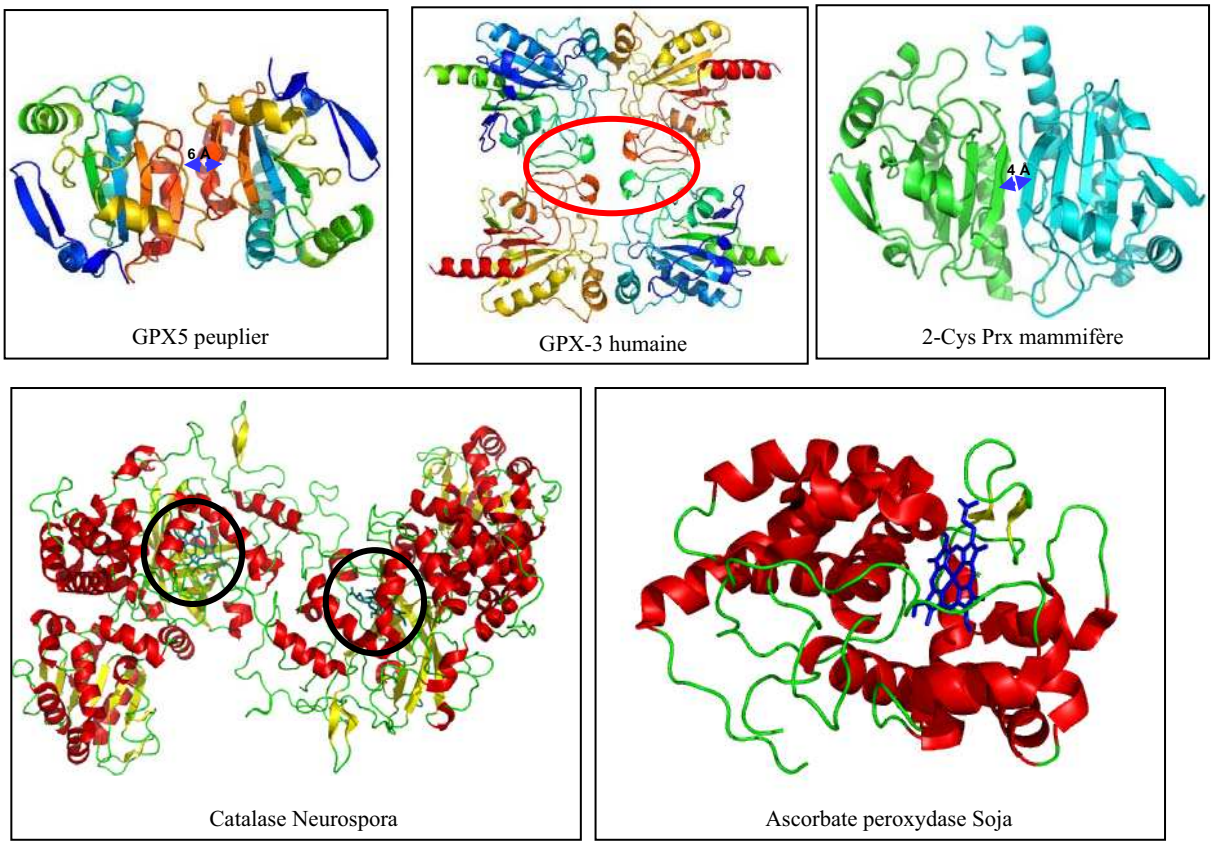


Figure 10 : Convergence fonctionnelle des peroxydases: relation structure-fonction.
Les protéines des systèmes de détoxication et de protection contre le stress oxydant et plus particulièrement les peroxydases présentent des structures variées alors qu'elles ont le même rôle. Elles peuvent s'organiser en dimère (Gpx5, Prx 2-cys), en tétramère (Gpx3) ou bien encore posséder un hème au sein de son site actif (catalase et ascorbate).

parmi les 6 isoformes connues, les Gpx1 et 3.2 sont localisées dans le chloroplaste, cette dernière étant également co-localisée dans les mitochondries (Navrot *et al*., 2006).

Chez *A. thaliana*, les Gpx1 et 7 sont chloroplastiques alors que la Gpx 3 est cytosolique (Miao *et al*, 2006) (Dayer *et al*., 2008). La transcription de la plupart des gènes codant ces protéines est augmentée en condition de stress chez *Arabidopsis thaliana*: les gènes codant les Gpxs 1, 2, 4 et 6 sont surexprimés lors d'un stress salin, en réponse au mannitol, au fer et au cuivre (Rodriguez Milla *et al*., 2003). En revanche, seule la Gpx 6 voit son taux d'ARNm augmenter avec un stress au « froid ». Ce gène est le plus régulé par différents stress abiotiques et le plus exprimé durant le développement de la majorité des tissus de la plante. La protéine qu'il code semble être dirigée vers la mitochondrie et le cytosol. De plus, ces Gpxs semblent être régulées par de nombreux facteurs comme le montrent les effets causés par des hormones de plantes telles l'acide salicylique, l'acide jasmonique, l'acide abscisique ou l'auxine sur le taux de transcrits (Rodriguez Milla *et al*., 2003). Au niveau protéique, certaines Gpxs voient leur abondance augmenter en réponse à l'application d'un stress métallique (cadmium et cuivre) chez *A. thaliana* et à une infection du peuplier par le champignon *M. larici populina* (Navrot *et al*., 2006). Alors qu'elles arborent toutes la même fonction physiologique, les peroxydases présentent donc des structures variées dépendantes de leur propention à multimériser, voir à fixer un cofacteur (Figure 10).

Plusieurs autres fonctions, quelquefois non reliées à leur activité peroxydase, ont été proposées pour l'une ou l'autre des thiol-peroxydases. Par analogie à la situation chez d'autres organismes modèles, il apparait que les Prxs 2-Cys eucaryotes sont susceptibles de s'inactiver par suroxydation de la cystéine catalytique en acide sulfinique ou sulfonique en présence de concentrations suffisantes de peroxyde d'hydrogène (Konig *et al*., 2003). Ceci serait important dans la signalisation cellulaire en réponse à l'H_2O_2 (Wood *et al*., 2003). Toujours dans le registre du sensing et de la signalisation par H_2O_2, il a été démontré chez plusieurs champignons, en

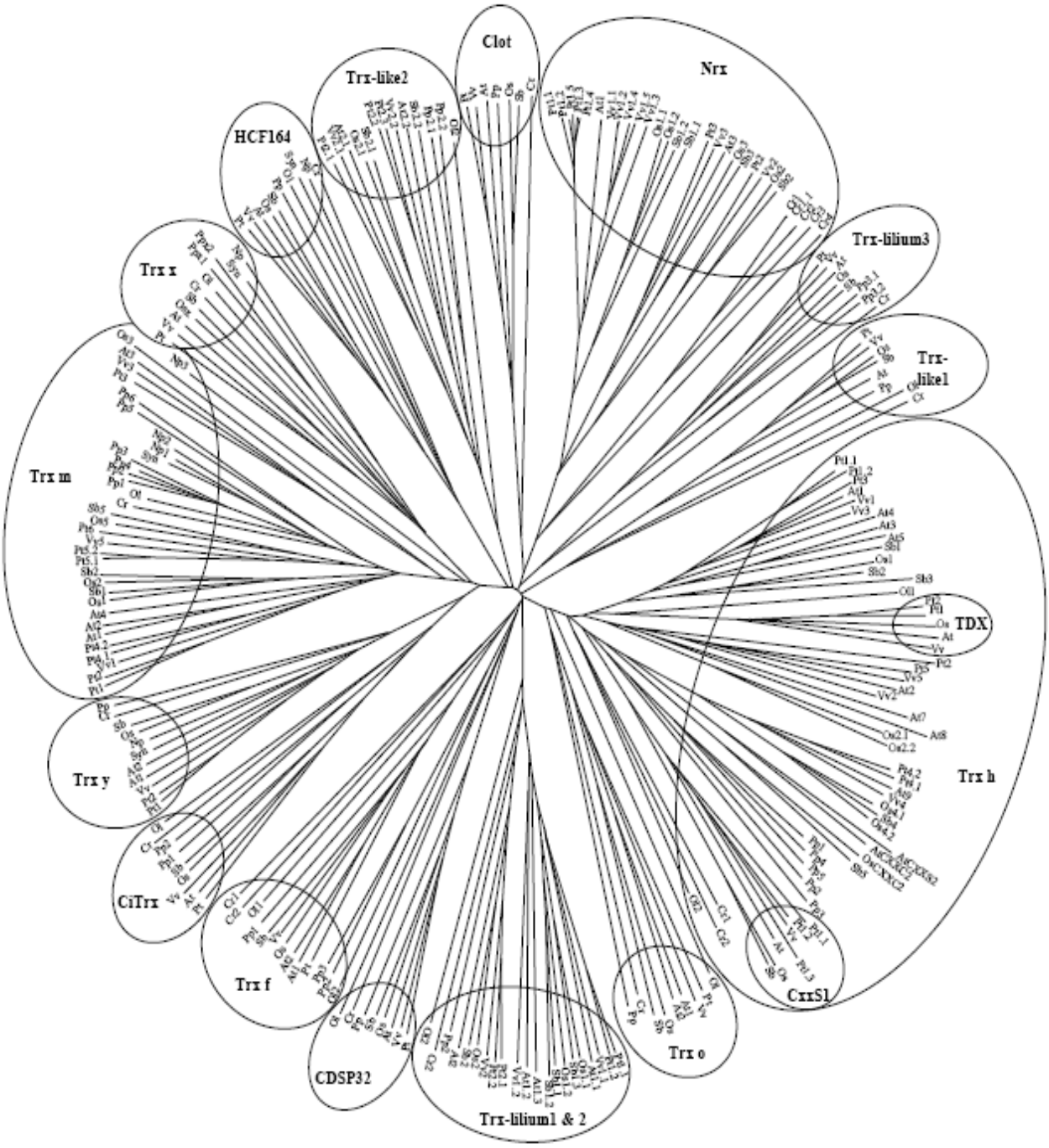


Figure 11 : Arbre phylogénétique des thiorédoxines de plante.
La classification des Trxs est complexe et se décompose en 17 groupes. Tous ces groupes ne sont pas retrouvés au niveau de chaque espèce (Chibani *et al*., 2009).

particulier les levures, que les Tpxs (Prx ou Gpx) participent à la signalisation cellulaire en réponse à l'H_2O_2, au travers de la régulation du facteur de transcription de type AP1 ou Cad1 (Delaunay *et al*., 2002; Iwai *et al*., 2010 ; Vivancos *et al*., 2005). En effet, l'oxydation de ces Tpxs se transmet à AP1 qui devient capable d'activer un certain nombre de gènes en réponse à la présence d'H_2O_2. Une fonction assez similaire pourrait exister chez les plantes où la Gpx 3 pourrait réguler une phosphatase en réponse à diverses conditions menant à l'initiation d'un stress oxydant (Miao *et al*., 2006).

Un autre exemple concerne la capacité des Prx 2-Cys de levure ou de mammifères à s'oligomériser pour former des complexes de gros poids moléculaires qui ne possèdent plus d'activité peroxydase mais une activité chaperonne (Yang *et al*., 2004). Il n'est pas encore certain que les orthologues de plante possèdent une telle propriété (Muthuramalingam *et al*., 2009).

III Les oxydoréductases à thiol

A Les thiorédoxines

a Classification

Les thiorédoxines sont des petites protéines, de taille classiquement comprise entre 10 et 15 kDa, possédant un repliement appelé motif thiorédoxine, caractérisé par la présence de 4 à 5 brins β qui s'organisent en un feuillet entouré par 4 à 5 hélices α. Ce motif est partagé par de nombreuses autres protéines (Dsb, Grx, PDI, Gpx, Gst). Grâce à un site actif généralement de la forme CxxC, elles participent à la régulation redox de la cellule via la réduction de ponts disulfure sur un large panel d'enzymes cibles (Gelhaye *et al*., 2005). Les Trxs classiques possèdent généralement un site catalytique très conservé du type WC(G/P)PC (Gelhaye *et al*., 2005) mais de nombreuses isoformes diffèrent énormément. Ainsi, on trouve 40 à 50 isoformes de Trx ou « Trx-

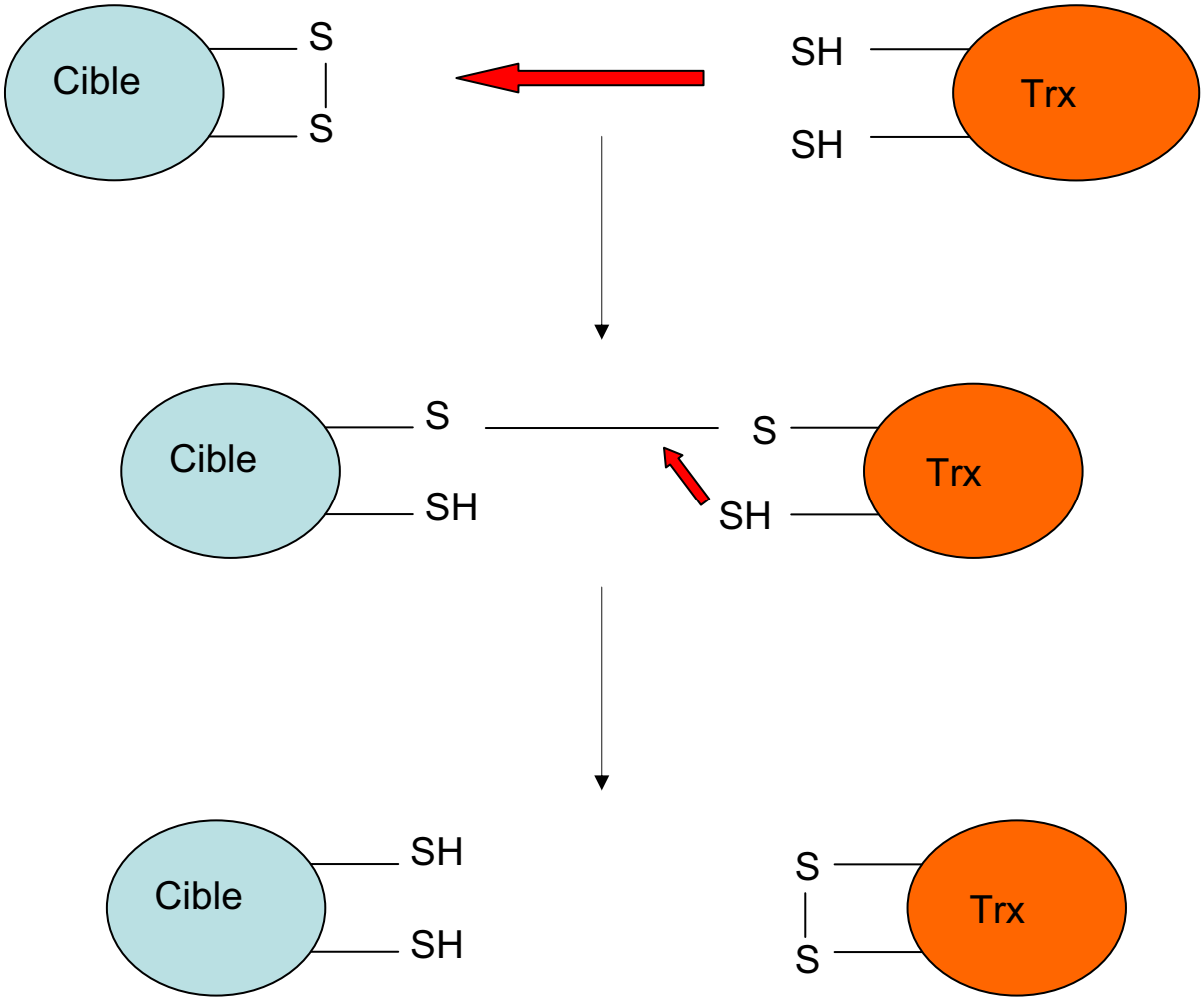


Figure 12 : Mécanisme d'action des thiorédoxines.
Les thiorédoxines sont de petites molécules spécialisées dans la réduction de ponts disulfure. La plupart d'entre elles possèdent deux cystéines au sein de leur site actif. La première effectuer une attaque nucléophile du pont disulfure de la protéine cible à réduire ce qui va aboutir à la formation d'un pont disulfure intermoléculaire, qui sera ensuite réduit par l'attaque de la deuxième cystéine catalytique. La thiorédoxine oxydée, comportant un pont disulfure intramoléculaire sera régénérée par l'action de la NTR qui tire son pouvoir réducteur du NADPH ou bien par l'action du système Fd/FTR (figure suivante).

like » chez *Arabidopsis thaliana* et les autres plantes terrestres (Chibani *et al*., 2009; Meyer *et al*., 2005). La cystéine catalytique est habituellement située au début de la première ou de la deuxième hélice α.

Les principaux groupes actuellement retrouvés et bien caractérisés chez les plantes supérieures sont les Trx *f,* Trx *h*, Trx *m*, Trx *o*, Trx *x* et Trx *y* (figure 11). Les Trx *f*, *m, y* et *x* sont localisées au niveau du chloroplaste comme des études de fusion avec la Green Fluorescent Protein (GFP) le montrent (Collin *et al*., 2003). On trouve généralement les Trxs *o* au niveau des mitochondries (Laloi *et al*., 2001) alors que les Trxs *h* ont une distribution variée ; elles peuvent être secrétées (Ishiwatari *et al*., 1998; Juarez-Diaz *et al*., 2006) mais elles ont aussi été trouvées dans le noyau (Serrato and Cejudo, 2003) et dans les mitochondries (Gelhaye *et al*., 2005) ou au niveau des membranes (Meng *et al*., 2010), les autres isoformes caractérisées semblent être cytosoliques. De nombreux autres groupes ont été maintenant inclus dans la famille des Trx, incluant des protéines multimodulaires telles que CDSP32, les nucléorédoxines ou les thiorédoxines contanant un domaine tétratricopeptide (TDX) (Chibani *et al*., 2009).

b Mécanismes d'action

La cystéine catalytique des Trx possède un pKa d'environ de 6 et le potentiel redox de ces protéines varie de -285 et -350 mV à pH7, à l'exception de la Trx*h*4 du peuplier qui possède un mécanisme de régénération particulier faisant intervenir le système GSH/Grx grâce à un potentiel redox de l'ordre de -200 mV (Brehelin *et al*., 2004; Koh *et al*., 2008). Les protéines qui possèdent un potentiel redox égal ou inférieur seront des protéines potentiellement réductibles par les Trx. Les 2 cystéines sont normalement impliquées dans la réduction d'un pont disulfure cible : on parle de mécanisme dithiol, par opposition au mécanisme monothiol souvent employé par les Grxs (Figure 12). La cystéine N-terminale du site actif CxxC effectue une attaque nucléophile sur le

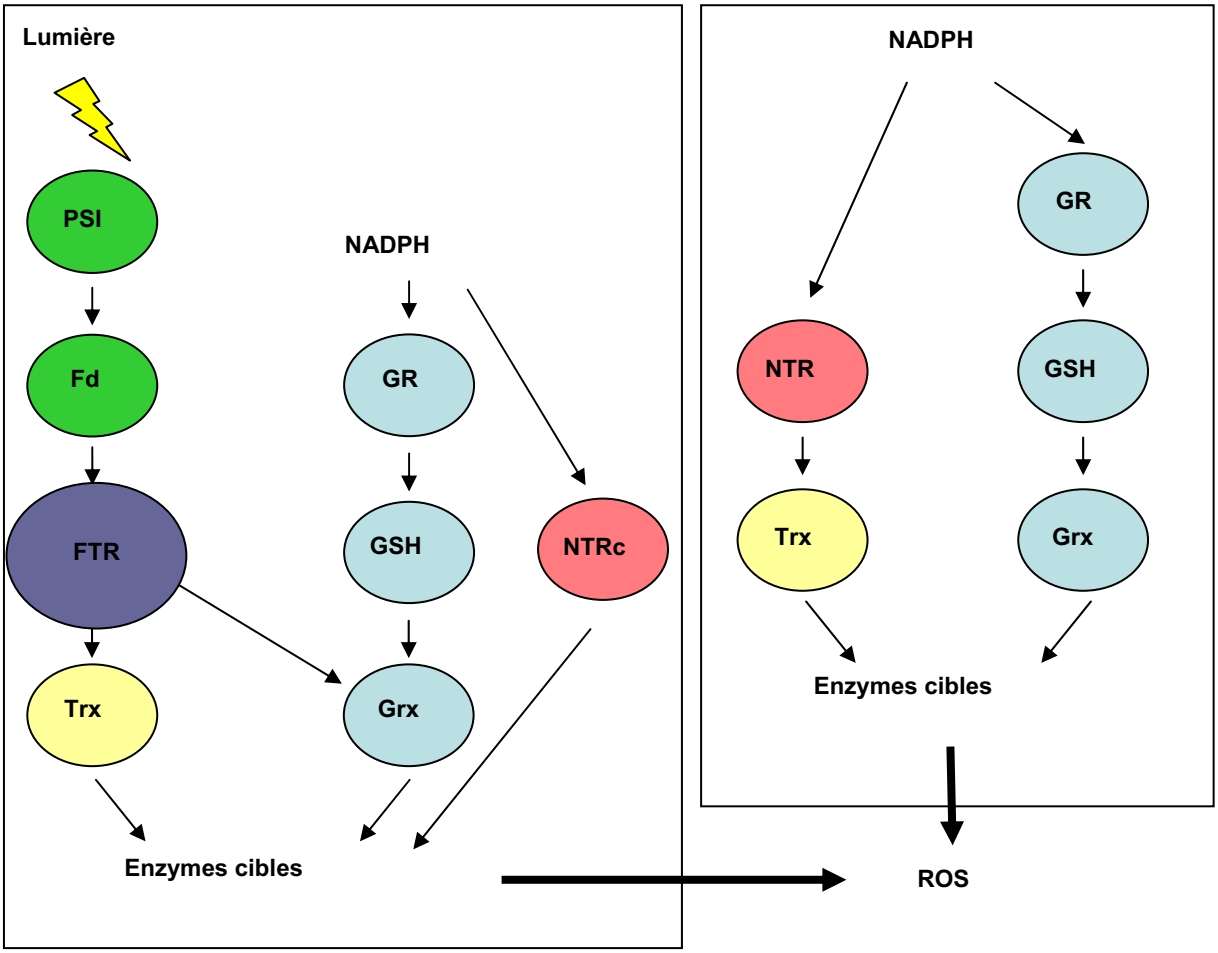


Figure 13 : Les différentes voies de réduction des thiorédoxines. Elles sont réduites par une NADPH thiorédoxine réductase qui tire son pouvoir réducteur du NADPH. Dans le chloroplaste, elles sont réduites par une ferrédoxine thiorédoxine réductase, elle-même réduite par la ferrédoxine qui tire son pouvoir réducteur du photosystème I en présence de lumière. Fd : ferrédoxine, Trx : thiorédoxine, NADPH : Nicotinamide adénine dinucléotide phosphate, GR : glutathion réductase, GSH : glutathion, Grx : glutarédoxine, NTR : NADPH-thiorédoxine réductase, NTRc : NADPH thiorédoxine réductase chloroplastique.

pont disulfure cible, conduisant à la formation d'un intermédiaire covalent transitoire entre les deux partenaires. Celui-ci sera résolu par attaque de la deuxième cystéine. L'activité des thiorédoxines va donc conduire à la formation d'un pont disulfure intramoléculaire qui devra ensuite être réduit pour les régénérer.

Dans le cytosol et les mitochondries, elles sont principalement régénérées par la NADPH thiorédoxine réductase qui tient elle-même son pouvoir réducteur du NADPH (Laloi *et al*., 2001; Reichheld *et al*., 2005). Cependant, certaines isoformes telles que la Trx h4 de peuplier sont réduites par le système GSH/Grx et non pas par le système NTR (Gelhaye *et al*., 2003; Koh *et al*., 2008; Reichheld *et al*., 2007).

Dans le chloroplaste, les Trxs simple module (Trx *f*, *m, y* et *x*) sont maintenues réduites par la ferrédoxine-thiorédoxine réductase qui elle-même reçoit ses électrons de la lumière, via les photosystèmes grâce à l'accepteur terminal qu'est la ferrédoxine (figure 13) (Jacquot *et al*., 2002; Lemaire *et al*., 2007). Ces mécanismes sont à l'origine de la régulation d'un grand nombre de voies métaboliques chloroplastiques par la lumière. Cependant, un système alternatif dépendant du NADPH existe également dans ce compartiment. Il s'agit d'une protéine de fusion entre un module NTR et un module Trx appelé Ntrc (Perez-Ruiz *et al*., 2006; Serrato *et al*., 2004). A priori, les deux modules ne fonctionnent qu'entre eux, ce qui fait que cette voie pourrait être spécifiquement utilisée en absence de lumière en gardant toutefois à l'esprit que le NADPH est produit à la lumière dans le chloroplaste. Bien que récemment décrite, cette voie semble importante pour un nombre croissant de voies métaboliques comme la réponse au stress.

c Signalisation et cibles des Trx

Les cibles de ces Trxs sont très variées et ne se limitent pas aux Tpxs décrites ci-dessus et aux enzymes chloroplastiques régulées par la lumière. Grâce à de nombreuses études

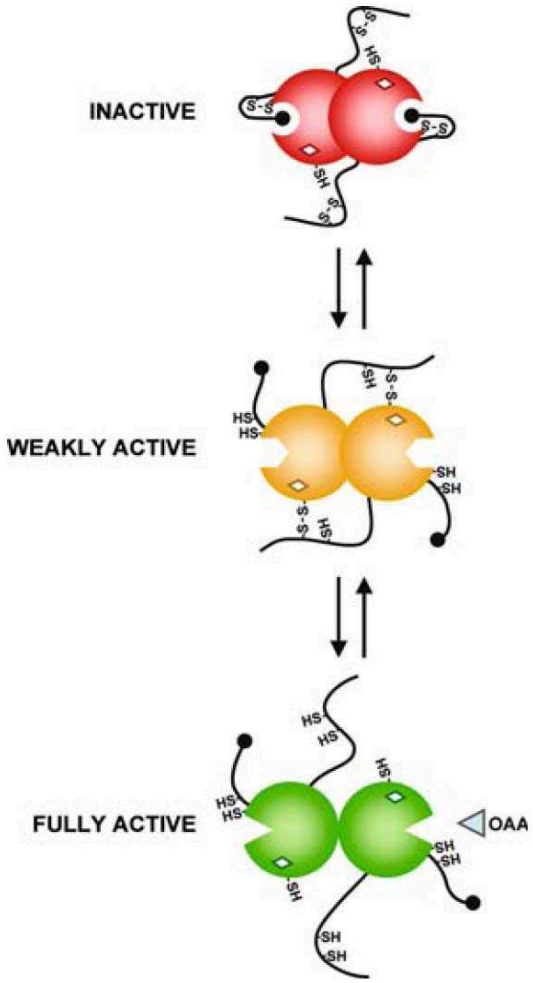


Figure 14 : Mécanisme d'activation de la NADP-MDH. Son mécanisme d'activation est complexe. Un pont disulfure au niveau N-terminal serait responsable d'un changement de conformation de la protéine faisant ainsi varier son activité sans toutefois la supprimer totalement (orange). En vert, l'enzyme est totalement réduite et peut accueillir la molécule OAA et permet ainsi son activité (Lemaire *et al*., 2007).

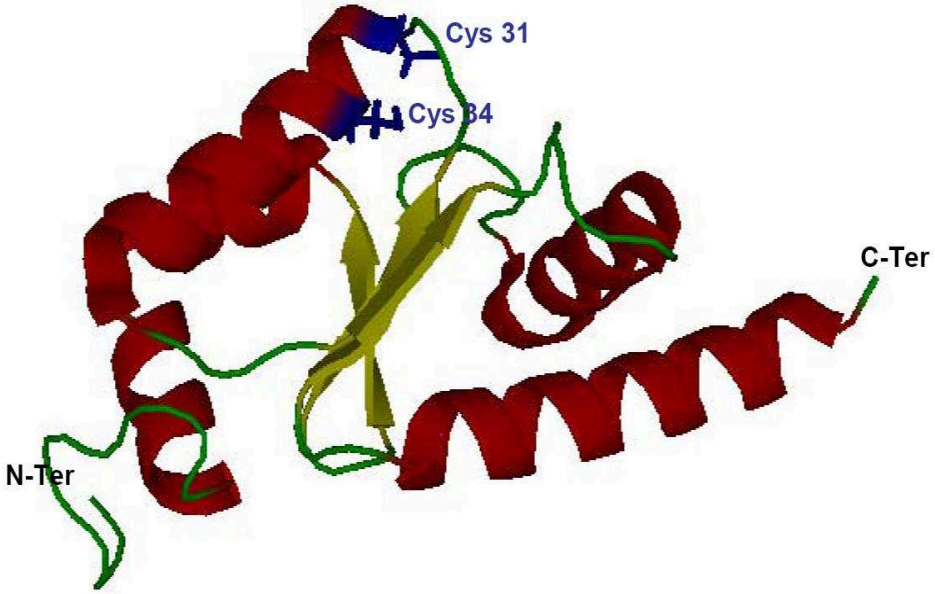
protéomiques, leur nombre pourrait se situer aux alentours de 500 (Montrichard *et al*., 2009). Outre leur participation à la réponse aux différents stress environnementaux via la réduction des Tpxs et des méthionine sulfoxyde réductases (Msr) (Konig *et al*., 2002) (Rodriguez Milla *et al*., 2003; Rouhier *et al*., 2007a; Vieira Dos Santos and Rey, 2006), les thiorédoxines interviennent dans des processus aussi importants que la photorespiration, le cycle de l'acide citrique, le métabolisme des lipides, le transport des électrons, la synthèse d'ATP, le transport membranaire, le métabolisme de l'azote et du soufre, la synthèse hormonale, et peuvent également jouer un rôle de protéines chaperonnes (Balmer *et al*., 2003) (Balmer *et al*., 2004a; Balmer *et al*., 2004b). En particulier, il a été démontré dans l'équipe que la Trx h2 de peuplier peut activer l'alternative oxydase (AOX) mitochondriale, en réduisant le pont disulfure qui lie les sous-unités de la forme inactive de cette enzyme (Gelhaye *et al*., 2004). Les thiorédoxines mitochondriales sont un facteur clé du maintien global de l'état redox de la cellule et de la mitochondrie (Robson and Vanlerberghe, 2002).

Au niveau des chloroplastes, historiquement la Trx*m* a été identifiée pour sa capacité à activer la NADP malate déshydrogénase (NADP-MDH) (Figure 14) alors que la Trx*f*, elle, est un activateur de la fructose, 1-6, biphosphatase (FBPase), cette activité spécifique leur donnant ainsi leur nom (Lemaire *et al*., 2007; Schurmann and Jacquot, 2000). Il est important de noter que les données génomiques acquises plus récemment ont permis de se rendre compte qu'il existe plusieurs Trxs m et f et que de nombreuses autres protéines participant au cycle de Calvin, si ce n'est la plupart, sont également régulées par ces Trxs (Schürmann & Buchanan, 2008). Cependant, toutes les Trx*m* ne peuvent pas activer la NADP-MDH, c'est notamment le cas de la Trx*m*3. En étant très schématique, il semble, que dans le chloroplaste, les Trx m et f régulent les enzymes métaboliques et les Trx x et y participent plutôt à la régénération des enzymes impliquées dans la réponse au stress oxydant.

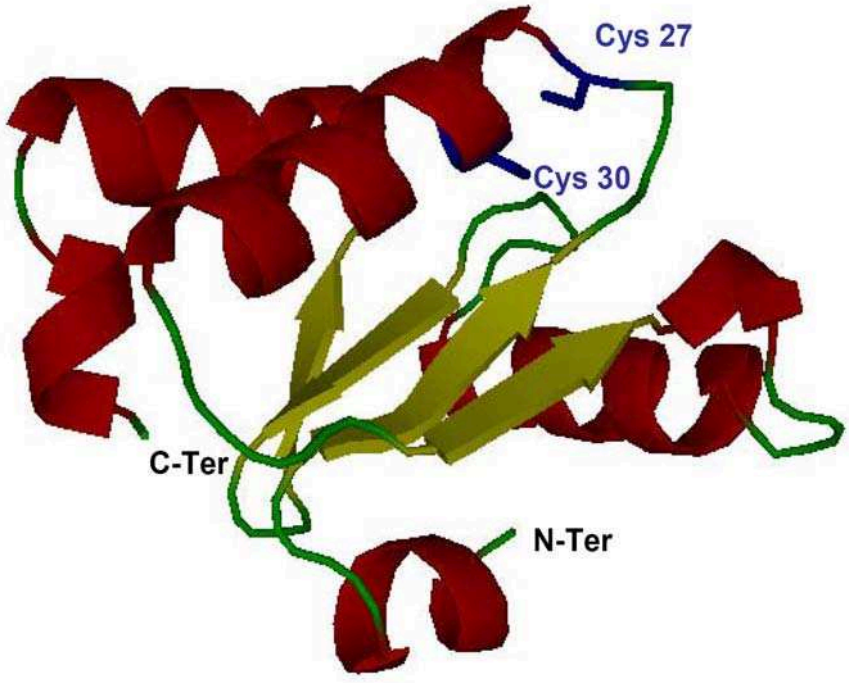
Il existe des protéines dont la régulation, qui dépend des Trxs, est rendu complexe par le nombre important de cystéines qu'elles contiennent. Toutes n'interviennent pas forcément dans ce mécanisme, les exemples de la FBPase, de la NADP-MDH et de la GAPDH sont présentés ci-dessous :

La FBPase est un tétramère de sous-unités identiques qui contiennent chacune quatre cystéines conservées, et chez l'enzyme de pois sous forme inactive (oxydée) les Cys153 et 173 forment un pont disulfure responsable de la régulation de son activité (Jacquot *et al*., 1995). L'activation de l'enzyme nécessite la réduction des ponts disulfures sur chacun des monomères suivie de changements conformationnels dans la protéine qui conduisent à l'établissement de sites actifs fonctionnels (Chiadmi *et al*., 1999). Le mécanisme d'activation de la NADP-MDH est également complexe puisqu'elle contient huit cystéines dont cinq seraient impliquées dans le mécanisme de régulation redox. L'enzyme est un dimère de sous-unités identiques et chaque monomère possède deux ponts disulfures, un situé du côté N-terminal et l'autre du côté C-terminal. Le pont disulfure N-terminal est situé à l'interface entre les sous-unités et il est responsable d'un changement de conformation de la protéine faisant ainsi varier son activité sans toutefois la supprimer totalement. Dans la forme oxydée et inactive de l'enzyme, l'extension C-terminale occupe le site actif permettant en principe de lier l'oxaloacétate. De ce fait, le substrat ne peut se lier à la protéine. Après réduction, le partie C-terminale change de conformation et ouvre le site actif de l'oxaloacétate et l'enzyme devient ainsi active après des réarrangements de ponts disulfure qui nécessitent une cystéine interne (Goyer *et al*., 1999; Issakidis *et al*., 1996; Krimm *et al*., 1999; Ruelland *et al*., 1998).

Une isoforme de la glycéraldéhyde-3-phosphate déshydrogénase (GAPDH), catalysant la conversion du 1,3-bisphosphoglycérate en glycéraldéhyde-3-phosphate est également activée par les Trxs. Cette GAPDH, composée de 2 sous-unités A et B, existe majoritairement sous forme hétérotétramérique (A2B2) mais peut se retrouver sous forme hétérohexadécamérique à l'état



A



B

Figure 15 : Structure tridimensionnelle de type thiorédoxine (« Trx-fold ») de la GrxC1 (A) et de la GrxC4 (B) de peuplier.
Ce motif est caractérisé par la présence de 4 à 5 feuillets β (en jaune) entourés par des hélices α (en rouge). Les cystéines sont représentées en bleu. PDB GrxC1: 1Z7R, numéro accession GrxC4: EEF03801.

oxydé. Le site de régulation est un pont disulfure intramoléculaire situé du côté C-terminal de la sous-unité B. Néanmoins, la forme oxydée n'est pas totalement inactive, mais ne peut plus utiliser le NADPH comme cofacteur (Lemaire *et al*., 2007). D'autres protéines chloroplastiques ont une régulation dépendante des Trxs, c'est notamment le cas de la phosphoribulokinase (Geck and Hartman, 2000), l'ATP synthase (Schwarz *et al*., 1997) ou la glucose-6-phosphate déshydrogénase (G6PDH) (Wenderoth *et al*., 1997).

B Les Glutarédoxines

a Classification

Ce sont des protéines généralement de petite taille (10 à 25 kDa) contenant un site actif très conservé, du type CxxC ou CxxS, qui permet l'activité oxydoréductase, et qui possèdent une structure tridimensionnelle « Trx-fold » (figure 15). Comme pour les Trxs, l'existence de domaines Grxs dans des protéines de fusion modifie quelque peu leur définition (Couturier *et al*., 2009). Les Grxs sont dites monothiols ou dithiols selon qu'elles possèdent 1 ou 2 cystéines au niveau de leur site actif. Cependant, on retrouve parfois une ou plusieurs cystéines additionnelles qui peuvent être impliquées dans le mécanisme réactionnel. La nature du site actif est à l'origine de la classification puisque les Grx dithiol sont dénommées Grx C_x alors que les Grx monothiol sont dénommées Grx S_x (Rouhier *et al*., 2004). Chez les organismes modèles non-photosynthétiques, on trouve généralement entre 4 et 7 Grxs. Ainsi, on dénombre deux Grxs dithiols chez l'homme et *S. cerevisiae*, 3 chez *E. coli* et 1 Grx monothiol chez *E. coli*, 2 chez l'homme et 5 chez *S. cerevisiae*. Jusqu'à relativement récemment, on dénombrait 3 différentes classes de Grxs chez les organismes photosynthétiques eucaryotes (Lemaire, 2004; Rouhier *et al*., 2004c) avec pas moins de 31 gènes identifiés chez *Arabidopsis thaliana*, 17 codant pour des Grxs dithiols et 14 pour des Grxs monothiols. Cependant, des études phylogénétiques et génomiques

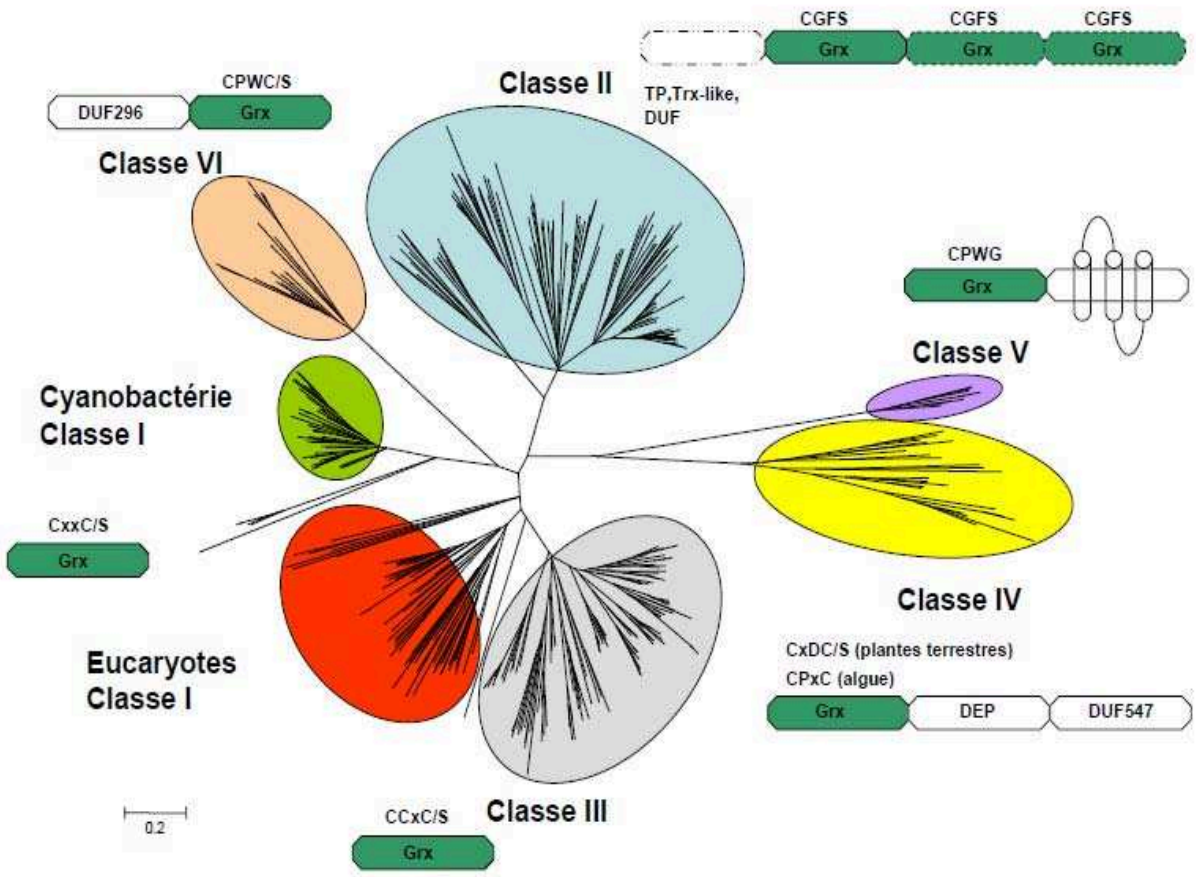
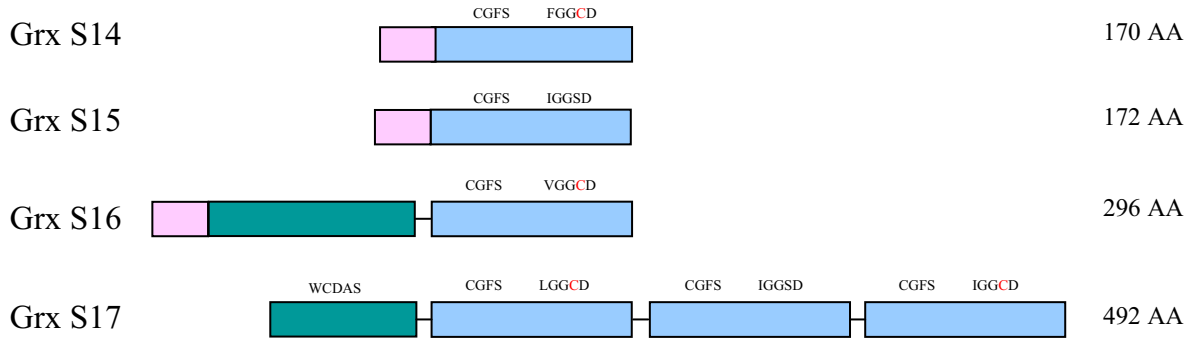


Figure 16 : Arbre phylogénétique des glutarédoxines de plantes terrestres, algues et cyanobactéries. (D'après Couturier *et al*., 2009). Les plantes supérieures possèdent des Grxs réparties dans les classes I, II et III. Les classes IV, V et VI sont composées de Grxs présentant plusieurs domaines distincts. La quatrième classe est spécifique des eucaryotes alors que les classes V et VI sont spécifiques des cyanobactéries. En vert, sont représentés les modules Grx.

plus récentes basées à la fois sur le site actif des Grxs et sur la conservation des acides aminés impliqués dans la liaison du GSH, a montré l'existence d'une autre classe chez les organismes photosynthétiques eucaryotes, portant ce nombre à 4 (Couturier *et al*., 2009). La figure 16 montre un arbre phylogénétique des Grxs de plantes terrestres, algues et cyanobactéries dont le génome est complètement séquencé. Chez le peuplier, on dénombre à présent 36 gènes codant des enzymes possédant au moins un module Grx. Les trois premières classes identifiées ont été définies en se basant essentiellement sur la séquence de leur site actif. La classe 1 comporte les Grxs C1 à C5 et S12 avec des sites actifs de la forme CxxC/S, la classe 2 regroupe les Grxs S14 à S17 qui ont un site actif de la forme CGFS, enfin, la classe 3, la plus vaste, inclut les Grxs S1 à S11 ainsi que S13 et les Grxs C6 à C14 qui possèdent des sites actifs le plus souvent de la forme CCxC/S (Rouhier *et al*., 2004c) (Rouhier *et al*., 2006). La classe 4, nouvellement identifiée, regroupe des protéines de fusion contenant un module Grx en position N-terminale. Les classes 5 et 6 comportent des protéines multi-modulaires, spécifiques des cyanobactéries, le site actif des modules Grxs étant du type CPWG ou CPWC/S (Couturier *et al*., 2009). Ces cyanobactéries possèdent également au moins 1 Grx dans chacune des classes 1 et 2, suggérant un rôle ancestral pour ces Grxs et assez conservé.

La figure 17 est une comparaison de séquences des Grxs CGFS qui ont fait l'objet d'une partie de ce travail de thèse. On remarque au moins 3 zones très conservées, outre le site actif, le motif WPTFPQ est strictement conservé dans les quelques séquences utilisées pour cet alignement et le motif GG[S/C]DI. Ces 2 motifs contiennent des résidus important pour la fixation du glutathion. Il est à noter que les GrxS16 et S17 sont plus grandes que les autres protéines, ceci étant dû à la présence d'une extension N-terminale pour la Grx S16 et d'un module « Trx-like » associé à trois modules Grx pour la Grx S17 des plantes supérieures.

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AtS17                     PVSTADALKSRLEKLTNSHPVMLFMKGIPEEPRCGFSRKVVDI 188
PtS17                     QPGLSDALKNQLQQLIGSHPVMLFMKGNAEAPKCGFSRKVVDI 191
AtS14                     ASALTPQLKDTLEKLVNSEKVVLFMKGTRDFPMCGFSNTVVQI 106
PtS14                       ALTPALKTTLDKVVTSHKVVLFMKGTKDFPQCGFSQTVVQI 104
AtS16                   RHVELTVPLEELIDRLVKESKVVAFIKGSRSAPQCGFSQRVVGI 167
PtS16                   RHVQLTVPLEELIDRLVKENKVVAFIKGSRSAPMCGFSQKVVGI 230
AtS15                   STDS----LKDIVENDVKDNPVMIYMKGVPESPQCGFSSLAVRV 100
PtS15                   SGSCSGINIKELVDKDVKEHPIVIYMKGYPDLPQCGFSALAVRV 101

AtS17     LKEVNVDFGSFDILSDNE---VREGLKKFSNWPTFPQLYCNGELLGGADIAIAMHESGEL 245
PtS17     LKGENVKFGTFDILSDIE---VREGLKLFSNWPTFPQLYCKGELLGGCDIVIALHESGEL 248
AtS14     LKNLNVPFEDVNILENEM---LRQGLKEYSNWPTFPQLYIGGEFFGGCDITLEAFKTGEL 163
PtS14     LKSLNAPFESVNILENEL---LRQGLKEYSSWPTFPQLYIDGEFFGGCDITVEAYKSGEL 161
AtS16     LESQGVDYETVDVLDDEYNHGLRETLKNYSNWPTFPQIFVKGELVGGCDILTSMYENGEL 227
PtS16     LESEGVDYESVDVLDEEYNYGLRETLKKYSNWPTFPQIFMNGELVGGCDILTSMHEKGEL 290
AtS15     LQQYNVPISSRNILEDQE---LKNAVKSFSHWPTFPQIFIKGEFIGGSDIILNMHKEGEL 157
PtS15     LKQYNVPLTARNILEYPD---LRTGVKAYSNWPTFPQIFIKGEFIGGSDIIMNMHQSGEL 158


AtS17     KDAFKDLGITTVGSKES 262
PtS17     KEVFRDHGIDTIGSNEA 265
AtS14     QEEVEKAMCS        173
PtS14     QEQVEKAMCS        171
AtS16     ANILN             232
PtS16     AGHFKK            296
AtS15     EQKLKDVSGNQD      169
PtS15     KEKLQDIAGKEESE    172
```

Figure 17 : Structure protéique et alignement des séquences en acides aminés des Grxs S14, S15, S16 et S17 d'*Arabidopsis thaliana* et *Populus trichocarpa* faisant partie du sous-groupe « CGFS ». Alignement réalisé avec Clustalw. On aperçoit le module Grx commun pour chaque isoforme, la GrxS15 étant la seule à ne pas avoir de cystéine additionnelle. La GrxS17 comporte 3 modules Grx (sur cet alignement il s'agit du premier module) et la GrxS16 possède un module additionnel en position N-terminale. En rouge sont représentés les acides aminés strictement identiques.

b Mécanismes d'action

Les Grxs agissent par échange dithiol-disulfure et sont donc capables en théorie de réduire des ponts disulfures aussi bien intramoléculaires qu'intermoléculaires ou des liaisons covalentes entre une protéine et une molécule de glutathion, réaction appelée déglutathionylation. Toutefois, cette dernière réaction semble être la réaction principale et presque exclusive des Grxs. Ceci a du sens car il existe déjà un système *a priori* plus efficace (système thiorédoxine) dépendant de la même source de réducteur, le NADPH ou de la ferrédoxine et, de plus, les Trxs ont un potentiel redox plus bas que les Grxs (autour de -290 mV pour les Trxs et -160 mV pour les Grx C1 et C4 à pH7) (Rouhier *et al*., 2007b). Il existe potentiellement plusieurs mécanismes d'action pour les Grxs qu'elles soient mono- ou di-cystéiniques. Pour un échange dithiol-disulfure sans intervention du GSH, le mécanisme est identique à celui utilisé par les Trxs, sauf que la régénération de la Grx oxydée se fait normalement à l'aide du GSH (voir figure 12), lui-même maintenu réduit par une glutathion réductase (GR) NADPH dépendante (Rouhier *et al*., 2008; Rouhier *et al*., 2003).

Lorsque la protéine cible se trouve être une protéine glutathionylée, la cystéine en position N-terminale peut attaquer ce pont disulfure. La Grx se retrouve ainsi glutathionylée à son tour. Elle sera ensuite régénérée par l'attaque d'une autre molécule de glutathion, ce qui libérera une Grx réduite et active, et une molécule de GSSG. Dans le cas de Grx dithiol, on pourrait imaginer que la 2ème cystéine du site actif ou une autre cystèine puisse aussi effectuer cette réaction. Les réactions de déglutathionylation peuvent donc être effectuées à la fois par des Grx monothiols ou dithiols. Pour le moment, les travaux effectués concernent le plus souvent les Grx de la classe 1. Un mécanisme légèrement différent pourrait être utilisé par les Grx CGFS chloroplastiques (classe 2). En effet, les premières étapes de la déglutathionylation se dérouleraient de manière similaire jusqu'à la formation d'une Grx glutathionylée. L'adduit peut alors être réduit par une cystéine assez conservée du côté C-terminal de la protéine incluse dans le motif GG[S/C]DI, formant un

pont disulfure intramoléculaire sur la Grx (Zaffagnini *et al*., 2008). Le potentiel redox de ce disulfure (-230 mV à pH7) rend une réduction par le glutathion défavorable mais la FTR peut catalyser cette réaction *in vitro*.

Au niveau structural, il a été démontré que les Grxs de plantes comme celles d'autres organismes possèdent un repliement de type Trx fold, où la cystéine catalytique se situe également généralement au début d'une hélice α, généralement l'hélice α2 pour les Grxs eucaryotes.

c Signalisation/glutathionylation et fonctions physiologiques

Chez les plantes, encore peu d'informations sont connues à propos des différentes fonctions des Grxs autres que l'intervention des Grxs de classe I dans la réponse au stress oxydant à travers la réduction des Prxs de type II, de méthionine sulfoxyde réductase B ou encore de la Trxh4 (Gelhaye *et al*., 2003; Rouhier *et al*., 2001; Tarrago *et al*., 2009). Il a été montré que les Grxs CC jouent un rôle spécifique dans le développement des fleurs et la réponse aux pathogènes (Wang *et al*., 2009). En revanche, chez les organismes autres que les plantes, les Grxs sont connues pour jouer un rôle dans divers processus tels l'apoptose (Chrestensen *et al*., 2000) ou l'assemblage des centres fer-soufre dans la mitochondrie (Muhlenhoff *et al*., 2003; Rodriguez-Manzaneque *et al*., 2002). De plus, elles ont une importance dans la transduction de signaux, elles sont en effet capables de réguler de nombreux facteurs de transcription, des kinases et des phosphatases. Elles sont également capables de réguler la formation du cytosquelette en particulier via la glutathionylation de l'actine. Les Grxs sont capables d'interagir avec la kinase 1 régulant le signal apoptotique (ASK1) (Song and Lee, 2003) chez les animaux, mais également chez les plantes pour, ce qui a été montré avec une tyrosine-phosphatase (Dixon *et al*., 2005a). Par ailleurs, il a été montré chez plusieurs organismes dont une plante (*Oryza sativa* et *Saccharomyces*

```
PtGrxC1         MGSLLSSSIKASKQELDAALKKAKELASSAPVVVFSKTYCGYCNRVKQLLTQVGASYKVV 60
PtGrxC4             MAGSPEAT---------FVKKTISSHQIVIFSKSYCPYCKRAKGVFKELNQTPHVV 47
                    ::.* :*:          .*:  **  :*:***:** **:*.* ::.::. : :**

PtGrxC1         ELDELSDGSQLQSALAHWTGRGTVPNVFIGGKQIGGCDTVVEKHQRNELLPLLQDAAATA 120
PtGrxC4         ELDQREDGHNIQDAMSEIVGRRTVPQVFINGKHIGGSDDTVEAYESGELAKLLG-VASEQ 106
                ***: .** ::*.*::. .** ***:***.**:***.* .** :: .**  **  .*:

PtGrxC1         KTSAQL  126
PtGrxC4         KDDFKLE 113
                * . :*
```

Figure 18 : Alignement des séquences en acides aminés des glutarédoxines C1 et C4 de *Populus trichocarpa*.
Bien qu'elles soient très proches et que leur site actif ne diffère que d'un acide aminé, YCGYC pour Grx C1 contre YCPYC pour Grx C4, elles présentent quelques caractéristiques différentes. En rouge et soulignés d'une étoile sont représentés les acides aminés strictement identiques. La présence de la proline à la place de la glycine après la cystéine catalytique empêche la fixation d'un centre fer-soufre sur la protéine.

cerevisiae), que certaines Grxs pouvaient réduire des peroxydes en alcool (Collinson *et al*., 2002; Lee *et al*., 2002). Cependant, cette activité est extrêmement faible et ne semble pas physiologique.

Plus d'une centaine de protéines cibles ont été identifiées par spectrométrie de masse chez les plantes et les cyanobactéries si l'on considère les protéines identifiées sur des colones d'affinités (Bedhomme *et al*., 2009; Li *et al*., 2009b; Rouhier *et al*., 2007b) et les protéines glutathionylées (Dixon *et al*., 2005b; Michelet *et al*., 2008). Toutefois, l'interaction avec les Grxs doit encore confirmée pour la plupart d'entre elles.

d Formation de centres fer soufre dans les Grxs

Deux des premières Grxs de peuplier étudiées (GrxC1 et GrxC4) possèdent des sites actifs YCGYC et YCPYC respectivement et appartiennent à la classe 1. Le motif YCGYC n'est pour ainsi dire jamais retrouvé chez d'autres organismes que les plantes terrestres. La figure 18 nous montre que, bien que ce soient deux Grxs dithiols appartenant à la même classe, elles présentent seulement 30% d'identité et diffèrent, en dehors de la séquence de leur site actif, sur un point majeur. La GrxC1 peut intégrer un centre [2Fe-2S] dans un homodimère, le centre étant lié par la cystéine catalytique de chaque monomère et stabilisé par deux molécules de glutathion (figure 19). La GrxC4 ne peut pas intégrer de centre fer-soufre, la proline présente dans le site actif occupant l'espace nécessaire pour la ligation du centre Fe-S alors que le faible encombrement de la glycine de la GrxC1 le permet (Rouhier *et al*., 2004c; Rouhier *et al*., 2007b). Il est très important de noter que le centre Fe-S présent dans Grx C1 est somme toute assez stable et ne peut être réduit sans être dégradé. De plus, le centre est stabilisée par du GSH. Cependant, bien qu'elle présente un centre fer-soufre, la Grx C1 ne peut pas complémenter une levure délétée pour le gène grx5 (site actif CGFS), protéine impliquée dans la formation des centres fer-soufre mitochondriaux dans la levure (Bandhyopadhyay *et al*., 2008). Il est possible donc, qu'elle joue un rôle de senseur de

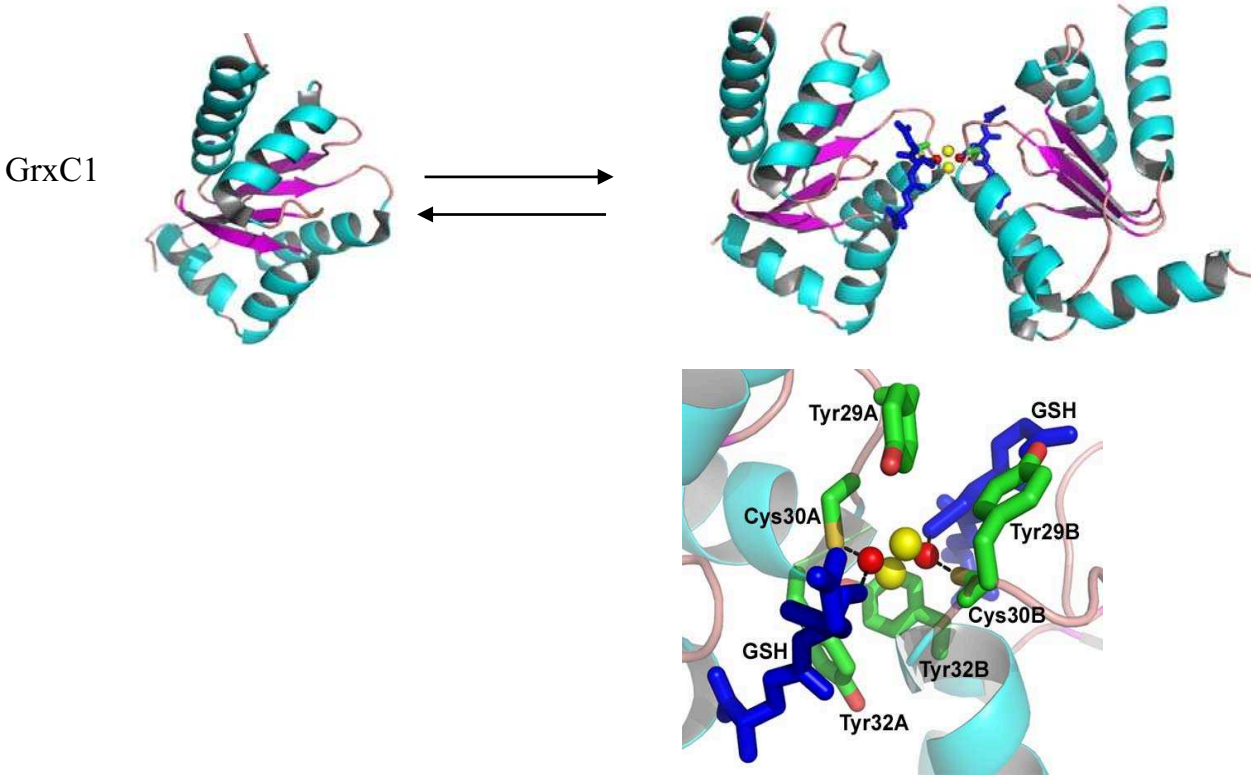


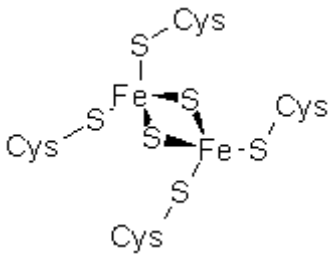
Figure 19 : Equilibre entre la forme monomérique apoprotéine et dimérique holoprotéine de la Grx C1 de *Populus trichocarpa*.
On observe bien le motif thiorédoxine composé d'un brin β composés de 4 feuillets entourés d'hélices α. La forme dimérique contient un centre fer-soufre (atome de fer en jaune, soufre en rouge) le tout stabilisé par deux molécules de glutathion en bleu foncé et les deux cystéines catalytiques des deux monomères en jaune foncé.

l'état redox du fer dans le cytosol des cellules de plante. L'incorporation d'un centre Fe-S dans la Grx C1 a été identifié à peu près au même moment et parallèlement à la démonstration que le même type de centre était intégré dans la Grx2 humaine mitochondriale qui possède un site actif SCSYC (Lillig *et al*., 2005). Le travail de mutagenèse dirigée effectué sur Grx C1 a aussi permis de montrer qu'une Grx avec un site actif CGFS devrait pouvoir aussi incorporer un centre fer-soufre (Rouhier *et al*., 2007b). Ceci a constitué la base d'une partie de mon travail de thèse. Brièvement, nous avons effectivement pu montrer que les Grxs CGFS de plantes produite sous forme mature recombinante dans *E. coli*, ou après des expériences de reconstitution, pouvaient incorporer un centre Fe-S mais qui était beaucoup plus labile (Bandyopadhyay *et al*., 2008).

Un article de revue faisant le lien entre les thiol-oxydoréductases (Grx, Trx et protéine disulfure isomérases (PDI)), la nature de leur site actif (CXXC ou CXXS) et leur propension à incorporer un centre métallique a été publié dans le volume 52 d'Advances in Botanical Research consacré aux systèmes redox. Dans ce chapitre d'ouvrage, la capacité de certaines Grxs à fixer un centre Fe-S grâce à la cystéine catalytique normalement impliquée dans les réactions d'oxydo-réduction est discutée par comparaison avec l'organisation structurale de protéines proches, les Trx et les ferrédoxines. Il ressort que le motif CXXC présent dans de nombreuses métalloprotéines et contenant les résidus cystéines nécessaires à la fixation de cofacteurs métalliques n'est pas strictement obligatoire pour les Trxs et Grxs. Des mutants d'*E. coli* avec des sites actifs CACC ou CACA peuvent incorporer des centres fer-soufre (Masip *et al*., 2004). Ainsi, certaines Grxs possédant un site actif de type CXXS ont contourné l'absence d'une deuxième cystéine en utilisant du glutathion comme ligand non protéique (Feng *et al*., 2006; Iwema *et al*., 2009; Johansson *et al*., 2007; Rouhier *et al*., 2007b).

IV Structure et assemblage des centres fer-soufre

A Les types de centres fer-soufre



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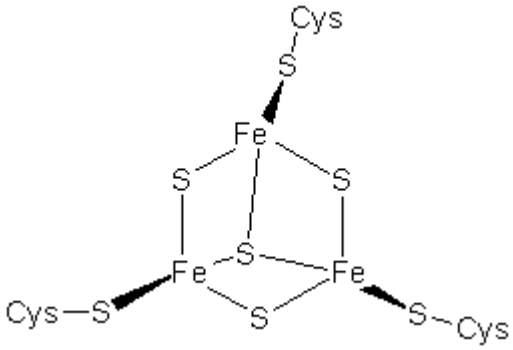

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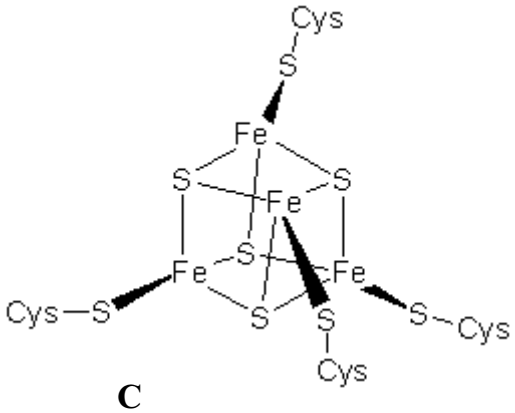

C

Figure 20 : Les différents types de centres fer-soufre.
A: centre [2Fe-2S], B: [3Fe-4S], C: [4Fe-4S]. Dans ces cas, les atomes de fer sont toujours liés à quatre atomes de soufre. Il existe également les centres de type Rieske, nom donné en rapport à la protéine de même nom présente dans le complexe III de la chaîne de transport d'électron mitochondriale ou dans le cytochrome b6f chloroplastique. Il s'agit d'un centre [2Fe-2S] avec une liaison spéciale impliquant deux cystéines et deux histidines via des atomes d'azote présents dans le noyau imidazole des ces acides aminés. Ce changement leur confère un potentiel redox plus élevé qu'avec un centre [2Fe-2S] classique.

Introduction

Les centres fer-soufre sont des cofacteurs protéiques composés d'atomes de fer et de soufre qu'on retrouve chez tous les organismes vivants. Ces centres fer-soufre font partie des plus anciens groupements prosthétiques, ce qui expliquerait pourquoi on retrouve une machinerie de biosynthèse conservée dans tous les règnes (Ayala-Castro *et al*., 2008; Balk and Lobreaux, 2005). Leur assemblage dépend de machineries protéiques complexes fournissant les atomes de fer et de soufre. Il a été montré récemment que des mutations dans les gènes de ces voies d'assemblage conduisaient souvent à des maladies génétiques (ataxies, myopathies, etc.) chez les mammifères ou des défauts de croissance chez les plantes voire à la non-viabilité de ces organismes. En effet, les protéines renfermant des centres FeS sont essentielles à de très nombreux processus ou voies métaboliques. Il existe différents types de centres Fe-S, ce sont principalement les centres [2Fe-2S], [4Fe-4S] ou [3Fe-4S] (figure 20). Une même protéine peut éventuellement intégrer plusieurs centres Fe-S voire différents types de centre, c'est notamment le cas de la biotine synthase qui peut intégrer un centre [2Fe-2S] et un [4Fe-4S], de la succinate déshydrogénase qui, elle, peut incorporer les trois différents types de centres (Balk and Lobreaux, 2005).

Le tableau 1 suivant présente une liste non exhaustive des protéines de plantes qui contiennent des centres fer-soufre ainsi que leur localisation avérée ou présumée. On s'apperçoit que ces protéines aux fonctions diverses sont retrouvées dans tous les compartiments cellulaires. La nature du centre n'a pas toujours été identifiée pour chacune de ces protéines. Ce tableau a été complété à partir de Balk et Lobréaux, 2005.

Protéine	Type de centre	Localisation
Aconitase	[4Fe-4S]	Mitochondrie, cytosol
Adénosine 5'-phosphosulfate réductase	[4Fe-4S]	Chloroplaste
Adrénodoxine	[2Fe-2S]	Mitochondrie
Aldéhyde oxidase	2[2Fe-2S]	Cytosol
Amidophosphoribulosyltransférase	[4Fe-4S]	Chloroplaste
Biotine synthase	[2Fe-2S], [4Fe-4S]	Mitochondrie
Chlorophylle a oxygénase	Rieske	Chloroplaste
Choline monooxygénase	Rieske	Chloroplaste
Cnx2 (Nitrate réductase et xanthine déshydrogènase cofacteur 2)	inconnu	Cytosol
Dihydroxy-acide déshydratase	[4Fe-4S]	Noyau
ADN glycosylase	[4Fe-4S]	Noyau
(E)-4-Hydroxy-3-methyl-but-2-enyl diphosphate réductase	[3Fe-4S]	Chloroplaste
Endonucléase III	[4Fe-4S]	Chloroplaste
Ferrédoxine	[2Fe-2S]	Chloroplaste
Ferrédoxine thiorédoxine réductase	[4Fe-4S]	Chloroplaste
Glutamate synthase	[3Fe-4S]	Mitochondrie, Chloroplaste
Ind1	[4Fe-4S]	Chloroplaste
Lipoate synthase	[2Fe-2S], [4Fe-4S]	Mitochondrie
NAD(P)H déshydrogénase	inconnu	Chloroplaste
Sous-unités K, I et H de la NADH-plastoquinone oxydoréductase	inconnu	Chloroplaste
NADH-ubiquinone oxydoréductase	8 centres	Mitochondrie
NBP35	[2Fe-2S], [4Fe-4S]	Cytosol
NAR1	inconnu	Cytosol
Nitrite réductase	[4Fe-4S]	Chloroplaste
Phéophorbide a oxygénase	inconnu	Chloroplaste
Sous-unités PsaA et PsAB du photosystème I	[4Fe-4S]	Chloroplaste
Sous-unité PsaC du photosystème I	2[4Fe-4S]	Chloroplaste
Protéine Rieske du cytochrome b6f	Rieske	Chloroplaste
Protéine Rieske de l'ubiquinol-cytochrome c réductase	Rieske	Mitochondrie
RNAse inhibiteur-like	inconnu	Cytosol
Sirohydrochlorine ferrochélatase	[2Fe-2S]	Chloroplaste
Sous-unité 2 de la succinate déshydrogénase	[2Fe-2S], [3Fe-4S], [4Fe-4S]	Mitochondrie
Sulfite réductase	[4Fe-4S]	Chloroplaste
THIC (Thiamine C)	inconnu	Chloroplaste
Tic55	Rieske	Chloroplaste
Xanthine déshydrogénase	[2Fe-2S]	Cytosol

Tableau 1 : Types de centre fer-soufre de différentes protéines de plantes et leur localisation cellulaire.

B Rôles des centres fer-soufre

Les protéines à centre fer-soufre sont impliquées dans de nombreux processus physiologiques tels la photosynthèse, la respiration ou encore les métabolismes du carbone, de l'hydrogène, de l'azote ou du soufre (Beinert *et al*., 1997; Johnson *et al*., 2005). Ces centres sont des catalyseurs qui participent à des réactions qui peuvent être ou non redox. Les mécanismes non redox sont notamment l'activité de l'aconitase qui catalyse l'isomérisation spécifique du citrate en isocitrate (Beinert and Kennedy, 1993; Tsuchiya *et al*., 2009), ou des mécanismes de régulation de l'expression des gènes aux niveaux transcriptionnel et traductionnel. Les exemples les plus connus sont la régulation traductionnelle des gènes du métabolisme du fer par IRP1 et 2 chez les mammifères et les régulations transciptionnelles bactériennes telles que FNR, ISCR ou SOXR/S. Les réactions redox concernent les chaînes de transfert d'électrons (respiration, photosynthèse), la biosynthèse des désoxyribonucléotides, la réparation de l'ADN, ou la biosynthèse de cofacteurs et de vitamines. En général, les protéines fer-soufre sont sous forme mature ou active lorsqu'elles ont acquis ce centre. Au niveau de la chaîne de transport d'électrons mitochondriale, on retrouve bon nombre de protéines à centre Fe-S. Dans le complexe I, 8 centres sont présents, 2 [2Fe-2S] et 6 [4Fe-4S]. La protéine Rieske dans le complexe III présente, elle, un centre [2Fe-2S] de type Rieske, c'est-à-dire avec une liaison spéciale impliquant deux cystéines et deux histidines via des atomes d'azote présents dans le noyau imidazole des ces acides aminés. Ce changement serait à l'origine du potentiel redox plus élevé que celui des centres [2Fe-2S] habituels (Schneider and Schmidt, 2005). En ce qui concerne la chaîne de transport d'électron chloroplastique, on retrouve un centre [2Fe-2S] pour la ferrédoxine, plusieurs centres [4Fe-4S] dans le photosystème I, ou bien encore un centre [2Fe-2S], de type Rieske au niveau du cytochrome b6f (Balk and Lobreaux, 2005). Ces protéines à centre Rieske semblent être spécifiques du transfert d'électrons (Schneider and Schmidt, 2005). Une carence en fer ou en soufre, ou bien une mutation affectant l'intégration

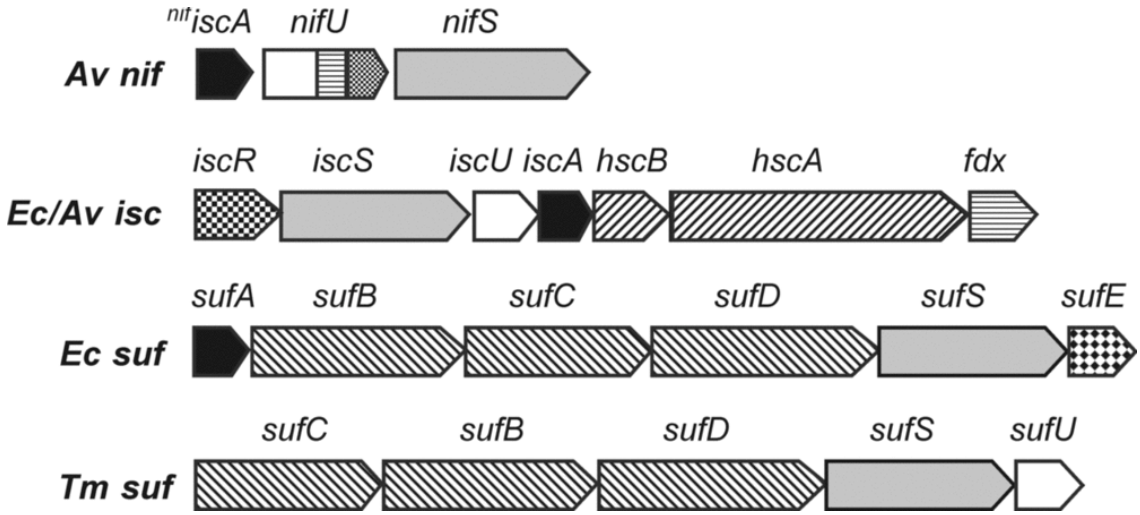

Figure 21 : Schéma des opérons Isc, Suf et Nif de bactéries, responsables de la biosynthèse et de l'intégration des centres fer-soufre (Bandyopadhyay *et al*, 2008).
On s'aperçoit qu'il s'agit d'opérons complexes qui vont coder pour plusieurs protéines agissant en synergie. Av : *Azobacter vinelandii*, Ec : *Escherichia coli*, Tm : *Thermotoga maritima*.

de ces centres engendrera un dysfonctionnement de ces chaînes de transport d'électrons. Enfin, pour certaines protéines dont la fonction du centre fer-soufre n'est pas connue, il a été suggéré qu'il pouvait avoir un rôle purement structural. C'est les cas de la protéine Rad3, une hélicase impliquée dans la réparation de l'ADN (Pugh *et al*., 2008) et Rli 1(RNase L inhibitor), une protéine impliquée dans la biogénèse des ribosomes (Barthelme *et al*., 2007).

C Assemblage des centres fer-soufre

Les connaissances concernant l'assemblage des centres Fe-S chez les plantes sont à ce jour encore limitées. En revanche, beaucoup d'études ont été réalisées d'abord chez les bactéries et ensuite chez la levure *S. cerevisiae*, ce qui a permis de mettre en évidence l'existence d'au moins 3 systèmes principaux intervenant dans cette machinerie ; il s'agit des opérons ISC (iron-sulphur cluster), SUF (sulfur mobilization) et NIF (nitrogen fixation) (Johnson *et al*., 2005) (Figure 21). Un quatrième système appelé csd est présent chez certaines bactéries comme *E. coli* (Loiseau *et al*., 2005). Le système NIF est responsable de la maturation des centres au sein des organismes fixateurs d'azote, alors que le système ISC représente le système principal de cette biosynthèse chez les bactéries, mais également le système spécifique des mitochondries des eucaryotes. Enfin, le système SUF ne serait opérationnel chez les bactéries qu'en conditions limitantes en Fe ou en réponse au stress et chez les cyanobactéries et dans les plastes des autres organismes photosynthétiques (Balk and Lobreaux, 2005; Bandyopadhyay *et al*., 2008; Fontecave *et al*., 2005; Lill and Muhlenhoff, 2005; Takahashi *et al*., 1999). Que ce soit ISC, SUF, ou NIF, les différentes machineries nécessitent et ont en commun une cystéine désulphurase (IscS, SufS, et NifS). L'assemblage des centres [2Fe-2S] ou [4Fe-4S] se réalise sur des protéines dites « scaffold », de type U (IscU, SufU, NifU). La première étape est donc le transfert d'atome de Fe et de S sur les protéines scaffold. Les cystéine désulfurases permettent de mobiliser du soufre à partir des

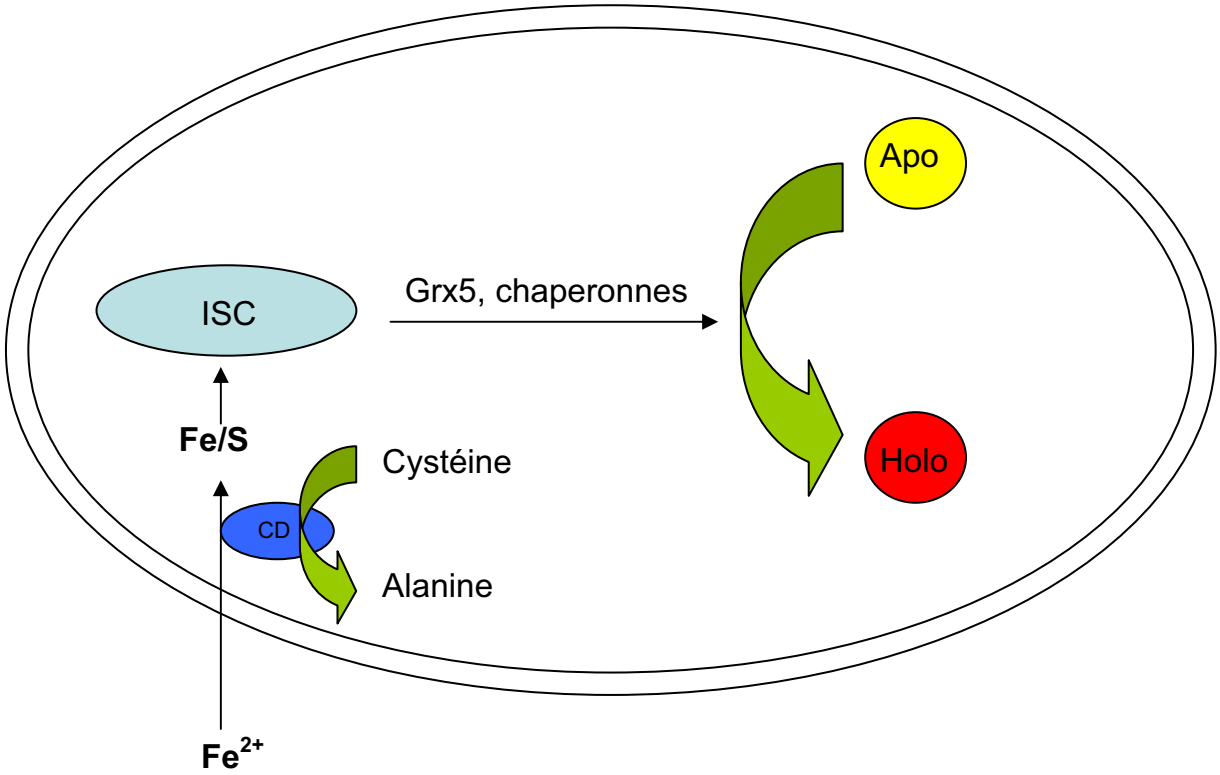

Figure 22 : Rôle de la Grx5 de levure dans la formation des centres fer-soufre au niveau des mitochondries.
L'ion Fe^{2+} est transféré dans la matrice à l'aide de transporteurs utilisant le potentiel de membrane. Le centre fer-soufre est produit à l'aide d'un complexe cystéine désulphurase (CD), composé des protéines NFS1 et Isd11, qui capte le soufre de la cystéine. Le centre sera ensuite pris en charge par les protéines de la machinerie de biosynthèse des centres fer-soufre ISC (Iron sulfur cluster), notamment Isu1 ou 2. Le centre sera ensuite transféré vers une protéine sous forme apo afin que celle-ci devienne mature (forme holo). Ce transfert est facilité par la Grx5, la protéine chaperonne Ssq1, la co-chaperonne Jac1 et le facteur d'échange de nucléotide Mge1.

cystéines afin de générer un persulfide. La source de fer n'est pas clairement établie mais il a été proposé pour le système mitochondrial que ce sont les frataxines qui jouent ce rôle. Finalement, une fois le centre FeS incorporé sur les protéines « scaffold » au travers de réactions de transpersulfuration, celui-ci est transféré vers les apoprotéines réceptrices. Pour le système ISC, le transfert des centres [2Fe-2S] nécessite de l'ATP et semble être facilité par des protéines cochaperonnes HscA et HscB ainsi que par des protéines « carrier » soit de type A (IscA, SufA, ^{Nif}IscA) soit de type Nfu (domaine C-terminal de NifU) (Chandramouli and Johnson, 2006). L'assemblage de centre [4Fe-4S] est réalisé à l'aide d'un complexe homodimérique formé par deux protéines IscU par la fusion de 2 centres [2Fe-2S] (Chandramouli *et al*., 2007).

Cependant, les connaissances sur les mécanismes d'assemblage et de transfert manquent encore de précision. Par exemple, il semble que la Grx5 de levure participe à ce processus mais son rôle exact est encore incertain (Muhlenhoff *et al*., 2003; Rodriguez-Manzaneque *et al*., 2002) (Figure 22). Chez les plantes, la machinerie d'assemblage mitochondriale, le système ISC, est composé des protéines Isu, IscA et Nfu4,5 comme protéines potentielles de l'assemblage, alors que, dans le chloroplaste, le système SUF utilise les protéines SufA1-4, SufB et Nfu1-3 comme protéines d'assemblage (Balk and Lobreaux, 2005; Layer *et al*., 2007; Ye *et al*., 2006).

D Rôle des glutarédoxines dans l'assemblage des centres fer-soufre mitochondriaux et dans l'homéostasie du fer

Comme évoqué précédemment, l'étude du mutant Grx5 de levure a permis de montrer que cette protéine était vraisemblablement impliquée dans l'assemblage mitochondrial des centres fer-soufre. En effet, cette mutation provoque une baisse sévère, voire la disparition totale, de l'activité de certaines protéines contenant un centre fer-soufre (Rodriguez Manzaneque *et al*., 2002). C'est notamment le cas de l'aconitase, une enzyme mitochondriale intervenant dans le cycle de Krebs,

et responsable de l'isomérisation du citrate en isocitrate et de la succinate déshydrogénase ou complexe II, qui catalyse l'oxydation du succinate en fumarate (Bandyopadhyay *et al*., 2008; Molina-Navarro *et al*., 2006; Molina *et al*., 2004). De plus, sans doute en raison de l'accumulation de fer, ce mutant devient plus sensible à un stress oxydant provoqué par l'ajout de diamide ou de peroxydes. Enfin, ce mutant présente une accumulation de fer ou de centres fer-soufre lié à la protéine Isu1, protéine scaffold faisant partie de la machinerie d'assemblage, ce qui a conduit à l'hypothèse que Grx5 joue le rôle de protéine « carrier » et intervient dans le transfert de centres Fe-S préformés sur les protéines scaffold vers des protéines receveuses (Muhlenhoff *et al*., 2003). La démonstration que plusieurs Grxs issues de différents organismes et appartenant à différentes classes (Grx C1, S14 et S16 de plantes, Grx4 d'*E. coli*, Grx2 humaine, Grx6 de levure, Grx1 de *Trypanosoma brucei*), intègrent un centre fer-soufre naturellement, renforce fortement l'hypothèse de l'intervention de ces protéines dans la maturation des protéines à centre fer-soufre (Comini *et al*., 2008; Lillig *et al*., 2005; Mesecke *et al*., 2008). Toutefois le rôle exact de ces protéines est encore assez vague. Une étude chez *Escherichia coli* avait suggéré que les Grxs 1, 2 et 3, en libérant des résidus cystéines par réduction de ponts disulfure, facilitaient l'intégration d'un centre [4Fe-4S] au sein de la forme apoprotéine de la Fumarate nitrate réductase (FNR) (Achebach *et al*., 2004). Les travaux actuels tendent plutôt à montrer que les Grxs présentes dans les organites sont impliquées dans l'assemblage des centres fer-soufre, alors que d'autres Grxs présentes en dehors de ces compartiments pourraient participer à la régulation de l'homéostasie du fer en particulier en jouant un rôle de senseur de l'état du fer dans la cellule. Cette dernière conclusion est basée sur le modèle développé chez *S. cerevisiae* où l'activité du principal facteur de transcription Aft1 (Activator ferrous transport 1) impliqué dans l'homéostasie du fer, est régulée par interaction avec deux Grxs cytosoliques, Grx3 et Grx4, appartenant à la classe II du fait de leur site actif CGFS (Ojeda *et al*., 2006). Ces deux Grxs font partie d'un hétérocomplexe incluant également les protéines appelées Fra1 et Fra2 (Fe repressor of activation-1 and 2) qui codent respectivement

pour une aminopeptidase de type P et pour une protéine de la famille BolA (Kumanovics *et al*., 2008; Li *et al*., 2009a). L'analyse des différents mutants a permis de proposer un modèle dans lequel cet hétérocomplexe est impliqué dans le transfert d'un signal venant de la machinerie mitochondriale d'assemblage des centres Fe-S vers Aft1 pour la régulation de la transcription des gènes de réponse au fer (Kumanovics *et al*., 2008). Ainsi, l'interaction d'Aft1 avec ce complexe dans le cytosol inhiberait son transfert vers le noyau empêchant l'activation des gènes impliqués notamment dans l'assimilation et le transfert du fer dans la cellule.

E Article I : "Glutaredoxin: the missing link between thiol-disulfide oxidoreductases and iron sulfur enzymes"

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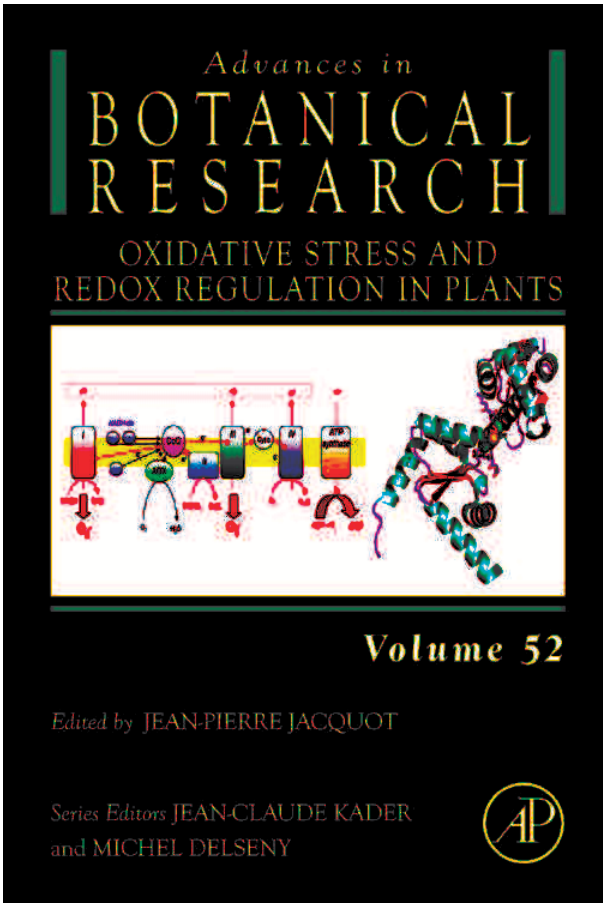

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Glutaredoxin: The Missing Link Between Thiol-Disulfide Oxidoreductases and Iron Sulfur Enzymes

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ABSTRACT

The CXXC motif is present in many disulfide oxidoreductases as thioredoxins, glutaredoxins, and protein disulfide isomerases. It is also present in several metal-binding structures including hemoproteins and iron sulfur proteins. Although the 3D structure of ferredoxins and thioredoxins is radically different, the presence of this motif in both proteins suggests that thioredoxins and their derivatives might be able to accommodate iron sulfur centers (ISCs) as well. Several studies have indeed proven the presence of metals, such as iron or zinc, in thioredoxin-like structures either as natural products or after mutagenesis as in *Escherichia coli* thioredoxin 1. Moreover, it was recently demonstrated that some glutaredoxin species with CGYC or CGFS active sites can assemble a [2Fe–2S] ISC in a homodimer. Quite surprisingly, the ligands are the glutaredoxin catalytic cysteine and an external glutathione molecule. As a yeast CGFS glutaredoxin is thought to be involved in the transfer of preassembled ISCs from scaffold to acceptor apoproteins, this suggests that glutaredoxins are involved in these pathways through their own capacity to assemble such centers and transfer them efficiently. Altogether, these data provide firm evidence that glutaredoxins are a link between the world of thiol-disulfide reductases and iron sulfur enzymes.

I. INTRODUCTION

A large number of biochemical reactions require the participation of metals, including the mitochondrial and plastidial electron transfer chains, the metabolic pathways leading to the assimilation of nitrogen or sulfur, the synthesis of ribosomes, or the process of DNA repair, to cite only a few of these processes. Overwhelmingly, the metal ligands in proteins are histidine (through its nitrogen atom) and methionine and cysteine residues via their sulfur atom. Occasionally, acidic residues as aspartate or glutamate are also encountered. Metals as diverse as copper, molybdenum, iron, manganese, zinc, and nickel or even vanadium and cadmium (Lane *et al.*, 2005; Messerschmidt and Wever, 1996) are assembled into apoproteins via post-translational reactions. With a few exceptions, most metalloenzymes play a redox role, transferring electrons to neighboring proteins or chemical compounds by shifting back and forth between reduced and oxidized states. The microenvironment of the metal is extremely important in determining the redox potential of the metallic structures and hence, their reactivity. Among the many metalloenzymes, iron-containing enzymes play a prominent role in biology, and thus we discuss thereafter more extensively the organization of these metallic centers in macromolecules.

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II. IRON-CONTAINING ENZYMES

Several types of iron-containing structures are present in proteins, the more frequently encountered being the di-iron centers, the hemes, and the iron sulfur centers (ISCs). It has been proposed that ISCs are the most primitive structures, as the primitive earth atmosphere is thought to have contained reduced sulfur and iron, the necessary components for ISC assembly (Milner-White and Russell, 2005). Interestingly, the Nest theory assumes the importance of glycine residues in early iron sulfur-binding peptides (see later the glutaredoxin (Grx) requirements for iron sulfur binding). It is postulated that hemes appeared later in evolution.

A. DI-IRON CENTERS

Di-ferric iron centers are present in a number of proteins, most notably ribonucleotide reductase (RNR), a key enzyme necessary for the biosynthesis of deoxyribonucleotides. In aerobic type RNR, the two irons are μ-oxo-linked and coordinated to the protein via ligands that include four carboxylic residues and two histidines. The di-iron site is close to the Tyr radical of the enzyme and serves for the generation and stabilization of the radical. Unlike the situation of cytochromes of the *c* type and of iron sulfur proteins, there is no direct link between this di-iron site in RNR and thioredoxin (Trx) except that RNR is an enzyme that requires thioredoxin or glutaredoxin for its catalytic activity (Avval and Holmgren, 2009). The regeneration of the active form of RNR involves the successive reduction of disulfide bonds, one situated at the C-terminus with the sequence CESGAC is more specifically the thioredoxin or glutaredoxin target (Persson *et al.*, 1997). Interestingly, some model compounds have been created where the two iron atoms are not oxo-linked but rather dithiolate bridged (Borg *et al.*, 2004).

B. HEMES

Several types of hemes are present in proteins, including the a, b, and c classes. All hemes are inserted posttranslationally into the apoproteins but their binding differs depending on the class considered. The a and b hemes are noncovalently attached, and are bound mostly via hydrophobic bonds. On the other hand, the c-type hemes are linked via thioether bonds that include two vicinal cysteine residues separated by two amino acids with the general formula CXXCH. The two X residues are variable, for example, in

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horse heart cytochrome *c*, the attachment sequence is CAQC (Abriata *et al.*, 2009). It is CAAC in *Chlamydomonas reinhardtii* cytochrome *c*6 (Merchant and Bogorad, 1987), CSQC in chicken cytochrome *c* (Chan and Margoliash, 1966), and CANC in cytochrome *f* either from *Mastigocladus laminosus* or from *C. reinhardtii* (Baniulis *et al.*, 2008). In all c-type cytochromes, the iron atom in the center of the heme possesses two axial ligands, a histidine, and a methionine. It is well known that spacing two cysteines with two amino acids results in having the two sulfur atoms in close proximity in the 3D space, and thus in the possibility of creating a disulfide bond between the two cysteines following translation of the polypeptide. This is probably the reason why heme assembly requires the participation of thioredoxin-like molecules, for example, CCMH for *Arabidopsis thaliana* mitochondrial cytochrome *c* (Meyer *et al.*, 2005). In this case, the thioredoxin-like protein is required for the reduction of the disulfide prior to heme assembly.

C. ISC AND IRON SULFUR PROTEINS

1. Nature of the center

There are many types of ISCs in proteins, from the most simple type containing a single iron and no heterosulfur (rubredoxin-like) to more complex structures as [2Fe–2S] centers (chloroplastic ferredoxin, chloroplastic, and mitochondrial Rieske protein, mitochondrial adrenodoxin), [3Fe–4S] centers (e.g., in aconitase), and [4Fe–4S] centers (e.g., in chloroplastic photosystem I, nitrite reductase, sulfite reductase, glutamate oxoglutarate aminotransferase, and also in mitochondrial complex I) (Sazanov and Hinchliffe, 2006).

2. Nature of the ligands, position in the primary structure, and the CXXC motif

Except for the Rieske protein in which the ISC is ligated via two cysteines and two histidines, the amino acid ligands for ISCs are overwhelmingly cysteines and the ligation involves the sulfur atom of the side chain. In the rubredoxin mono iron type, the ligands coordinate with the iron atom in a tetrahedral organization. Interestingly for the *Clostridium pasteurianum* rubredoxin, the four cysteines are organized in two pairs separated by two amino acids with the sequences CTVC and CPLC (Mathieu *et al.*, 1992). In [2Fe–2S] ISCs, the four coordinating sulfur atoms are in the same plane than the two iron atoms, but in addition there are two inorganic sulfur atoms (also called labile sulfur) linking the two irons above and below the above defined plane. In [3Fe–4S] and [4Fe–4S] centers, the iron atoms and the hetero sulfur atoms are alternating in a cubane-like structure, the iron atoms being themselves covalently bound via the sulfur atoms of the ligand cysteines. In chloroplastic

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ferredoxins, the position of the cysteines is conserved across species (in position 38, 43, 46, and 76 in *C. reinhardtii*) with a pair of cysteines in a CXXC motif which is absolutely conserved (Stein *et al.*, 1993). In general, the positions of the cysteine ligands are quite variable depending on which enzyme is considered, but examples abound where a CXXC motif is necessary for the binding of either [2Fe–2S] or [4Fe–4S] ISCs (Amman *et al.*, 2004; Brandt, 2006; Bych *et al.*, 2008; Raux-Deery *et al.*, 2005; Song and Lee, 2008; Yabe *et al.*, 2008; Zhang *et al.*, 2008). The remark concerning the cysteine spacing in cytochrome *c* and their potential oxidation into a disulfide after protein synthesis and before heme insertion obviously also applies to the many ISC-containing proteins with the CXXC motifs. Overall, nearly all of the ISC-containing proteins contain multiple iron atoms (except for the rubredoxin-type) and sometimes even multiple ISCs are present in a single poplypeptide, as in PsaC of photosystem I (Takahashi *et al.*, 1991). Nevertheless, although they contain multiple iron atoms, the ISCs are able to transfer only one electron at a time, this being possibly related to the position of the iron atoms in the 3D structure. For example, for a spinach chloroplastic ferredoxin (pdb accession number 1A70), it is indeed clear that one of the iron atoms is located closer to the surface of the protein and the other more deeply buried and thus unable to participate in electron transfer reactions (Binda *et al.*, 1998).

3. *Pathways where iron sulfur enzymes are required*

It has been briefly mentioned that nitrite reductase and glutamate synthase contain an ISC, and that nitrogen assimilation requires the participation of iron sulfur enzymes (Swamy *et al.*, 2005). Likewise, the iron sulfur-containing sulfite reductase, an enzyme with high analogy to nitrite reductase, and APS reductase are involved in sulphate assimilation (Hirasawa *et al.*, 2004; Kim *et al.*, 2006). As noted above the electron transfer chains of mitochondria and chloroplasts contain multiple ISCs (Complex I, II, and III in mitochondria, and cytochrome *b6f* and PSI in chloroplasts). The energetic metabolism and formation of ATP are thus dependent on these enzymes and so is the carbon assimilation in plants. In the cytosol, one enzyme involved in leucine biosynthesis, isopropylmalate isomerase (Leu1p) also contains an ISC (Sipos *et al.*, 2002). Scaffold proteins such as CnfU, a key iron sulfur cluster biosynthetic scaffold that is required for biogenesis of ferredoxin and photosystem I in chloroplasts also contain ISCs (Yabe *et al.*, 2008). This is also the case for the proteins Dre2, Nar1, and SirB which are involved in cytosolic iron sulfur biogenesis and siroheme biosynthesis, respectively (Raux-Deery *et al.*, 2005; Song and Lee, 2008; Zhang *et al.*, 2008). These examples are certainly not exhaustive but are demonstrative of how essential

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these structures are for central metabolism, be it carbon, nitrogen, or sulfur metabolisms (and autotrophy in plants) or amino acid biosynthesis and iron sulfur assembly.

4. 3D structure of [2Fe–2S]-containing ferredoxins

Experiments described subsequently in this chapter have indicated that the thioredoxin and glutaredoxin molecules can harbor ISCs of the [2Fe–2S] type, either in "natural" proteins or in engineered mutagenized versions of the proteins and thus become "ferredoxin-like," so we describe here the 3D structure of most well-known ferredoxins from cyanobacteria or chloroplasts. These proteins are extremely well conserved with 96–99 amino acids in their mature form and the ligand cysteines in invariant positions. The protein is held together by several β-strands forming a β-sheet at the back of the molecule (Fig. 1). To the front of the molecule, three short α-helices surround the iron sulfur cluster, with one of the iron atoms positioned closer to the surface and thus better suited for transferring electrons. In the three helices lie key acidic residues that are required for protein–protein interaction (Binda *et al.*, 1998; Jacquot *et al.*, 1997). Other types of structures harboring a low potential [2Fe–2S] center are thioredoxin-like proteins present in bacteria such as *Azotobacter vinelandii*, *C. pasteurianum*, and *Chlorobium tepidum* (Meyer, 2001). Interestingly, these proteins form dimers, each

Fig. 1. Crystal structure of the E92K mutant of [2Fe–2S] ferredoxin I from *Spinacia oleracea* (Binda *et al.*, 1998; pdb accession number 1A70). Sulfur atoms belonging to cysteine residues 38, 43, 46, and 76 are represented by green spheres, iron atoms by red, and labile sulfur atoms by yellow.

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monomer having a half "thioredoxin-like" architecture with a central pleated β-sheet surrounded by α-helices (Fig. 2) (Yeh *et al.*, 2000). In the dimer, the two β-sheets face one another and two of the helices surrounding them in more traditional thioredoxins are missing.

D. CURRENT MECHANISM OF ISC ASSEMBLY IN PLANT PLASTIDS AND MITOCHONDRIA

Two of the three bacterial ISC assembly systems, nif, suf, and isc have been retained in photosynthetic eukaryote organisms, the suf machinery being present in plastids and the isc machinery in mitochondria. In addition, nonplant eukaryotes possess an ISC export machinery and a cytosolic

Fig. 2. Crystal structure of a thioredoxin-like [2Fe–2S] ferredoxin from *Aquifex aeolicus* (Yeh *et al.*, 2000; pdb 1F37). Sulfur atoms belonging to cysteine residues 9, 22, 55, and 59 are represented by green spheres, iron atoms by red and labile sulfur atoms by yellow.

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assembly machinery for cytosolic and nuclear FeS proteins (Lill and Mühlenhoff, 2008). Based on the conservation of the proteins involved in the two latter systems, it is likely that these pathways also exist in plants (Balk and Lobreaux, 2005). It has been suggested from genetic studies in yeast that Grx5 is involved in the transfer of preassembled clusters from Isu proteins to acceptor proteins (Muhlenhoff *et al.*, 2003). As most Grxs from mammals, plants, and cyanobacteria can complement the defects associated with the deletion of Grx5 in yeast, this suggests that they fulfil similar functions in their respective organelles (Molina-Navarro *et al.*, 2006; Picciocchi *et al.*, 2007; Rouhier *et al.*, 2008). The latest developments concerning the involvement of Grxs in plant ISC biogenesis will be described in a subsequent section.

III. THIOL-DISULFIDE OXIDOREDUCTASES

The family of oxidoreductases includes disulfide reductases called thioredoxins, glutaredoxins, disulfide oxidases, or isomerases belonging to the sulfhydryl oxidase (SOX) and protein disulfide isomerase (PDI) families. Grxs, Trxs, and PDIs belonging to the large Trx superfamily possess many features in common, the active site, the 3D structure, the reaction mechanism. They are all ubiquitous or nearly ubiquitous redox proteins with conserved redox-active sites CXXC/S possessing generally oxidoreductase activity in dithiol-disulfide exchange reactions (Holmgren, 1985). The major difference lies in the redox potential of their active pair of cysteines, Trxs having a more electronegative redox potential than Grxs and even more than PDI. Furthermore, Chivers and collaborators have shown that the modification of the CXXC motif and consequently of the redox potential can modify the oxidoreductase properties of the protein. For example, a *Sc*Trx mutant with a higher redox potential can efficiently replace *Sc*PDI1 null mutant (Chivers *et al.*, 1996, 1997).

Photosynthetic and nonphotosynthetic organisms contain a large number of genes encoding these three classes of enzymes, for example, in poplar, around 40 Trx- and Grx-encoding genes and 13 PDI encoding genes (Chibani *et al.*, 2009; Couturier *et al.*, 2009b; Houston *et al.*, 2005; Morel *et al.*, 2008). All these proteins have variable subcellular localizations. In eukaryotic cells, Trxs and Grxs are rather present in compartments with a reducing environment, whereas PDI and SOX are present in compartments supposed to have an oxidizing environment. For example, PDIs are mostly found in the endoplasmic reticulum and their function is to fold properly other proteins via cysteine rearrangement.

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A. THE THIOREDOXIN MODEL

1. Active-site sequence

Most frequently, Trxs have a highly conserved classical dicysteinic CXXC active-site sequence (overwhelmingly WC[G/P]PC), comprising two vicinal cysteines separated by two variable amino acids. Besides, in plants, several genomic studies have highlighted the presence of thioredoxin-like proteins with dicysteinic or monocysteinic active sites (CXXS), but little is known about their biochemical properties (Chibani *et al.*, 2009; Gelhaye *et al.*, 2004; Meyer *et al.*, 2007). In fact, Serrato and colleagues have demonstrated that some *Arabidopsis* CXXS Trxs have a disulfide reductase activity (Serrato *et al.*, 2008).

2. Redox potential and reaction mechanism

In Trxs containing a pair of cysteine residues, both cysteines play different roles. The first cysteine is involved in the nucleophilic attack on disulfide bonds present in target proteins, leading to the formation of a disulfide bond between the target protein and Trx. The second cysteine, called the backup/resolving or recycling cysteine, subsequently cleaves the disulfide formed between Trx and its target. In general, Trxs with a conventional active site WCGPC, have a low redox potential comprised between − 270 and − 330 mV compared to the other redoxins (Bréhélin *et al.*, 2004; Collin *et al.*, 2003). The two residues located between the two cysteines in the active-site motif are important in controlling the redox properties of the proteins. Changing one residue of the active site by site-directed mutagenesis affects the redox potential of the protein. For example, swapping the proline to histidine in the *Escherichia coli* Trx active-site sequence induces a higher redox potential (− 235 mV) than the wild type (Krause *et al.*, 1991). This mutation confers the ability to function as a disulfide isomerase and also has an impact on its interaction with folding protein substrates (Eklund *et al.*, 1991; Holmgren, 1995). Recently, it has also been demonstrated that the residue preceding the *cis* Pro conserved in all Trx superfamily members is crucial in determining the redox potential of the oxidoreductases (Ren *et al.*, 2009). A recent study showed that atypical chloroplastic Trxs called Trx-lilium from *Arabidopsis* with CGSC, CGGC, or CASC active sites display higher redox potentials between −237 and −240 mV, suggesting they cannot reduce all the usual Trx partners but that they could instead have specific functions (Dangoor *et al.*, 2009).

3. Subcellular localization and physiological roles

Thioredoxins are involved in a wide variety of fundamental biological functions including dithiol hydrogen donation to RNR, regulation of the activity of photosynthetic enzymes including fructose-1,6-bisphosphatase and

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NADP malate dehydrogenase and of some eukaryotic transcription factors (Schürmann and Buchanan, 2008; Schürmann and Jacquot, 2000). In addition, it has been recently well documented that thioredoxins serve as regenerating systems for peroxiredoxins (Prx) and methionine sulfoxide reductases, enzymes in which the catalytic cysteine becomes oxidized into a sulfenic acid residue upon catalysis (Rouhier *et al.*, 2001, 2007a, 2008). In addition to these well-described interactions, the list of Trx targets involved in many metabolic pathways and cellular processes is growing with their identification by proteomic studies (for a review see Montrichard *et al.*, 2009 and also the paper by Nishiyama and Hisabori in this issue). There are multiple Trx isoforms localized in different compartments such as the cytosol (h), chloroplast (f, m, x, y, CDSP32, lilium), mitochondria (o, h2), nucleus (nucleoredoxin), apoplasm (h), endoplasmic reticulum (s), and Trxs with unknown localization (Trx-like and clot) (Alkhalfioui *et al.*, 2008; Chibani *et al.*, 2009; Meyer *et al.*, 2006). In chloroplasts, Trxs are essentially reduced by ferredoxin via ferredoxin-thioredoxin reductase (FTR), whereas cytosolic or mitochondrial Trxs are reduced by NADPH via a NADPH-Trx reductase (NTR) (Jacquot *et al.*, 2009). An alternative chloroplastic Trx reduction pathway involves NADPH and the hybrid enzyme NTRC (it contains a built-in Trx module in the C-terminal part of a NTR module). This protein might be specifically devoted to the reduction of some Prx types. A recent review summarizes the evolution and properties of thioredoxin reductases in photosynthetic organisms (Jacquot *et al.*, 2009).

4. 3D structures of thioredoxins

The first 3D Trx structure is the one of the *E. coli* Trx1 and it has been solved by X-ray crystallography (Holmgren *et al.*, 1975). Subsequently, several structures of Trxs from different organisms have been determined, and they display a high degree of homology, most of these proteins being rather homogeneous in length with ca. 110–120 amino acid residues (Fig. 3). Trx structures are also described in this volume in a chapter by Hägglund and colleagues. All Trx structures have a well-conserved hydrophobic core and most amino acid variants are located on the surface of the protein, affecting surface patches only locally. Thioredoxins and glutaredoxins are characterized by a common fold, the thioredoxin fold, which is a central five stranded β-sheet flanked by three or four α-helices and a CXXC active-site motif. The secondary elements are in the order β1, α1, β2, α2, β3, α3, β4, β5, α4. The conserved redox-active site forms the link between the second β-strand and the subsequent α2 helix, and the cysteines are located at the N-terminus of the helix and rather exposed at the surface of the molecule, especially the catalytic one (Dai *et al.*, 2000; Mössners *et al.*, 1998). Concerning plant Trxs, the

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Fig. 3. Crystal structure of thioredoxin h1 from *Arabidopsis thaliana* (Peterson *et al.*, 2005; pdb 1XFl). Sulfur atoms belonging to cysteine residues 40 and 43 are represented in spheres (green in the web version).

structures of some Trxs f, m, and h have been determined in several organisms and solved by X-ray crystallography and NMR. The crystal structure of recombinant spinach Trx m has been solved in the oxidized and reduced state at 2.1 and 2.3 Å resolution, respectively (Capitani *et al.*, 2000). The structure of *C. reinhardtii* thioredoxin *m* has been solved by NMR and it is very similar to the spinach protein (Lancelin *et al.*, 2000). The spinach Trx f structure shares much similarity with the m, however, the f protein is more positively charged with some of these charges surrounding the active site where they must be instrumental in orientating the protein correctly upon interaction with its targets. Despite their structural similarities, a striking difference is the presence of a conserved third Cys73 in the C-terminal part of the f sequence. This cysteine is structurally exposed on the surface of the structure, 9.7 Å away from the first Cys of the active site (Brandes *et al.*, 1993; del Val *et al.*, 1999). Thus, the overall structure of the spinach Trx f and m does not differ markedly from the *E. coli* model (Schürmann and Buchanan, 2008). The structures of h-type Trxs have been determined for *C. reinhardtii,* barley, *A. thaliana*, and poplar enzymes (Coudevylle *et al.*, 2005; Koh *et al.*, 2008; Maeda *et al.*, 2008; Menchise *et al.*, 2001; Peterson *et al.*, 2005). In general, the h structure presents one major difference compared to other thioredoxins, namely an elongated α1 helix. Analysis of the 3D-structure of the *C. reinhardtii h* together with calorimetric studies showed that thioredoxin h has a much reduced thermal stability compared to thioredoxin m and has more similarity to the mammalian protein-type (Lemaire *et al.*, 2000;

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Richardson *et al.*, 2000; Stein *et al.*, 1995). This is in agreement with the phylogenetic analyses that proposed an eukaryotic origin for thioredoxin f and h and a prokaryotic origin for thioredoxin m (Meyer *et al.*, 2002). These hypotheses were recently strengthened in a study reporting the effect of applying force to individual thioredoxin molecules from various sources (Perez-Jimenez *et al.*, 2009). A recent study has allowed the comparison of structures of two barley Trx h isoforms (Trxh1 and Trxh2). Barley Trx h1 and h2 have been solved in the oxidized state at 1.7 Å and Trxh2 in the reduced state at 2 Å resolution. The Trxh1 Arg101 can play a particularly crucial role in the association with target proteins by forming electrostatic interactions with a protein motif bound in the substrate-binding loop motif. The presence of Arg101 in Trxh1 and the uncharged Ile107 in Trxh2 may therefore give rise to differential isoform interactions with some redox patterns (Hagglund *et al.*, in this issue; Maeda *et al.*, 2008).

B. PDIs DERIVE FROM THIOREDOXINS

PDIs are generally multidomain proteins sharing structural and amino acid sequence similarities with thioredoxins (Ferrari and Söling, 1999). ''Classical'' PDI are constituted by five independent domains (a-b-b′-a′-c). The a, a′, b, and b′ domains have homologies to thioredoxins, while the a and a′ domains generally possess the CXXC active site, the b and b′ domains do not. The c domain is a short, acidic amino acid sequence (Edman *et al.*, 1985; Freedman *et al.*, 1994; Kemmink *et al.*, 1999). Most PDI also share an endoplasmic reticulum retention signal (K/HDEL) at the C-terminal end (Freedman *et al.*, 1994). Other classes of PDI differ from the ''classical'' representative member by the number and distribution of active and inactive TRX modules and the presence of additional domains sharing no similarity with Trxs (Appenzeller-Herzog and Ellgaard, 2008). Concerning plants, PDIs were first detected in higher plants at the end of the 1970s (Grynberg *et al.*, 1977) and then in the photosynthetic alga *C. reinhardii* (Myllylä *et al.*, 1989). Some comparative genomic studies performed on different photosynthetic organisms (*C. reinhardii*, *A. thaliana*, *Oryza sativa*, *Zea mays*) highlighted the complexity of the plant PDI family, similar to the situation in mammals and yeast (Houston *et al.*, 2005; Lemaire and Miginiac-Maslow, 2004).

1. Active site

The plant PDIs characterized so far generally possess one or two TRX active domains with a catalytic WCGHC motif that can be extended to the EFYAPW*CGHC*K/Q sequence, based on amino acid sequence comparisons

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(Houston *et al.*, 2005; Lemaire and Miginiac-Maslow, 2004; Xu *et al.*, 2002). However, as for the thioredoxin and glutaredoxin families, a large variability exists concerning the amino acids present between the cysteines and even the number of cysteines in the catalytic modules leading to CxxS or SxxC active-site motifs (Maattanen *et al.*, 2006). Although plants have such isoforms, none of them has been characterized yet. As mentioned before, an *Arabidopsis* atypical thioredoxin h with a CXXS active site has been shown to catalyze isomerization of scrambled RNAse (Serrato *et al.*, 2008). This study suggests the existence of plants atypical PDI with efficient isomerase activity.

2. Redox potential

The global redox potential of PDI is around − 175 mV, with values ranging from − 188 mV for the a domain to − 155 mV for the a′ domain. These data are coherent with the fact that the redox state of the two modules can be different, the active site of the a domain being natively oxidized while the one of the a′ domain is reduced (Tian *et al.*, 2006). The prokaryotic periplasmic oxidase, DsbA, which has a function similar to eukaryotic PDI has a redox potential of around − 130 mV. This could explain why PDIs, which have a redox potential similar to Grxs, can also be efficient reductases (Hawkins *et al.*, 1991; Wunderlich and Glockshuber, 1993).

3. Subcellular localization and physiological role

As mentioned previously, PDIs are generally residents of the endoplasmic reticulum and they present all features required and sufficient for its targeting and retention into the ER. Nevertheless it was shown that RB60, a *C. reinhardtii* PDI member with a KDEL retention signal was partitioned between the stroma and thylakoids of chloroplasts (Trebitsh *et al.*, 2001). Concerning their physiological role, PDI is probably one of the most versatile members of the thioredoxin superfamily as it is able to catalyze *in vitro* disulfide oxidation, reduction, and isomerization (Kaska *et al.*, 1990; Myllylä *et al.*, 1989; Shorrosh *et al.*, 1993; Wadahama *et al.*, 2007). Some data obtained *in vivo* with mammalian and yeast PDI confirmed oxidase and isomerase activities (Frand and Kaiser, 1999; Laboissiere *et al.*, 1995), and also chaperone activities in the absence of an active Trx domain (Song and Wang, 1995).

4. 3D structures of PDIs

The 3D structures of some nonplant eukaryotic PDI members have been solved, essentially from human and yeast (Fig. 4). Only a few of them represent whole proteins (*Hs*ERp29, *Hs*ERp18, *Sc*PDI1p, and *Sc*Mpd1 with respective pdb accession numbers: 2QC7, 1SEN, 2B5E, 3ED3) while some others only describe isolated protein domains (Barak *et al.*, 2009; Rowe

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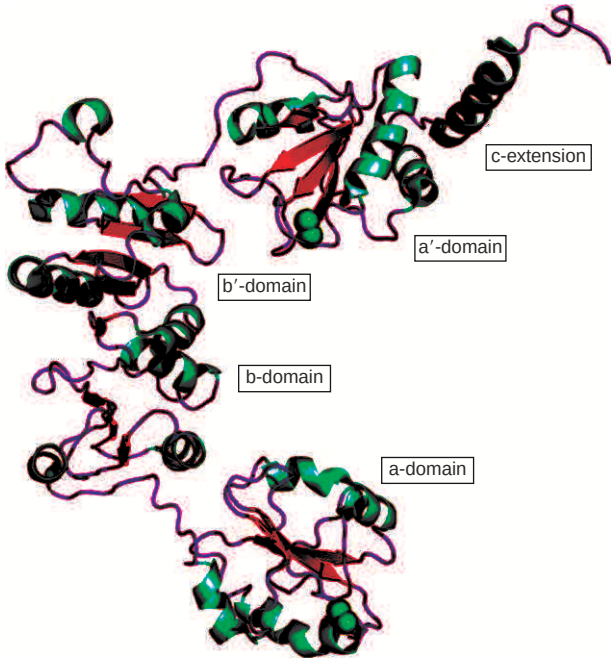

Fig. 4. Crystal structure of protein disulfide isomerase *Sc*PDI1p from Baker's yeast *Saccharomyces cerevisiae* (Tian *et al.*, 2006; pdb accession number 3BOA). Active Trx domains are named a and a′, inactive Trx domain b and b′ and acidic C-terminal extension c. Sulfur atoms belonging to cysteines 43, 46, 388, and 391 are represented by green spheres.

et al., 2009; Tian *et al.*, 2006; Vitu *et al.*, 2008). These four proteins vary by the domain composition, the number of "active" Trx modules, and exemplify various PDI classes. Here, we will focus our attention on the 3D structure of *Saccharomyces cerevisiae* PDI1p, a "classical" PDI. This protein is composed of the four domains a, a′, b, and b′ with thioredoxin fold. The four Trx domains and the acidic C-terminal tail of *Sc*PDI form a twisted "U" shape with active sites (CGHC) of active TRX domain (a-a′) facing each other at the end of the U branch. As in thioredoxins, each active site is located at the N-terminal side of the second α-helix. The inactive Trx domain (b-b′), forming the base of the "U," is present at the same relative position as the hydrophobic patch forming a continuous hydrophobic surface at the inside face of the "U." This hydrophobic "pocket" seems to be important for substrate recognition, particularly when PDI acts as a folding and chaperone protein (Kozlov *et al.*, 2006; Zheng and Gilbert, 2001). The connections between the Trx modules are different. The noncatalytic b and b′ modules are connected by a sequence of 17 amino acid residues in an

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extended conformation susceptible to rigidity. The link between a-b and b′-a′ domains are shorter and presumably more flexible, suggesting that PDI can accommodate diverse substrates (Tian *et al.*, 2006).

C. GLUTAREDOXINS ARE GLUTATHIONE-DEPENDENT PROTEINS DERIVED FROM THIOREDOXINS

1. Active-site sequence

Glutaredoxins are ubiquitous proteins present in most prokaryotes and eukaryotes (Couturier *et al.*, 2009b; Fernandes and Holmgren, 2004). They were initially categorized, based on the active-site sequence, into two groups, a dithiol (CPY/FC motif) and a monothiol (CGFS motif) subgroup (Rodríguez-Manzaneque *et al.*, 1999). In plants, these two types of Grxs represent what we defined as class I and II, respectively. Classes I and II have been further divided into several subgroups called Grx C1, C2, C3, C4, and C5/S12 for class I and GrxS14, S15, S16, and S17 for class II. The specificity of these subgroups has been detailed previously (Couturier *et al.*, 2009b; Rouhier *et al.*, 2004a, 2006). A third Grx class, which contains proteins with a CCxx active-site motif, was also identified specifically in terrestrial plants (Lemaire, 2004; Rouhier *et al.*, 2004a), see the chapter by Li and Zachgo in this volume concerning the Roxy protein of this group. More recently, it has been demonstrated, notably in eukaryotic photosynthetic organisms, that there is a fourth class comprising multidomain proteins containing in their N-terminal part one Grx module with atypical active sites (essentially, CRDC in higher plants and CPHC in algae) (Couturier *et al.*, 2009b). Moreover, genome analysis of cyanobacterial genomes identified two new Grx classes composed of multidomain proteins containing a Grx domain with atypical active site, either CP[W/Y]G in class V, or CPWC/S in class VI (Couturier *et al.*, 2009b). With some exceptions, Grxs from other kingdoms fit well with the above-defined classification.

2. Redox potential and mechanism

Basically, whatever the organism considered, half of the Grxs possess a CxxC active site whereas the remaining sequences have a CxxS active site. Hence, this sequence property makes Grxs very efficient in reducing glutathionylated proteins (the topic of the chapter by Gao and colleagues in this volume). Only in a few cases, has it been shown that Grxs reduce intra- or intermolecular disulfide bonds on target proteins. For the reduction of protein-glutathione adducts, two distinct mechanisms have been elucidated, a monothiol or a dithiol one. In both cases, the N-terminal cysteine of the active site is employed for reducing mixed disulfides between glutathione and the target protein.

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The striking difference between the two mechanisms is the involvement of a second cysteine for solving the intermediate disulfide bridge in the case of dithiol mechanism, whereas a glutathione molecule is used for solving the intermediate disulfide bridge in the case of monothiol mechanism. Until recently, the Grxs were considered as glutathione (GSH)-dependent oxidoreductases, but the identification of some Grxs in organisms lacking glutathione, especially in Gram negative bacteria, the identification of fusion proteins between FTR and Grx modules and the biochemical evidence that some Grxs from various sources are reduced by NADPH- or ferredoxin-dependent thioredoxin reductases have moderated this assumption (Jacquot *et al.*, 2009; Johansson *et al.*, 2004; Jordan *et al.*, 1997; Reynolds *et al.*, 2002; Zaffagnini *et al.*, 2008). These observations also suggest that Grxs derived from a Trx ancestor by the acquisition of a glutathione binding site, but some of these Grxs, although possessing the necessary residues, kept the possibility to be regenerated by thioredoxin reductases. This is exemplified by the human Grx2, which is reduced both by GSH and NTR, depending on the protein oxidation state that has been generated during its catalytic cycle (Johansson *et al.*, 2004). A redox potential can be measured for Grxs with two cysteine residues involved in the formation of a disulfide bridge. This led to the conclusion that Grxs are less efficient reductants than Trxs as they possess redox potential values around − 170 mV at pH 7 for poplar GrxC4 and GrxC1, compared to values around − 300 mV for thioredoxins (Rouhier *et al.*, 2007b). In the case of a class II Grx (*C. reinhardtii* Grx3 possessing a CGFS active site), a disulfide bond is formed during its catalytic cycle between the active-site cysteine and a cysteine positioned in the C-terminal part. A lower redox potential has been measured for this protein (around − 323 mV at pH 7.9), explaining that this protein is reduced by FTR but not by glutathione (Zaffagnini *et al.*, 2008). Assuming this is a two-electron process, the value at pH 7, extrapolated from the one at pH 7.9, would be − 269 mV, still much more negative than those determined for classical Grxs with regular dithiol active sites.

3. Subcellular localization and function

There are multiple Grx isoforms localized in different compartments, such as the cytosol, chloroplast, mitochondria, nucleus, and probably apoplasm (Rouhier *et al.*, 2008). The functions of Grxs in plants have been reviewed recently and are detailed in the chapter by Li and Zachgo. Basically, these proteins emerged as stress response proteins involving either their specific deglutathionylation activity or not. They are involved in some developmental processes such as petal development and most likely participate in iron sensing and/or iron sulfur biogenesis (reviewed in Rouhier *et al.*, 2008). Indeed, they are able to reduce dehydroascorbate and are involved in the

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regeneration of thiol-dependent antioxidant enzymes, especially type II peroxiredoxins and methionine sulfoxide reductases (Msr) (Rouhier *et al.*, 2001, 2002, 2004b, 2007a; ; Tarrago *et al.*, 2009; Vieira dos Santos *et al.*, 2007). Normally, they do not reduce the same Msr and Prx classes than Trxs.

4. 3D structure of Grxs

Grx structures from various organisms have been solved both by NMR and X-ray crystallography. In general, most X-ray determined structures have been obtained with mutated proteins on the second active-site cysteine which allowed the irreversible binding of a glutathione molecule on the first active-site cysteine, most likely stabilizing the protein. The solved Grx structures (Grx1, 2, 3, and 4 from *E. coli*, pig, and human Grxs, Grxs from viruses) generally indicate a monomeric organization and the arrangement of secondary structures is quite similar to Trx structures, with β-strands forming a β-sheet flanked by several α-helices. Interestingly, some eukaryotic Grxs possess two supplementary α-helices in the N- and C-terminal regions compared to most prokaryotic Grxs. Several of the nonphotosynthetic 3D structures of Grx are discussed in the following reviews (Fernandes and Holmgren, 2004; Qin *et al.*, 2000). To date, three structures of plant Grxs (poplar GrxC1, C4, and S12) have been solved, GrxC1 and GrxC4 existing in a monomeric and a dimeric form, whereas GrxS12 exists only in the monomeric form (Figs. 4 and 5) (Couturier *et al.*, 2009a; Feng *et al.*, 2006; Noguera *et al.*, 2005; Rouhier *et al.*, 2007b). These different studies have notably demonstrated that dimeric GrxC1 bridges an iron sulfur cluster, whereas dimeric GrxC4 cannot ligate such a cluster due to the presence of an active-site proline residue (Glycine in GrxC1). The negative impact of another conserved *cis* proline residue for ISC incorporation into disulfide oxidoreductases has also been described by Su and colleagues (Su *et al.*, 2007). Some differences exist between the dimers of GrxC1 and GrxC4, since in GrxC4 the monomers are arranged in a head-to-tail orientation, while in GrxC1 the monomers are in a mirrored conformation. We discuss in more detail in the next section the arrangement of the iron sulfur cluster insertion in glutaredoxins as it is relevant to the function in iron sulfur assembly and to the CXXC motif described throughout this chapter.

IV. EARLY EXPERIMENTS SUGGESTING A LINK BETWEEN IRON SULFUR ENZYMES AND REDOXINS

Early experimental evidence for a connection between the thioredoxin world and proteins of the iron sulfur world originated from the laboratory of Jacques Meyer. As explained above Meyer and colleagues have isolated an

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Fig. 5. RMN structure of *Populus tremula* GrxC4 exhibiting a CPYS active site (Noguera *et al.*, 2005). The sulfur atom belonging to cysteine 27 is shown as a sphere (green in the web version).

unusual ferredoxin from *Aquiflex aeolicus* that they have named ferredoxin of the third kind. Instead of having an architecture similar to traditional ferredoxins, this protein incorporates a [2Fe–2S] center in a thioredoxin-like molecule which forms dimers, each monomer containing an ISC (Fig. 2; Yeh *et al.*, 2000, 2002). Similar proteins have been identified in *C. pasteurianum*, *A. vinelandii*, and *C. tepidum* (Meyer, 2001). These studies have been instrumental in indicating that a thioredoxin fold could incorporate an ISC.

That thioredoxin was able to incorporate metals was also recognized in several other instances. In 2003, Collet and colleagues have observed that *E. coli* thioredoxin 2, an elongated form of thioredoxin, can bind zinc with a high affinity. Compared to traditional thioredoxins, Trx2 contains an

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N-terminal extension with two CxxC motifs (CTHC and CGRC) that provide the zinc binding site (Collet *et al.*, 2003). The presence of this zinc center influences the reactivity and redox properties of the protein (El Hajjaji *et al.*, 2009).

Another approach that has led to the incorporation of metals in thioredoxin was through rational design and site-directed mutagenesis. In a pioneering work, Hellinga and colleagues have introduced a series of mutations in *E. coli* thioredoxin allowing the insertion of metals as copper or mercury (Hellinga *et al.*, 1991). Likewise, the substitution of W28 and I75 in *E. coli* thioredoxin into cysteines has led to the creation of a rubredoxin-like center with one iron atom linked to four cysteines (Benson *et al.*, 1998). Similar strategies have led to the creation of a mononuclear Cys2His2Zinc binding site in *E. coli* thioredoxin (Wisz *et al.*, 1998) and to the incorporation of a variety of transition metals with possible gain of enzymatic function (e.g., superoxide dismutase activity linked to zinc insertion) (Benson *et al.*, 2000).

In the present context of discussing the CXXC motif in redoxins and iron sulfur proteins, the most significant achievement has been the introduction of a [2Fe–2S] into *E. coli* thioredoxin by manipulating the WCGPC active-site sequence and altering it into a WCACA active site (Fig. 6.) (Masip *et al.*, 2004). This has been achieved by random mutagenesis and the structure of the CACA mutant has suggested that an exposure of a second cysteine (it is generally buried) allows the bridging of a 2Fe–2S cluster into a homodimer, the sulfur atoms of the two cysteines of each subunit serving as ligands to the iron atoms (Collet *et al.*, 2005).

Except for the bacterial ferredoxins of the third kind, all of the experiments described above create *de novo* artificial metal-containing thioredoxins by rational design and site-directed mutagenesis and it was unclear whether these manipulations were related to the ability of molecules with a thioredoxin fold to bind ISCs *in vivo*.

V. GLUTAREDOXINS BIND ISCs

The simultaneous observation that two glutaredoxins, one human with a SCSYC active-site sequence and the other from poplar with a YCGYC active site sequence, were able to bind [2Fe–2S] clusters *in vitro* and *in vivo* was a remarkable discovery (Feng *et al.*, 2006, Lillig *et al.*, 2005; Rouhier *et al.*, 2007b). Site-directed mutagenesis, together with the elucidation of the 3D structure of the two proteins, has indicated that the ISC is inserted at the interface of dimers with only the first Cys of the active site being a ligand. Remarkably, in the two structures, the additional ligands are two external

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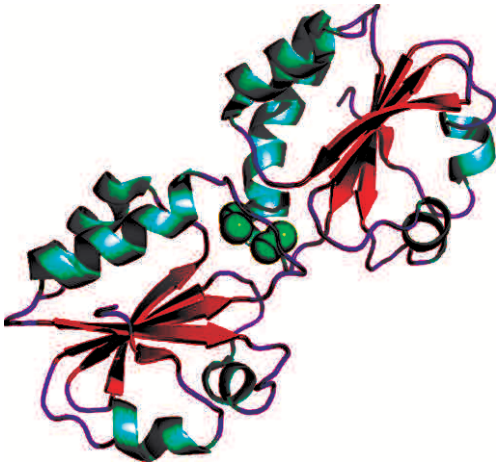

Fig. 6. Crystal structure of the *E. coli* Trx A CACA mutant dimer (Collet *et al.*, 2005; pdb 1ZCP). Sulfur atoms belonging to protein cysteine residues 32 and 34 are shown as green spheres.

glutathione molecules situated in *trans* with respect to the ISC (Fig. 7.; Johansson *et al.*, 2007; Rouhier *et al.*, 2007b). Further investigations with poplar Grxs (GrxC1, YCGYC active site; GrxC2, YCPFC active site; GrxC3, YCPYC active site; GrxC4, YCPYC active site; and GrxS12, WCSYS active site) have established that the ISC assembly in these structures requires a Tyr or another small amino acid residue before the first Cys residue and a small amino acid (Gly or Ser but not Pro) after the first Cys of the active site. On the other hand, the second Cys of the active site is dispensable, and as a consequence, Grxs with a CGFS active site sequence could accommodate an ISC in a dimer as poplar GrxC1 (Rouhier *et al.*, 2007b). This finding is remarkable in many respects, (i) it shows that glutaredoxins which possess a thioredoxin fold are able to incorporate an ISC, thereby providing a link between disulfide oxidoreductases and iron sulfur proteins; (ii) it indicates that the prediction of the binding of an ISC to a given polypeptide is complex. So far, it has essentially been based on sequence comparisons and the presence of CXXC motifs in proteins or conserved Cys and His for the Rieske type ISC. The possibility for CGFS Grxs to incorporate ISC in a homodimer with external glutathione molecules as ligands would have been impossible to predict in this context; (iii) it brings forth a hypothesis that glutaredoxins of the CGFS type can actually be involved in reactions related to iron homeostasis rather than in redox reactions. Many CGFS Grxs (*E. coli* Grx4, yeast Grx5 and, *A. thaliana* and polar GrxS14 and S16) characterized so far are indeed able to bind a labile

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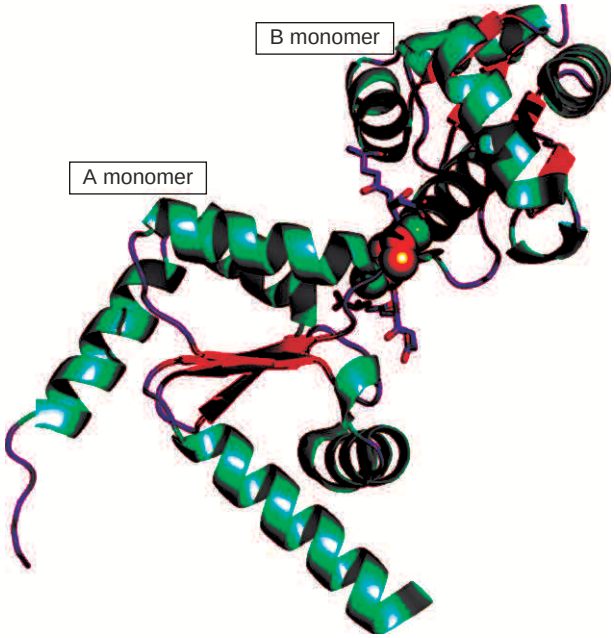

Fig. 7. Crystal structure of the holo form of glutaredoxin C1 from *Populus Tremula X Tremuloides* (Rouhier *et al.*, 2007b; pdb 2E7P). Sulfur atoms belonging to protein cysteine residues 30 and 33 of each monomer (A and B) are shown as green spheres, iron atoms as red, labile sulfur atoms as yellow and glutathione in stick.

[2Fe–2S] cluster when overexpressed in *E. coli*, presumably, as in GrxC1, with the catalytic cysteines and external glutathione molecules, but some differences in the spectroscopic signature of these proteins suggest that the cluster environment might be different (Bandyopadhyay *et al.*, 2008; Picciocchi *et al.*, 2007). No structure for ISC-containing CGFS Grxs has been solved yet. The recent biochemical and structural characterization of the *A. thaliana* CNFU scaffold protein indicated that the protein, similar to Grxs, bridged an all cysteinyl-ligated and labile [2Fe–2S] cluster into a dimer but that the ligands are two cysteines of each monomer comprised in a CXXC motif (Yabe *et al.*, 2008).

The study of Grxs from various sources led to the characterization of other ISC-containing Grxs, as the yeast Grx6 or the *Trypanosoma brucei* Grx1 (Comini *et al.*, 2008; Mesecke *et al.*, 2008). Considering that different Grxs, located in various subcellular compartments, can incorporate ISCs with variable stabilities, different putative roles have been proposed. First, based on the observation that GSH can stabilize the cluster in human Grx2 and poplar GrxC1, it has been proposed that the cluster might, similar to some

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bacterial ISC-containing transcription factors, serve as a redox sensor, which would respond to cellular variation in the GSH/GSSG ratio (Lillig *et al.*, 2005; Rouhier *et al.*, 2007b). Alternatively, these ISC-containing Grxs could sense the iron status of a given compartment or of the cells. It has been shown in yeast that Grx3 and 4 can regulate the iron regulon in response to the cellular iron status through their interaction with the transcriptional activator Aft1 (Kumánovics *et al.*, 2008; Ojeda *et al.*, 2006). Last but not least, additional evidence indicates that CGFS Grxs participate in the ISC assembly (see Section VI).

VI. GLUTAREDOXINS HELP TRANSFER ISCs IN APOPROTEINS

The first evidence for a role of Grxs in ISC assembly came from the study of the yeast null *grx5* mutant strain (Rodríguez-Manzaneque *et al.*, 2002). In this mutant, the activity of two mitochondrial enzymes requiring iron sulfur clusters, aconitase and succinate dehydrogenase, was considerably decreased, whereas the content and activity of cytochrome *c*, a heme-containing protein is not affected. Other deficiencies include inability to grow in respiratory conditions and iron accumulation in their mitochondria. The latter observation might be responsible for the hypersensitivity of this Δ*grx5* mutant to external oxidants. It has been proposed later, still in yeast, based on the accumulation in this mutant of high amount of Fe/S clusters bound to the Isu1p scaffold protein that this mitochondrial Grx5 would help in the transfer of preassembled clusters from scaffold to acceptor proteins (Muhlenhoff *et al.*, 2003). With a few exceptions, almost all CGFS Grxs, either from prokaryotes or eukaryotes, are able to complement the yeast Grx5 deleted strain, suggesting that they could play similar roles in their own cells (Bandyopadhyay *et al.*, 2008; Molina *et al.*, 2004; Molina-Navarro *et al.*, 2006). The presence of a labile ISC in many CGFS Grxs and the possibility of the poplar chloroplastic monothiol Grx, GrxS14, to efficiently transfer intact [2Fe–2S] cluster to chloroplastic *Synechocystis* apoferredoxin, then supported the view that CGFS Grxs could also act directly as scaffold proteins (Bandyopadhyay *et al.*, 2008). The fact that GrxC1, which contains a more stable cluster and a different active site, was not able to transfer its cluster suggests that only Grxs with CGFS active sites and containing a labile cluster could be involved in ISC biogenesis (Bandyopadhyay *et al.*, 2008). The presence of CGFS Grxs in all cellular compartments (chloroplast, mitochondria, cytosol), where an ISC assembly machinery exists, supports the hypothesis that the function of these Grxs is conserved. Nevertheless, as

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many scaffold proteins of the SUF, NFU, or ISC types have already been characterized in chloroplasts and mitochondria, one may wonder what the roles of these Grxs would be (for an exhaustive list, see Balk and Lobreaux, 2005). In addition, it is not known whether other Grxs with less classical active sites can also incorporate a cluster, whether all Grxs containing a labile cluster can transfer it to various acceptor proteins. Clearly, there is a pressing need to investigate if Grxs can transfer their cluster to other acceptor proteins and if some specificity exists for these reactions, both *in vitro* and *in vivo*.

VII. CONCLUDING REMARKS

At first sight, disulfide oxidoreductases and iron sulfur proteins have little in common, for example, the 3D architecture of ferredoxin is radically different from those of thioredoxin, both having a pleated β-sheet, central in thioredoxin but in the back of the molecule for ferredoxin and the arrangement of the α-helices is completely different. Nevertheless, they have in common to possess CxxC motifs that participate either in disulfide/dithiol exchange (thioredoxin) or in ISC binding (ferredoxin). The recent advances made with glutaredoxins in animal, plant, and bacterial systems have finally firmly connected these two separate worlds. The observation that some glutaredoxins bind ISCs in a way that has never been observed before for other ISC-containing proteins, together with the capacity to transfer those centers with high efficiency to apoferredoxin have absolutely transformed our vision of this research domain. Much remains to be done, however, for example, it is not known if Grxs are able to bind ISCs different from the [2Fe–2S] type. Can they bind [4Fe–4S] centers for example? Would they be able to transfer those? What about more complex proteins such as nitrogenase that contains both iron sulfur and iron molybdenum centers? All these questions are still unanswered and much remains to be done to further clarify the role that glutaredoxins play in ISC synthesis and assembly.

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V Les organismes d'étude:

A *Populus trichocarpa*

L'espèce *Populus trichocarpa* (ou peuplier de l'ouest) est une des principales essences forestières françaises. Appartenant à la famille des *Salicaceae*, elle est intéressante pour ses qualités physiques et mécaniques, mais également en terme de rentabilité due à sa croissance rapide. Elle est aussi intéressante pour la création et l'étude de plants transgéniques, certaines espèces de peuplier étant facile à cultiver *in vitro,* régénérer rapidement à partir de cals ou de boutures, et pouvant être transformables par *Agrobacterium tumefaciens*. De plus, le génome de l'espèce *Populus trichocarpa* a été totalement séquencé (Tuskan *et al*., 2006). Il est composé de presque 500 millions de paires de bases réparties sur 19 chromosomes et plus de 45000 gènes codant des protéines ont été identifiés. De ce fait, le peuplier est devenu le modèle d'étude végétal principal de notre UMR et de nombreux autres laboratoires à travers le monde.

B *Arabidopsis thaliana*

Arabidopsis thaliana, également appelé arabette des dames, est une plante appartenant à la famille des Brassicacées. Elle est utilisée comme organisme modèle pour la recherche par de très nombreux laboratoires, aussi bien en génétique qu'en physiologie. En 2000, elle a été la première plante à avoir son génome entièrement séquencé. Ce choix comme organisme modèle s'explique aisément par les nombreux avantages qu'elle présente, y compris par rapport au peuplier. Elle est de petite taille et donc on peut cultiver de nombreux plants pour un minimum de surface. Son cycle de développement est court, environ deux mois, de plus, un seul plant produit plusieurs

milliers de graines. C'est un des plus petits génomes connus chez les végétaux : 157 millions de paire de bases sur 5 chromosomes pour environ 25000 gènes. De plus, des banques de mutants d'insertion sont disponibles et sa transformation est maîtrisée.

VI Présentation du travail de recherche :

L'équipe « Réponse aux stress et régulation redox » dirigée par le Professeur Jean-Pierre Jacquot s'est spécialisé dans l'étude des systèmes de régulation redox chez les plantes. Cette régulation est essentielle pour la plante puisque de très nombreux processus physiologiques semblent dépendre à un moment ou un autre de ce type de régulation post-traductionnelle. Il est maintenant clairement reconnu que, par rapport à d'autres organismes tels que les mammifères, bactéries ou levures, les plantes constituent un système complexe pour l'étude de ces phénomènes puisque ces protéines sont codées par des familles multigéniques plus larges que chez les autres espèces vivantes. Ainsi, de nombreuses études sont consacrées à la caractérisation des différentes protéines, pour comprendre pourquoi une telle variété. C'est ainsi que dans le cadre de ma thèse, j'ai étudié en premier lieu certaines protéines de la famille des peroxyrédoxines, en particulier les Prx IIE et IIF présentes respectivement dans les chloroplastes et les mitochondries et qui n'avaient pas encore été caractérisées chez le peuplier. Des études biochimiques se sont concentrées sur l'étude du mécanisme catalytique utilisé par ces enzymes en se focalisant en particulier sur les différents substrats et réducteurs potentiels que pouvaient utiliser ces protéines. Par ailleurs, l'expression de ces protéines en condition physiologiques « normales » et en conditions de stress a également été analysée afin d'obtenir une vision plus fonctionnelle. Ainsi, dans le but de réaliser une étude la plus exhaustive possible, il a été nécessaire de produire un grand nombre des

réducteurs potentiels, appartenant notamment à la famille des Grxs, puisque les Prx de type II semblaient préférentiellement utiliser ce système réducteur. Je me suis naturellement tourné au cours de la thèse vers la production des Grx de classe II, GrxS14, S15 et S16, qui étaient prédites comme étant localisées soit dans la mitochondrie, soit dans le chloroplaste. La production des protéines recombinantes dans *E. coli* a permis de mettre en évidence l'incorporation de centres Fe-S labiles dans certaines de ces protéines (GrxS14 et S16). La caractérisation biochimique et spectroscopique des formes apo et holo de ces protéines, la détermination de leur localisation subcellulaire a constitué une part importante de ce travail de thèse pour comprendre le rôle de ces protéines dans les plantes. Par ailleurs, l'observation que certaines de ces Grxs pouvaient transférer leur centre Fe-S vers des protéines acceptrices ou qu'elles pourraient transférer des centres pré-assemblés sur des protéines scaffold, nous a conduit à également produire un certain nombre d'autres métalloprotéines pour des expériences de transfert.

RESULTATS

Résultats

I Etude des Peroxyrédoxines de type II de peuplier

A Construction et propriétés catalytiques de protéines de fusion artificielles

Des protéines hybrides naturelles, et composées d'un module Prx de type II du côté N-terminal et d'un module Grx du côté C-terminal, appelées PrxGrx sont retrouvées dans de nombreuses bactéries pathogènes et cyanobactéries. Ces fusions naturelles constituent un système analogue à celui décrit chez le peuplier pour deux enzymes isolées, la Prx IIB et la Grx C4 (Rouhier *et al*., 2001). Les deux systèmes sont en effet capables de réduire le peroxyde d'hydrogène mais également des alkyles ou des peroxydes de lipides, en présence de glutathion ou d'un analogue de glutathion, le glutathion amide (Cha *et al*., 2004; Pauwels *et al*., 2003; Rouhier *et al*., 2002; Rouhier *et al*., 2001; Rouhier and Jacquot, 2003; Vergauwen *et al*., 2001). La résolution de la structure 3D de la protéine d'*Haemophilus influenzae* par cristallographie a montré que l'enzyme forme un tétramère avec des interfaces entre les modules Prx, les modules Grx et également entre les modules Prx et Grx (Kim *et al*., 2003). Pour voir si on pouvait former des enzymes catalytiquement actives et ainsi obtenir des informations sur les interactions moléculaires entre ces partenaires, nous avons, à partir de ce modèle, fusionné les gènes codant la Prx IIB et les Grx C4 et Trx h3 de peuplier, puis produit dans *E. coli* plusieurs types de protéines de fusion artificielles entre ces protéines, en utilisant la séquence de liaison APDWKA(QE) que l'on avait identifiée dans l'enzyme de *Neisseiria meningitidis* (Rouhier and Jacquot, 2003). La Trx h3 ayant également été identifiée comme un réducteur potentiel de cette Prx II, nous avons ainsi construit un hybride composé du module Prx et du module Trx h3 appelé PrxTrx (PT) et deux hybrides où le module Prx II est fusionné au module GrxC4 et appelés PrxGrx1 (PG1) et PrxGrx2 (PG2). Dans le cas de l'hybride PG2, la séquence codant la première hélice α de la Grx C4 a

été éliminée pour mimer le module Grx bactérien naturellement plus court. Des études de dichroïsme circulaire et des expériences de filtration sur gel nous ont permis de confirmer que les protéines (qui étaient solubles) possèdent une structure repliée et que leurs masses apparentes correspondaient à celles de dimères. Les propriétés enzymatiques de ces 3 fusions ont été mesurées à l'aide de trois types de tests : la réduction de l'insuline, du déhydroascorbate (DHA) et de peroxydes.

Enfin, grâce à des études par spectrométrie de masse, nous avons pu mettre en évidence que le module Prx pouvait être glutathionylé *in vitro* en présence de GSSG mais pouvait certainement aussi être suroxydé. Cette suroxydation semblait à l'époque plutôt spécifique des Prx 2-Cys (Biteau *et al*., 2003; Georgiou and Masip, 2003; Woo *et al*., 2005).

Ces résultats ont été publiés dans l'article suivant: Article 2: " Engineering functional artificial hybrid proteins between poplar peroxiredoxin II and glutaredoxin or thioredoxin"

I Etudes des peroxyrédoxines de type II, la Prx IIF mitochondriale et la Prx IIE chloroplastique de peuplier

A Construction et propriétés catalytiques de protéines de fusion artificielles entre des modules Prx et Trx ou Grx

Article 2 : “Engineering functional artificial hybrid proteins between poplar peroxiredoxin II and glutaredoxin or thioredoxin”

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Engineering functional artificial hybrid proteins between poplar peroxiredoxin II and glutaredoxin or thioredoxin ☆

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Abstract

The existence of natural peroxiredoxin–glutaredoxin hybrid enzymes in several bacteria is in line with previous findings indicating that poplar peroxiredoxin II can use glutaredoxin as an electron donor. This peroxiredoxin remains however unique since it also uses thioredoxin with a quite good efficiency. Based on the existing fusions, we have created artificial enzymes containing a poplar peroxiredoxin module linked to glutaredoxin or thioredoxin modules. The recombinant fusion enzymes folded properly into non-covalently bound homodimers or homotetramers. Two of the three protein constructs exhibit peroxidase activity, a reaction where the two modules need to function together, but they also display enzymatic activities specific of each module. In addition, mass spectrometry analyses indicate that the Prx module can be both glutathiolated or overoxidized in vitro. This is discussed in the light of the Prx reactivity.
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Keywords: Glutaredoxin; Hydroperoxide; Peroxiredoxin; Poplar; Thioredoxin

In plants, the thioredoxin peroxidase family includes four types of peroxiredoxins (Prx), called 1-Cys Prx, 2-Cys Prx, Prx Q, and Prx II, and many glutathione peroxidase (Gpx) isoforms, which use thioredoxin (Trx) as an electron donor instead of glutathione (GSH) [1–4]. All these groups differ by the number and the position of conserved catalytic cysteines [1]. The Prx II class is unique as it is able to use glutaredoxin (Grx) and sometimes Trx, although with a lower efficiency, as electron donors [5–7]. Grx and Trx are small disulfide reductases involved in dithiol–disulfide exchange using a catalytic CxxC/S active site, but Grxs possess also a more specific function in glutathiolation/deglutathiolation processes using only the first of the two active site cysteines [8]. Prxs II possesses two cysteines, the so-called peroxidatic cysteine, common to all Prxs and a putative resolving cysteine, 25 amino acids downstream, which is not conserved in all plant sequences. Mutagenesis studies indicated that the peroxidatic cysteine of Prx II and only the first cysteine of the Grx active site are absolutely essential [7], suggesting that either Grx or GSH is able to directly attack the sulfenic acid formed on the peroxidatic cysteine of Prx II during catalysis.

Interestingly, natural fusion proteins, found in many bacteria, are constituted of Prx II followed by Grx modules. These enzymes are able to use GSH or sometimes glutathione amide as electron donor and various oxidized substrates; i.e., hydrogen peroxide or various alkyl and lipid hydroperoxides as electron acceptor [9–12]. The three-dimensional structure of the fusion found in *Haemophilus influenzae* has been obtained by cristallography and indicates that the enzyme is a tetramer [13], in which each

☆ *Abbreviations:* DHA, dehydroascorbate; DTT, dithiothreitol; Grx, glutaredoxin; Prx, peroxiredoxin; Trx, thioredoxin.

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monomer is constituted of Grx and Prx subdomains connected by a flexible linker. The different subunits are linked together mostly through electrostatic interactions implicating Prx–Prx, Grx–Grx, and Prx–Grx subdomain interactions. Moreover, Kim and coworkers have identified some amino acids responsible for the Prx–Grx interaction.

In order to get a better understanding of the structural determinants of the interactions between these enzymes, we have engineered and purified artificial hybrid proteins containing poplar PrxII and two identified donors, poplar Grx C4 or poplar Trx h3. These three protein components were taken from the same plant (poplar) where they are located in the same cellular compartment (cytosol) and, in addition, we have shown that they function biochemically well together. All the chimeric recombinant proteins are soluble, correctly folded and active, but display sometimes different efficiencies compared to the isolated recombinant proteins. In addition, the Trx and Grx modules are active in the fusion enzymes as judged by specific activity tests.

Materials and methods

Plasmids and strains

Plasmids used for expression in *Escherichia coli* BL21(DE3) were pET-3d and pSBET which carry the resistances for ampicillin and kanamycin, respectively [14]. The cloning strain was *E. coli* DH5α.

ORF cloning in fusion

The method used to fuse the two proteins is similar to the PCR site-directed mutagenesis strategy. It is based on successive PCR amplifications, a first step is to amplify two overlapping DNA fragments, which are then used in a second step to synthesize the full-length open reading frame. Three different constructions, which encode artificial fusions between poplar Prx II and poplar Grx C4 or Trx h3 modules (respective GenBank Accession Nos.: AAL90751, AAL90750, and BU822062), have been engineered [6,15,16]. These various constructions differ in particular by the structure of the linker sequence. This linker, identical to the one of *Neisseria meningitidis* PrxGrx sequence (GenBank Accession No.: NP_273984), contains the sequence **APDWKA** for PrxGrx1 (PG1) and PrxTrx (PT) or **APDWKAQES** for PrxGrx2 (PG2) (Table 1 and Fig. 1). The two PrxGrx constructions also differ from one another by the N-terminal sequence of the Grx module introduced. PG1 possesses the entire poplar Grx C4 sequence (linker in bold and Grx sequence underlined) (**APDWKA**AGSPEA), whereas PG2 contained a shortened N-terminus part of the Grx module, mimicking the size of the *N. meningitidis* Grx sequence (**APDWKAQES**IVIFSK). Cloning and fusion oligonucleotides are described in Table 1. The amplification of the Prx II module including the linker sequence was the same for the three constructions and was done using the primers called Prx II for and Prx(G)Trx rev. The second overlapping fragment is different in the three constructions and necessitated the use of primers PrxTrx for and Trx h3 rev for the PrxTrx construction, PrxGrx1 for and Grx C4 rev for the PrxGrx1 construction, and PrxGrx2 for and Grx C4 rev for the PrxGrx2 construction.

Expression and purification of the recombinant enzymes

Escherichia coli strain BL21(DE3) containing the helper plasmid pSBET [14] was transformed with the various recombinant plasmids. LB cultures of 2.6 L were grown at 37 °C and induced in exponential phase by 100 μM IPTG (isopropyl-β-D-thiogalactopyranoside). After 3 h induction, the bacteria were harvested by centrifugation for 15 min at 5000*g* and resuspended in buffer A (30 mM Tris–HCl, pH 8.0, 1 mM EDTA, and 200 mM NaCl). Bacteria were disrupted by sonication and centrifuged for 30 min at 30,000*g* to discard the insoluble fraction. The soluble part was then precipitated with ammonium sulfate successively up to 40% and 80% of the saturation.

PrxGrx1 precipitated mainly between 40% and 80%, whereas PrxGrx2 and PrxTrx between 0% and 40%. The fractions of interest were first separated onto an ACA 44 gel filtration column (Biosepra) equilibrated with buffer A, dialyzed and then onto a DEAE Sepharose column (Sigma) equilibrated with buffer A without NaCl. The proteins were eluted using a 0 to 0.4 M NaCl gradient, dialyzed again, concentrated, and finally stored in buffer A at −30 °C until further use. SDS–PAGE was used to check the homogeneity of the proteins.

Enzymatic assays

Insulin reduction. The reduction of insulin was measured at room temperature using a Cary 50 spectrophotometer (Varian) by following changes in absorbance at 650 nm, in 500 μL cuvettes containing 50 mM phosphate buffer, pH 7, 500 μM DTT, 8 U recombinant human insulin, and 5 μM of each protein tested.

Dehydroascorbate reduction. Dehydroascorbate (DHA) reduction (500 μM) was tested either in the presence of the Grx system (180 μM NADPH, 0.4 U glutathione reductase (GR), 500 μM GSH) for poplar GrxC4, PrxGrx1, and PrxGrx2 or in the presence of the Trx system (180 μM NADPH, 1 μM NADPH thioredoxin reductase (NTR)) for poplar Trx h3 and PrxTrx fusion. The reaction in 500 μL TE buffer (30 mM Tris–HCl, pH 8, EDTA 1 mM) was initiated after 1 min incubation by adding 50 nM to 1 μM enzyme.

Hydroperoxide reduction. Two different tests were used to measure hydroperoxide reduction activity of the various fusions. The first one is coupled to NADPH oxidation. When using the Trx reducing system, the reaction medium in 500 μL was the same as the one used for DHA reduction measurement except that DHA was replaced by 250 μM H_2O_2. Concentrations used were 5 μM of PrxTrx or 5 μM Trx h3 in combination with 5 μM Prx II. When the Grx reducing system (NADPH, 0.4 U GR and 1 mM GSH) was used, 500 μM *t*-BOOH was used instead H_2O_2 to

Table 1
Cloning primers

Name	Nucleotidic sequence	Amino acid sequence
Prx II for	5′ GGGGCCATGGCCCCGATTGCTGTTGGT 3′	MAPIAVG
GrxC4 rev	5′ GGGGGGATCCTTAAAAGTCATCTTTCTGCTC 3′	EQKDDF*
Trx h3 rev	5′ CCCCGGATCCTCAAGCAGAAGCAGTAGC 3′	ATASA*
PrxGrx1 for	5′ **GCTCCCGATTGGAAG**GCTGGCAGCCCTGAAGCT 3′	**APDWKA**AGSPEA
PrxGrx2 for	5′ **GCTCCCGATTGGAAGGCTCAAGAGTCT**ATCGTCATCTTCTCCAAG 3′	**APDWKAQES**IVIFSK
PrxTrx for	5′ **GCTCCCGATTGGAAG**GCTGCTGAAGATGGACAAGTG 3′	**APDWKA**AEDGQV
Prx(G)Trx rev	5′ AGCCTTCCAATCGGGAGCAAGATCCTTGAGGATATC 3′	DILKDL**APDWKA**

Cloning restriction sites, *Nco*I or *Bam*HI are underlined. In bold the linker sequence between Prx and Grx or Trx modules, either APDWKA for PrxGrx1 and PrxTrx fusions or APDWKAQES for the PrxGrx2 fusion.

* Corresponds to stop codons.

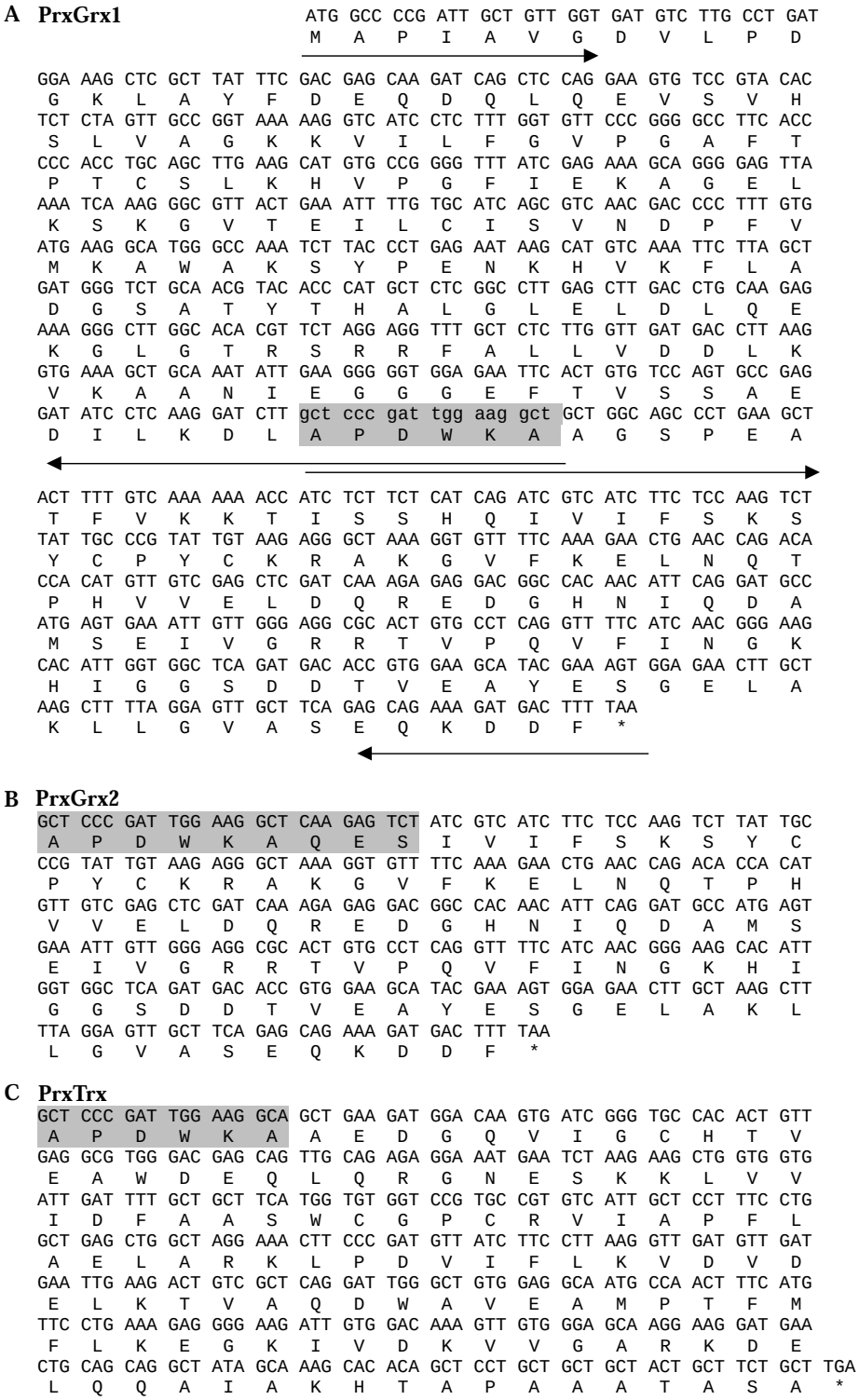

Fig. 1. Hybrid protein sequences. The linker sequence introduced was shaded for each fusion and oligonucleotides described in Table 1. (A) Artificial fusion protein (PrxGrx1) between poplar Prx II and GrxC4 modules. The entire Grx sequence was added downstream to the Prx II sequence using a linker sequence homolog to the one of the natural fusion enzyme from *Neisseria meningitidis*. Cloning primers are indicated by arrows. (B,C) C-terminal end of PrxGrx2 and PrxTrx, the Prx module until the linker sequence is similar to the one in PrxGrx1.

prevent direct reduction of H_2O_2 by ionised GSH. Concentrations used were 1 μM PG1 or PG2 and 1 μM GrxC4 plus 1 μM Prx II when using the separate catalysts. Background activity in these conditions was negligible.

In the second test used, H_2O_2 consumption was measured directly in the presence of DTT or GSH using the FOX (ferrous oxidation in xylenol orange) colorimetric detection method [17]. The 50 μL reaction medium contained 30 mM Tris–HCl, pH 8.0, 500 μM DTT or GSH, 500 μM H_2O_2, and 5 μM of enzymes. The reaction was stopped by adding 5 μL of the reaction mixture to 495 μL of the FOX reagent.

Conformational characterization

Circular dichroism spectra were recorded at 25 °C between 190 and 260 nm on a Jobin-Yvon CD6 spectro-dichrograph with a 2 s integration time for each 0.5 nm step and a bandwidth of 2 nm. Two spectra of each protein at a concentration of ca 50 μM in 10 mM Tris–HCl, 0.3 mM EDTA buffer were averaged and corrected from the baseline for buffer solvents. Analysis was performed using K2D program (http://www.embl-heidelberg.de/~andrade/k2d/) described by Andrade et al. [18].

Gel filtration chromatography experiments

The apparent molecular sizes of the three fusion proteins were determined by calibration against a standard curve generated using proteins of known molecular mass : chymotrypsinogen A, myoglobin, bovine serum albumin, and chicken egg ovalbumin (all from SIGMA) using an ACA 44 gel permeation chromatography (Biosepra).

In vitro glutathiolation and overoxidation experiments

In 50 μL reaction mixtures containing 30 mM Tris–HCl, pH 8.0, and 1 mM EDTA, 50 μM of PG1, PG2, PT, Trx h3, Prx II, and Grx C4 were incubated for 10 min with 1 mM DTT before adding 5 mM oxidized glutathione or 5 mM H_2O_2 for 30 min. Before Electrospray Mass Spectrometry analyses, samples were diluted 20-fold in 50:50 acetonitrile:0.1% formic acid solution. Acquisition of the electrospray ionization (ESI) mass spectra were performed using a Micromass Q-Tof Ultima (Waters Micromass MS Technologies) hybrid tandem mass spectrometer, equipped with a nanoflow electrospray source. The samples were infused into the mass spectrometer using nanoflow capillaries (Proxeon Biosystems, Denmark). The needle voltage was ~1800 V, and collision energy was 10 eV for MS analyses. Data analysis was accomplished with a MassLynx data system and Transform deconvolution software supplied by the manufacturer (Waters Micromass MS Technologies).

Results and discussion

Engineering artificial fusion proteins

One remarkable feature of poplar PrxII is its ability to use both Grx and Trx as electron donor for its regeneration. In order to study the structural interactions between poplar Prx and Grx or Trx, stable covalent interactions have been obtained, using cysteinic mutants of the active site of the different partners [7] but we were not able to further purify these heterodimers. Another approach was to characterize fusion enzymes naturally present in many bacteria. However, we only found PrxGrx but no PrxTrx prototypes. Here, we describe the construction of both PrxGrx or PrxTrx fusions using poplar enzymes. Based on the recent characterization of peroxiredoxin-glutaredoxin hybrid enzymes in several bacteria (*N. meningitidis*, *H. influenzae*, *Vibrio cholerae*, and *Chromatium gracile*) three different artificial fusion proteins between a poplar Prx II and two of its efficient electron donors, poplar Grx C4 or Trx h3 have been constructed by using a strategy described in Materials and methods. When comparing the fusion of *N. meningitidis* with the non-fused Prx and Grx, one detects the presence of a linker sequence APDWKA or even APDWKAQES when considering shorter bacterial Grx sequences as references [11]. Fig. 1 displays the three different hybrid sequences created here, called PrxGrx1 (PG1), PrxGrx2 (PG2), and PrxTrx (PT). The two different PrxGrx fusions (PG1 and 2) differ first by the length of the incorporated linker (APDWKA or APDWKAQES, respectively). Another difference is the length of the Grx module. Bacterial Grxs are generally shorter (75 to 90 amino acids) compared to eukaryotic sequences (110 to 140 amino acids). Thus, in PG1 the full-length poplar Grx sequence (109 amino acids) was introduced, whereas a shorter Grx (92 amino acids), mimicking bacterial Grxs, was introduced in PG2. In this shorter version, the first α-helix of poplar glutaredoxin was truncated (D'ambrosio et al., unpublished results). Indeed, most of the eukaryotic Grxs possess an additional α-helix in the N-terminus part, but it is not known whether it is required for biological activity. A PrxTrx fusion was also engineered by fusing the entire poplar Trx h3 sequence to Prx II and using the linker APDWKA. This construction is particularly interesting to understand the molecular contacts between Prx II and Trx since there are no homologous sequences in any database. Overall, the different constructions (PG1, PG2 and PT) contain respectively 276, 263, and 288 amino acids.

Purification and conformational organization

The three fusions were expressed in *E. coli* as folded and soluble proteins and purified to homogeneity with high yield (20, 35, and 73 mg protein can be obtained in a highly homogeneous state from 1 liter culture, respectively, for PT, PG2, and PG1). In order to determine whether these fusions were properly folded, they were analyzed by circular dichroism. Fig. 2 shows CD spectra recorded between 190 and 260 nm for each fusion. All spectra are indicative of structured proteins with a positive absorption around 195 nm and negative peaks around 208 and 222 nm.

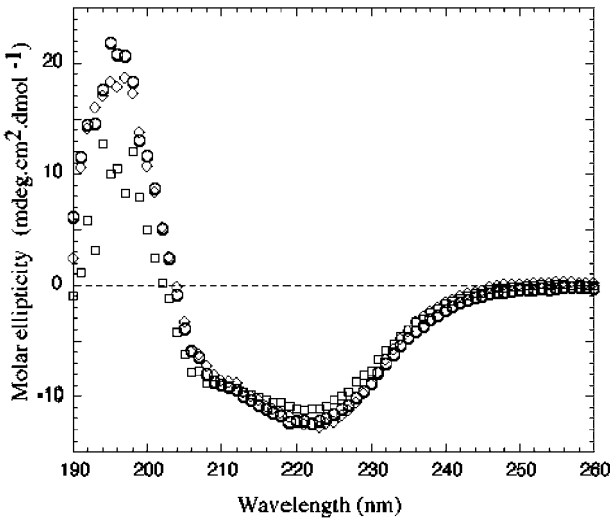

Fig. 2. Far UV circular dichroism spectra of the fusion proteins. PrxGrx1 fusion (open circles); PrxGrx2 fusion (open squares); PrxTrx fusion (open diamond). Spectra were recorded for each species at 5 μM and 25 °C. The negative absorption at 208 and 222 nm are indicative of structured species.

Deconvolution of each spectrum using K2d software is in agreement with the presence of more than a third of helical secondary structures in each protein. Gel filtration chromatography unambiguously indicates that in the absence of reductant, PG2 elutes as a tetramer, whereas PG1 and PT behave as dimers (Fig. 3). This result is very interesting when compared to the only known 3D structure of *H. influenzae* PrxGrx, which is a tetrameric protein either in reducing or oxidizing conditions [13]. Indeed, this indicates that the interactions between the subdomains in PT and PG1 are probably different.

In addition, previous reports on bacterial PrxGrx fusions are somewhat ambiguous. Indeed, the X-ray structure and analytical ultracentrifugation results clearly indicate that *H. influenzae* PrxGrx is a tetramer [13]. On the other hand, mass spectrometry and SDS–PAGE analyses demonstrated that *H. influenzae* PrxGrx exists either as a monomer in the glutathiolated form or as a dimer linked by a disulfide bridge [9,10]. In the same way, the PrxGrx fusions from *N. meningitidis* and *V. cholerae* generally exist as monomers under reducing or non-reducing conditions, but lines of evidences indicated that *V. cholerae* PrxGrx can also form an intramolecular disulfide bridge between the two cysteines of the Prx module [9,11]. In order to analyze the nature of the contacts within our oligomers, we performed SDS–PAGE separation both in reducing or non-reducing conditions (Fig. 4). The three hybrid proteins migrated as monomers under both conditions, indicating that these oligomers are not linked by disulfide bridges but more likely by charge interactions as described for *H. influenzae* PrxGrx [13].

Catalytic properties and overoxidation

The peroxidase activities of the fusions were measured and compared to those of the isolated modules mixed together. Using a spectrophotometric assay coupled to

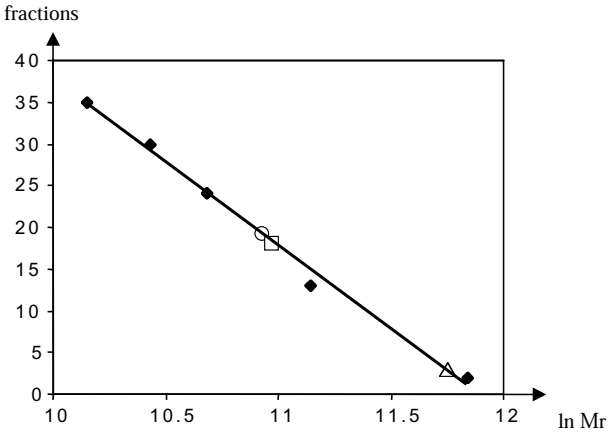

Fig. 3. Gel filtration analysis of the fusions. The molecular sizes of PG1 (open square), PG2 (open triangle), and PT (open circle) were determined using an ACA 44 exclusion size chromatography and proteins of known molecular weight (closed diamonds). Log of the molecular mass is reported as a function of the elution volume.

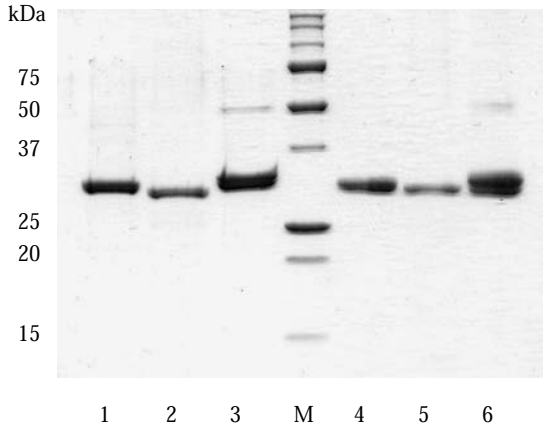

Fig. 4. Reducing or non-reducing SDS–PAGE of the recombinant proteins. 2.5 μg of PrxGrx1 (lanes 1 and 4), PrxGrx2 (lanes 2 and 5), and PrxTrx (lanes 3 and 6) were separated in reducing (10 mM DTT) (lanes 1–3) or non-reducing (lanes 4–6) conditions. M, molecular weight marker is the protein unstained standard marker from Bio-Rad. Band sizes (kDa) are indicated on the left.

NADPH oxidation, we measured PT activity in the presence of NTR (NADPH thioredoxin reductase). The initial velocity of PT is comparable to the one observed in the presence of the two isolated proteins, but the activity decreases rapidly, presumably because of a higher sensitivity to inactivation (Fig. 5A). Incidentally, these results indicate that NTR is able to recognize and access the Trx module within the fusion and that Trx is able to reduce the Prx module and subsequently to support its H_2O_2 reducing activity. We do not know presently if the electrons are transferred from Trx to Prx within a single fusion subunit, but the presence of a dimeric organisation rather suggests a head-to-tail organisation in which the Prx module could be reduced by a Trx from another subunit. PG2 was only slightly active within the range of concentrations tested, whereas initial velocity for PG1 was faster when compared to the activity obtained when mixing together the individual poplar proteins (Fig. 5B). Nevertheless, PG1 also seemed to be inactivated rather quickly by *t*-BOOH. One possibility to explain the inactivation is the formation of sulfinic or sulfonic acid intermediates. The formation of overoxidized Prx forms was checked by mass spectrometry after reaction with high concentrations (5 mM) of H_2O_2 (Table 2). After H_2O_2 treatment, the molecular mass of Prx II increased by 48 Da, a value which can be interpreted as resulting from the formation of a sulfonic acid. A similar mass increment was also found in PG1 and PT but not in PG2, in which the increase in mass was only about 32 Da (presumably arising from a sulfinic form). Although these overoxidized forms can also be formed on Prx II, it is likely that a lower rate of regeneration of Prx by the Trx or the Grx modules occurs, when engaged in the fusion. This suggests that the Prx sulfenic acid reduction or the subsequent steps are less efficient in the fusions compared to the isolated modules. Indeed, it has already been demonstrated for some enzymes (Prx or

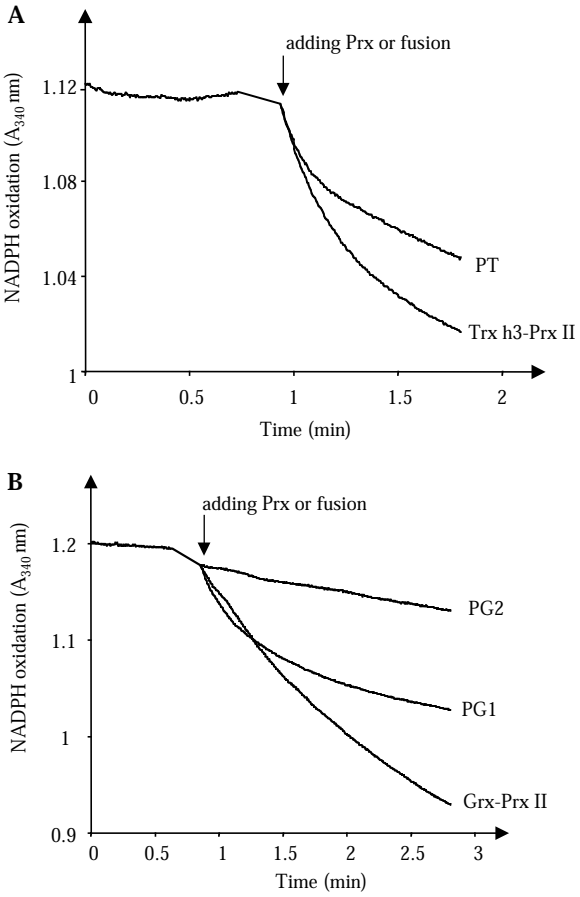

Fig. 5. Time course NADPH dependent reduction of peroxides. (A) Activity of PrxTrx (5 μM), or the isolated modules Trx h3 (5 μM), and Prx II (5 μM) in the presence of the Trx reduction system (180 μM NADPH, 1 μM NTR) and 250 μM H_20_2. (B) Activity of PrxGrx1 (1 μM), PrxGrx2 (1 μM), or the isolated modules GrxC4 (1 μM) and Prx II (1 μM) in the presence of the Grx reduction system (180 μM NADPH, 0.4 U GR, 500 μM GSH) and 500 μM *t*-BOOH.

methionine sulfoxide reductases) which use sulfenic acid chemistry for their catalysis, that the sulfenic acid reduction or rather the subsequent reduction steps by Trx are the rate-determining steps [19,20].

Fig. 6 shows the time-dependent H_2O_2 consumption by the three fusion enzymes using either DTT or GSH as reductants. With DTT as a donor, the reduction is independent of the Trx or Grx module and we thus measure the ability of the Prx module, either isolated or within the fusion, to directly reduce H_2O_2. All the constructions were able to reduce H_2O_2, but PG1 is more active than PG2 and PT which present similar efficiencies. Thus, either the Prx module of PG1 is more accessible to DTT or PG2 and PT are inactivated faster by H_2O_2. This second proposal is supported by the fact the peroxide reduction is not complete and tends to decline and stop with time.

With GSH as a donor, the reduction is dependent on the Grx module, and this test can thus provide valuable information concerning the low catalytic activity of PG2. PT is not able to reduce H_2O_2 using GSH as electron donor. This result was expected as GSH alone cannot support the activity of poplar Prx II and cannot be used by Trx h3 [7, unpublished results]. PG1 is almost as active with DTT or GSH, although the initial velocity is slower with GSH, but with elapsing time, the consumption of H_2O_2 is quite similar. As PG2 is only active using DTT but not GSH, the low efficiency of PG2 is likely to result from an impaired GSH recognition due to the first N-terminal α-helix deletion of Grx since the major difference between PG1 and PG2 is the presence or absence of this α-helix. Thus the low catalytic activity of PG2 observed in Fig. 2 is not due to a distorted Prx active site, which might have prevented the recognition of peroxides.

The catalytic activity of the Grx and Trx modules within the fusions was then investigated by using specific tests. Only poplar Trx h3 and PT were able to reduce insuline with similar efficiency (Fig. 7A), whereas poplar GrxC4, Prx II, PG1, and PG2 were inactive, indicating that the Trx module is fully active within the fusion. Alternatively, the activity of the Grx module can be evaluated using the DHA reduction test. In this case, PG1 is as efficient as Grx C4 alone (Fig. 7B), whereas PG2 is only slightly active at very high concentration (Fig. 7B and data not shown) and Trx h3 or PT were not active at all. These results again suggest that PG2 is unable to bind GSH and support catalysis.

In vitro glutathiolation experiments

In order to further investigate the catalytic mechanism of the Prx II and of the fusions and especially to test if the catalytic peroxidatic cysteine is glutathiolated or if Grx is able to directly attack the sulfenic acid, the glutathiolation state of the various proteins was tested in vitro and detected using mass spectrometry (Table 2). As expected, GrxC4 was able to bind one GSH, whereas Trx h3 does not bind any GSH, either on its two active site cysteines, or on its additional cysteine. All other enzymes (Prx II, PT, PG1, and PG2) were also able to bind GSH, although the reaction was not always complete since some non-modified proteins were still detected. For Prx II and PrxTrx, the results suggest that the catalytic cysteine of the Prx module is able to react with GSSG and thus become modified. For PG1 or PG2, the results seem to be in accordance with those described earlier for the *H. influenzae* PrxGrx [10]. Indeed, Pauwels and colleagues have demonstrated that the PrxGrx was glutathiolated on the peroxidatic cysteine whereas the two active site cysteines of the Grx module formed a disulfide bridge. This can be expected if the mixed-disulfide Prx-SG formed primarily is reduced by the first Grx active site cysteine making the catalytic cysteine available for another glutathiolation, whereas the Grx-SG adduct is reduced by the second cysteine, preventing additional reaction. The in vitro glutathiolation of the Prx module suggests that the sulfenic acid formed during Prx catalysis can be reduced by GSH, this GSH adduct being likely reduced by Grx. Nevertheless, if Trxs are the preferred electron donors in vivo or if Grxs are not

Table 2
Glutathiolation and hyperoxidation experiments

	Theoretical mass	DTT treated protein	GSSG treated protein	GSSG dependent mass increment	H_2O_2 treated protein	H_2O_2 dependent mass increment
PrxGrx1	29962	29762.4	29762		29810.6	48.2
			30066.8	304.2		
		29964.4 (203)	29964.6		30012.4	48
			30270.9	306.3		
PrxGrx2	28607	28408.3	28408.8		28440.9	32.6
			28714.2	305.4		
		28611.3(203)	28610.6		28643.3	32
			28917.2	306.6		
PrxTrx	31086	30886.3	30886.4		30934.5	48.2
			31190.6	304.2		
		31089.3 (203)	31089.6		31137.25	48
			31394.8	305.2		
PrxII	17358	17156.5	17156.4		17204.6	48.1
			17463.3	306.9		
		17358.9 (202.4)	17358.3		17407.2	48.3
			17664	305.7		
GrxC4	12526.1	12395.9 (130.2)	12700.4	304.5	n.d.	n.d.
Trxh3	13345	13142.9 (202.2)	13143	0	n.d.	n.d.

The theoretical mass was calculated from the primary nucleotide sequence. The analysis revealed that the initial N-terminus methionine is cleaved for Grx C4 (mass decrease of 130.22 Da), the methionine and an alanine are cleaved for Trx h3 (mass decrease of 202.22 Da), whereas we have a mixture of two populations for Prx II and thus for the three fusions, one without cleavage and a second with methionine and alanine cleaved (mass decrease around 203 Da). After reduction, around 50 μM proteins were treated with 5 mM GSSG or 5 mM H_2O_2 as described in the method section and analyzed by mass spectrometry. n.d., not determined.

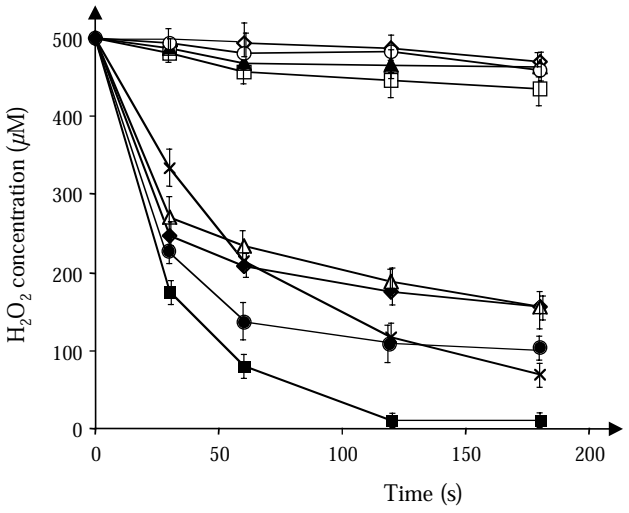

Fig. 6. DTT or GSH dependent reduction of H_2O_2 GSH dependent activity : (□) control, (○) PT, (×) PG1, (▲) PG2 or DTT dependent activity : (◊) control, (■) PG1, (♦) PG2, (●) Prx II, (△) PT. Five micromolar enzyme was used.

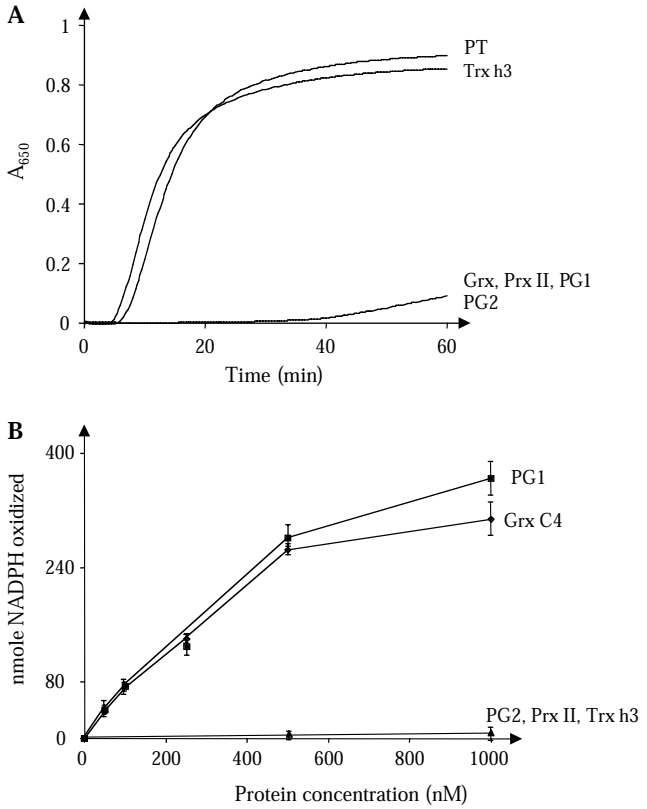

Fig. 7. Specific Trx or Grx activity tests. (A) Insulin reduction was followed at 650 nm in the presence of 500 μM DTT and the various proteins at 5 μM. (B) DHA reduction activity in function of protein concentration was measured by following NADPH oxidation at 340 nm in a coupled reaction system.

expressed in the same subcellular compartment, glutathiolation could be a way to transiently inactivate the protein but also to prevent overoxidation of the reactive cysteine.

Conclusion

These experiments were initiated to study the molecular contacts involved in Prx–Trx or Prx–Grx interactions, but

the prerequisite, for which we present evidence here, was to produce and purify folded and active enzymes. It is remarkable that all three chimeric constructions folded properly and could be purified to homogeneity with high yield. Out of the three chimeric enzymes, only the PT and PG1 constructs yielded enzymes that possessed catalytic efficiencies comparable to the combination of the individual poplar proteins. Our results suggest that in the PG2 construct, the truncation of the N-terminal part of the glutaredoxin module is responsible for the drop in activity of the fusion presumably because GSH recognition and binding is impaired. This observation strongly suggests that the N-terminus extension found in plant glutaredoxins and absent in many other species plays an important role in determining the cognate properties of this protein and is required for efficient catalysis. On the other hand, it is promising that the PrxTrx and PrxGrx1 fusions were correctly folded, active and organized as dimers rather than tetramers, which confirm that the molecular contacts between the partners should be different from those described in the known PrxGrx structure from *H. influenzae* [13]. In this enzyme, Prx–Prx, Prx–Grx, and Grx–Grx interfaces were described but the Prx module of one monomer is certainly reduced by the Grx module of another monomer. One question which thus remains to be elucidated is whether the electrons are transferred from the Trx or Grx modules to the Prx module within a monomer in electron channelling or not. Indeed, the dimeric organization could be suggestive of a head-to-tail orientation, in which the N-terminal Prx domain of one subunit is interacting with the C-terminal Trx or Grx domain of another subunit. Nevertheless, we cannot at this point rule out the possibility that the two N-terminal Prx domains and the two C-terminal domains face, and that the linker peptide is flexible enough to allow the two intra-chain domains to interact directly with one another. In order to solve this ambiguity, we are currently trying to solve the 3D structure of the artificial fusions. Finally, we also had evidence that Prx II can be glutathiolated or overoxidized as was already demonstrated for some eukaryotic Prx [21–24]. This kind of post-translational modification could thus represent a signalling process in plants as suggested in other organisms [25].

Acknowledgment

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B Etudes biochimiques et fonctionnelles des Prx IIE et IIF du peuplier

Au début de cette thèse, chez le peuplier, seule la Prx IIB, *a priori* cytosolique, avait fait l'objet d'études précises démontrant qu'elle pouvait être régénérée à la fois par des Trx de type h ou par des Grxs mais pas par le GSH seul (Rouhier *et al*. 2001) (Rouhier *et al*., 2002). Chez *A. thaliana*, la Prx IIB ne semblait pas être régénérée par les Trxs mais seulement par les Grxs alors qu'aucun donneur d'électron pour les Prx IIE (chloroplastique) et IIF (mitochondriale) n'avait été identifié (Brehelin *et al*., 2003). Un peu plus tard, il s'est avéré que le GSH était un réducteur efficace pour une Prx II de cyanobactérie (Hosoya-Matsuda *et al*., 2005) et pour la Prx IIF (Finkemeier *et al*., 2005), la présence de Grx permettant d'accroître l'efficacité de la réaction. La Prx IIF d'*A. thaliana* est également réduite par la Trx o mitochondriale alors que la PrxIIB ne peut pas être régénérée par la Trx h2 mitochondriale (Finkemeier *et al*., 2005).

La publication du génome de *Populus trichocarpa* a facilité l'identification de tous les gènes codant des Prxs chez cet organisme et permis de comparer cette famille multigénique avec les autres organismes modèles séquencés, à l'époque *Arabidopsis thaliana* et *Oryza sativa*. Malgré quelques différences concernant le nombre de gènes, toutes ces espèces contiennent des représentants des 4 groupes de Prx et pour ce qui concerne les Prx II, au moins une isoforme cytosolique, une chloroplastique et une mitochondriale. Nous avons ainsi entrepris de cloner et produire de façon hétérologue les Prx IIE (chloroplastique) et IIF (mitochondriale) de peuplier sous leur forme mature afin d'éclaircir la situation et de tester un maximum de réducteurs potentiels pour comparer l'efficacité des différents systèmes.

En parallèle à ces approches biochimiques, nous avons également mis l'accent sur les profils d'expression de ces protéines, en particulier en réponse à différentes conditions de stress. Les données obtenues à ce moment là étaient plutôt des études transcriptionnelles qui indiquaient que la Prx IIF est exprimée de façon constitutive (Brehelin *et al*., 2003) et qu'elle est essentielle à la croissance racinaire en condition oxydante (Finkemeier *et al*., 2005). La Prx IIE d'*A. thaliana* est principalement exprimée dans les tissues reproducteurs et son taux de transcrit augmente suite à une exposition bactérienne et diminue en présence d'ascorbate ou lors d'un stress salin (Brehelin *et al*., 2003; Horling *et al*., 2003). Nous avons donc étudié les profils d'expression de la Prx IIE et de la IIF tant dans *A. thaliana* que dans *P. trichocarpa*, en utilisant des anticorps reconnaissant les protéines d'*A. thaliana* (issus du travail de Claire Bréhelin et d'Iris Finkemeier) mais qui réagissaient également très spécifiquement avec les orthologues du peuplier. Afin d'obtenir une vue la plus complète possible sur l'ensemble des Prxs, les variations d'abondances d'autres Prxs (Prx Q, Prx 2 Cys et Prx IIB) ont quelquefois été mesurées. Ces analyses ont été effectuées sur toute une série d'organes et sur des feuilles soumises à des stress abiotiques (métaux lourds comme le cuivre ou le cadmium), sénescence, stress hydrique, photooxydant, et froid, tous appliqués sur *A. thaliana*) ou biotique (infection du peuplier par 2 races de *Melampsora larici populina* conduisant à des réactions compatible ou incompatible).

Toutefois, il est important de noter que ces analyses d'expression ont été effectuées principalement chez *A. thaliana* et il n'est pas certain que l'on puisse étendre les conclusions effectuées à tous les organismes photosynthétiques ou tout du moins aux plantes supérieures dans leur ensemble.

Résultats

Toutes ces données ont été publiées dans les 2 articles suivants:

Article 3 : “The mitochondrial type II peroxiredoxin from poplar”

Article 4 : “Biochemical and functional analysis of chloroplastic Prx IIE”

B Les Prx II chloroplastique et mitochondriale, Prx IIE et IIF, du peuplier

Article 3 : “The mitochondrial type II peroxiredoxin from poplar”

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The mitochondrial type II peroxiredoxin from poplar

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Mitochondria are a major site of reactive oxygen species production and controlling the peroxide levels in this compartment is essential. Peroxiredoxins (Prx) are heme-free peroxidases, which use reactive cysteines for their catalysis and reducing systems for their regeneration. One of the two Prxs present in poplar mitochondria, Prx IIF, expressed as a recombinant protein, was found to reduce a broad range of peroxides with electrons provided preferentially by glutaredoxin and to a lesser extent by glutathione, all the thioredoxins tested being inefficient. This protein is constitutively expressed because it is found in all tissues analyzed. Its expression is modified during a biotic interaction between poplar and the rust fungus *Melampsora laricii populina*. On the other hand, Prx IIF expression does not substantially vary under abiotic stress conditions. Nevertheless, water deficit or chilling and probably induced senescence, but not photooxidative conditions or heavy metal treatment, also led to a small increase in PrxIIF abundance in *Arabidopsis thaliana* plants.

Introduction

Aerobic life requires constant adjustments to molecular oxygen and its reactive oxygen species (ROS) derivatives. In eukaryotic cells, one major site of ROS generation is in the mitochondria because of the combination of powerful oxidants together with low potential reductants used to fuel the electron transfer chain. As ROS can damage most macromolecules, adaptation to aerobiosis has required the generation of multiple antioxidative and repair systems. Among the most documented systems for ROS detoxification in human and yeast mitochondria are the glutathione (GSH) peroxidase and other thiol-dependent peroxidases known as peroxiredoxins (Prxs). Both enzymatic systems are able to degrade peroxides into the corresponding alcohol and water in the presence of an exogenous donor containing cysteines [frequently thioredoxin (Trx)].

While mammalian Prxs have a low catalytic activity, they are present in large amounts in most subcellular compartments and play a prominent role in peroxide detoxication in these organisms. Human Prxs have been classified from I to VI, based on their amino acid sequence and mode of catalysis (they function either

Abbreviations – C, cytosol; CM, crude mitochondria; CuOOH, cumene hydroperoxide; EDTA, ethylenediaminetetraacetic acid; F, flower; GR, glutathione reductase; Grx, glutaredoxin; GSH, glutathione; L, leaf; M, mitochondria; ML, mature leaf; N, nucleus; NADPH, nicotinamide adenine dinucleotide phosphate; OL, old leaf; P, plastid; Prx, peroxiredoxin; R, root; ROS, reactive oxygen species; RT-PCR, reverse transcriptase–PCR; S, stem; *t*-BOOH, *tert*-butyl hydroperoxide; TE, total extracts; Trx, thioredoxin; WD, water deficit; YL, young leaf.

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with a single catalytic cysteine or with two cysteines, one catalytic or peroxidatic and one regenerating or resolving) (Wood et al. 2003). Both Prx III and V are present in human mitochondria (Banmeyer et al. 2005, Tien Nguyen-nhu and Knoops 2003). Mammalian or yeast Prxs are involved in many physiological processes as diverse as mitochondrially linked apoptosis signaling (Chang et al. 2004), hypoxia tolerance (Nonn et al. 2003), calcium-induced damage of mitochondria (Monteiro et al. 2004) and resistance to radiation (Wang et al. 2005). Of particular interest is human Prx V, which is distributed in virtually all subcellular compartments including the cytosol, peroxisomes, mitochondria and the nucleus (Banmeyer et al. 2005). The mammalian mitochondrial Prxs have been extensively studied in terms of catalytic mechanism and structure. Prx III requires two cysteines for its functioning, which are presumably reduced via Trx and is organized as a decameric toroid (Gourlay et al. 2003). On the other hand, Prx V functions with a single conserved cysteine, is organized as an homodimer and is reduced by a specific mitochondrial Trx, the two proteins showing three dimensional complementarity (Declercq et al. 2001, Smeets et al. 2005).

Organisms able to perform oxygenic photosynthesis, either prokaryotic or eukaryotic, contain additional photosynthetic/chloroplastic electron transfer chains, which constitute additional sites of ROS generation in the light. As a consequence, their Trx, glutaredoxin (Grx) and Prx equipment is generally much more complex than in non-photosynthetic organisms. The use of yeast and *Synechocystis* mutants has considerably facilitated the investigation of the Prxs functions in plants and elsewhere (Hosoya-Matsuda et al. 2005, Kobayashi et al. 2004, Pedrajas et al. 2000). In photosynthetic eukaryotes, the chloroplasts possess two types of Prx, one called 2-Cys Prx and another one referred to as Prx Q (Dietz et al. 2006, Rouhier and Jacquot 2005). Prx Q is monomeric, Trx dependent, involved in pathogenic response and also overexpressed under sulfur starvation (Kiba et al. 2005, Lamkemeyer et al. 2006, Rouhier et al. 2004a, Zhang et al. 2004). 2-Cys Prx is a homodimer, requires chloroplastic Trxs for its regeneration and is involved in peroxide detoxification (Broin and Rey 2003, Broin et al. 2002, Collin et al. 2003, Konig et al. 2002, Rey et al. 2005). In addition to Prx Q and 2-Cys Prx, higher plants chloroplasts also contain a type II Prx (Prx IIE), the equivalent of human Prx V (Horling et al. 2003).

Besides chloroplastic Prxs, plants also contain extra chloroplastic proteins as 1-Cys Prx present in seeds and involved in germination (Haslekas et al. 2003) but also in roots and linked to resistance to oxidative stress generated by exogenous application of arsenate (Requejo and Tena 2005). As its mammalian homolog, plant type II Prx is also present in several subcompartments, the chloroplast as mentioned above, but also the cytosol and mitochondria. The cytosolic type II Prxs are by far the best documented enzymes, and a poplar protein was found to accept both Trx and Grx as thiol regenerators, a special feature among all Prxs and of considerable interest for the study of protein–protein interactions (Rouhier et al. 2001). This property has been further confirmed by the existence of natural fusion products containing a Prx module linked to a Grx domain in pathogenic bacteria and the engineering of functional artificial fusions made with the individual poplar enzymes (Rouhier and Jacquot 2003, Rouhier et al. 2006). The structures of the cytosolic poplar type II Prx and of two of its potential donors, poplar Trx h1 and Grx C4 have recently been solved, providing essential tools to understand these protein–protein interactions (Coudevylle et al. 2005, Echalier et al. 2005, Noguera et al. 2005).

However, very little is known about Prx IIF, the mitochondrial isoform. Recently, Brehelin et al. (2003) have respectively reported the constitutive expression of the gene coding for Prx IIF in *Arabidopsis thaliana*. They did not identify the electron donor for its regeneration. Other reports, Finkemeier et al. (2005) and Horling et al. (2003) confirmed the presence of Prx IIF in mitochondria from *A. thaliana* and estimated its redox potential to −307 mV in the presence of the artificial donor dithiothreitol. The protein was efficiently reduced by a mitochondrial Trx o, but also by GSH in the presence or not of Grx. At this point, the identity of the physiological reductant of type II Prxs and especially of Prx IIF is still unclear. As for Prx IIF, various reducing compounds have been proposed for other type II Prxs including GSH in cyanobacteria (Hosoya-Matsuda et al. 2005), Trx and Grx in plants (Rouhier et al. 2002). We describe here the isolation and characterization of the mitochondrial Prx IIF from poplar and we investigate its kinetic characteristics and expression pattern during both biotic and abiotic stresses on either poplar or *Arabidopsis*.

Materials and methods

Heterologous production of recombinant Prx IIF in *Escherichia coli*

The open reading frame (ORF) of poplar Prx IIF (expressed sequence tag [EST] accession number: DV463589) was amplified from fruit via reverse transcriptase–PCR (RT-PCR) and cloned into the pET3d vector between *Nco*I and *BamH*I restriction sites (underlined) using the following two primers: Prx IIF forward: 5′ CCCCCCATGGCTACTGACATAGTCTCCGCT 3′ and Prx IIF reverse: 5′ CCCCGGATCCTTAGATTTGTCCCAGTATGACTTCGCC 3′. These primers allowed amplification of the sequence

corresponding to the mature form of the protein deprived of its N-terminal extension, which is predicted to direct the protein into mitochondria. For cloning facilities, codons corresponding to a methionine and an alanine were introduced in the 5′ part of the gene. The recombinant protein starts thus by MATDIVSA.

Escherichia coli strain BL21(DE3) containing the helper plasmid pSBET (Schenk et al. 1995) was transformed with the recombinant plasmid. Lucia Benteni cultures of 2 l were grown at 37°C and induced in exponential phase by 100 μ*M* isopropyl-β-D-thiogalactopyranoside. After a 4-h induction, the bacteria were harvested by centrifugation for 15 min at 5000 *g* and resuspended in buffer A [30 m*M* Tris–HCl pH 8.0, 1 m*M* ethylenediaminetetraacetic acid (EDTA), 200 m*M* NaCl]. Bacteria were disrupted by sonication and centrifuged for 30 min at 30 000 *g* to discard the insoluble fraction. The soluble part was then precipitated with ammonium sulfate successively up to 40 and 80% of the saturation. PrxIIF was mainly insoluble but an important part also precipitated between 40 and 80% of the saturation in ammonium sulfate. Around 80% of the expressed protein was insoluble. This fraction was first loaded onto an ACA 44 gel filtration column (Biosepra, Cergy Saint Christophe, France) equilibrated with buffer A. After eliminating the salt by dialysis, the second step was a diethylaminoethyl (DEAE) Sepharose column (Sigma, Saint Quentin, Fallavier, France) equilibrated with buffer A without NaCl. The protein was retained on the column and eluted using a 0–0.4 *M* NaCl gradient, dialyzed again, concentrated and finally stored in buffer A at –30°C until further use.

Peroxidase activity

The peroxidase activity of Prx IIF was tested using a spectrophotometric assay coupled to nicotinamide adenine dinucleotide phosphate (NADPH) oxidation. The reaction medium (500 μl) contained 200 μ*M* NADPH, 1 μ*M* Prx IIF and various concentrations (from 25 μ*M* to 3 m*M*) of peroxides [H_2O_2, *tert*-butyl hydroperoxide (*t*-BOOH), cumene hydroperoxide (CuOOH)]. When using the Trx reducing system, 0.8 μ*M A. thaliana* NADPH Trx reductase and 17 μ*M* Trx (Trx h1, h2 or h3 from poplar) were added. When using the Grx reducing system, the concentrations used were 0.4 unit of yeast glutathione reductase (GR) (Sigma), 1 m*M* GSH (Sigma) and 11 μ*M* poplar Grx C4. Background activity was subtracted and especially the direct reduction of H_2O_2 by ionized GSH.

Crude mitochondria extraction

Crude mitochondria from hybrid *Populus deltoides/trichocarpa* were extracted using method ''A'' described in Keech et al. (2005). However, in this case, about 20 g of young leaves freshly harvested was used as starting material and ground with a Warring Blender (five bursts of 5 s) and filtered through a 20-μm Nylon net. After two successive centrifugations (5 min at 2500 *g* where the pellet was discarded, and 15 min at 15 000 *g* to pellet the mitochondria both at 4°C), crude mitochondria (CM) were resuspended in 500 μl of washing buffer [0.3 *M* sucrose, 10 m*M* N-Tris (Hydroxymethyl) methyl-2-Aminoethanesulfonic Acid (TES), 2 m*M* EDTA and Protease Inhibitor Cocktail ''Complete'' (one tablet for 50 ml of buffer, Roche Applied Science, Meylan, France)]. Aliquots were finally frozen in liquid N_2.

Senescence and stress induction in *A. thaliana or P. trichocarpa*

Arabidopsis thaliana plants, ecotype Columbia 0, were grown for 7–8 weeks in short-day photoperiod [8-h light (250 μmol m^{-2} s^{-1}) at 22°C and 16-h dark at 17°C] and with a relative humidity of 75%. For cold treatment, plants were transferred to a climate chamber having the same growth conditions except for the temperature, which was set at 8°C/light and 5°C/dark. Plants were collected after 1, 5 or 10 days and frozen in liquid N_2. Leaf senescence was induced by modification of the photoperiod as described by Weaver and Amasino (2001) with the following modifications. A few leaves from the rosette were covered by ''mittens'' made with black plastic bag and covered by aluminum foil to reduce heat, whereas the rest of the plant remained in the light. Moreover, as described above, plants were grown in short-day photoperiod and senescence was monitored along a 6-day period. Water deficit and photooxidative treatments were applied on 6-week-old *Arabidopsis* plants as described in Vieira dos Santos et al. (2005). For Cu and Cd ion treatments, *Arabidopsis* plants were first grown on sand for 6 weeks and then transferred onto a solution containing either 75 μ*M* $CuSO_4$ or 75 μ*M* $CdCl_2$ for 7 days using a hydroponic system set up with the same light and temperature conditions than described above. The infection of *Populus x interamericana*, Beaupre plants with *Melampsora laricii populina* is described in Rouhier et al. (2004a).

Protein extraction and immunoblot analysis

For chilling and senescence experiments, frozen leaves from different treatments were ground in liquid N_2. Proteins were extracted from 200 mg of frozen leaf powder by adding 300 μl of extraction buffer [50 m*M* Tris–HCl pH 7.5, 5 m*M* ethylene glycolbis (beta

aminoethyl ethen) (EGTA), 5 m*M* $MgCl_2$, 100 m*M* NaCl, 10% glycerol, 1% Triton-X-100, 0.5% sodium dodecyl sulfate, 10 m*M* dithisthseitol N, N, N′, W′-tetraacetic acid (DTT) and a Protease Inhibitor Cocktail ''Complete'' (one tablet for 50 ml of buffer)]. After centrifugation (4°C, 15 min, 20 000 *g*), supernatants were collected and aliquots of each sample were frozen in liquid N_2. Western blots were performed on nitrocellulose membranes. Primary antibodies raised against Prx IIF, 2-Cys-Prx and H-protein (from Agrisera, Vännäs, Sweden) were used, respectively, at final dilutions of 1/2500, 1/3000 and 1/5000. Secondary antibodies (anti-rabbit Ig, horseradish peroxidase linked from Amersham Biosciences, Uppala, Sweden) were used at a final dilution of 1/8000.

Protein extraction and Western blot experiments for all other expression studies were as described in Rouhier et al. (2004a) for poplar extracts and in Vieira Dos Santos et al. (2005) for *Arabidopsis* extracts.

Results and discussion

Poplar genome analysis

With the release of the *P. trichocarpa* genome, we have identified all the genes encoding *Prx* in this species and compared the *Prx* gene families in the three sequenced genomes of land plants, *Arabidopsis*, rice and poplar. Although Gpx belongs to this family because they are reduced by Trx, they are not included in this study because of their more distant phylogenetic relationship with Prx (Rouhier and Jacquot 2005). Nine genes are present in *P. trichocarpa* and *A. thaliana* and eight in *Oryza sativa* (Table 1). The repartition into the four previously identified subgroups is different between these plants. Two genes coding for 1-Cys Prx are present in *O. sativa*, while there is only one in *Arabidopsis* and poplar. Whether the two rice genes are expressed only in seeds as earlier demonstrated for *Arabidopsis* and barley 1-Cys Prx (Haslekas et al. 2003) remains to be investigated but some ESTs for the two sequences were found for example in panicles. Two genes of the 2-Cys Prx family are present and expressed in *P. trichocarpa* and *A. thaliana*, but only one in rice. With regard to the Prx Q subgroup, the situation in the three species differs. Whereas poplar contains two expressed genes based on existing EST, *Arabidopsis* and rice have only one gene but in rice the gene is alternatively spliced. Finally, in the type II subgroup, the distribution is complex and distinct between plants. *A. thaliana* contains the highest number of Prx II (five), three being predicted to be cytosolic, one chloroplastic and one mitochondrial. In poplar and rice, four genes code for proteins of the three compartments mentioned above but in poplar the two Prx belonging to the same group are predicted to be cytosolic, whereas

Table 1. Peroxiredoxin (Prx) gene content in genomes of *Populus trichocarpa*, *Oryza sativa* and *Arabidopsis thaliana*. The gene names for *O. sativa* and *A. thaliana* are from the TIGR database. The entry codes for *P. trichocarpa* are expressed sequence tag (EST) accession numbers containing the full-length Prx sequences (in case of gene models changed). Gene models, coming from the version 1.1 of the poplar genome released by the Joint Genome Institute, US department of energy (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) are listed here: 1-Cys Prx: estExt_fgenesh4_pm.C_LG_VIII0405; 2-Cys Prx A: eugene3.00160660; 2-Cys Prx B: estExt_fgenesh4_pm.C_LG_VI0567; Prx Q1: eugene3.00280069; Prx Q2: estExt_Genewise1_v1.C_LG_X-VIII0612; Prx IIB: ESTEXT_FGENESH4_KG.C_LG_XIII0043; Prx IIC: ESTEXT_FGENESH4_PG.C_LG_XVIII0691; Prx IIE: GW1.41.572.1; Prx IIF: EUGENE3.00190240. To date, this version is restricted to annotators. Between parenthesis are successively the length of the proteins in amino acids, the localization, putative or confirmed experimentally (C, cytosol; P, plastid; M, mitochondria; N, nucleus) and the number of exons. One exception is the gene from *O. sativa* coding for Prx Q, which can be transcribed in two alternatively spliced transcripts.

Organism (number of genes)	Names			
	1-Cys Prx	2-Cys Prx	Prx Q	Prx II
P. trichocarpa (9)	BU866494 (220, C/N, 4)	DT509677 (A) (269, P, 7) DT495540 (B) (263, P, 7)	CV283265 (Q1) (214, P, 4) DT497438 (Q2) (214, P, 4)	DT520998 (IIB) (162, C, 3) DT515557 (IIC) (162, C, 3) DT493226 (IIE) (218, P, 1) DV463589 (IIF) (203, M, 5)
A. thaliana (9)	At1g48130 (216, C/N, 4)	At3g11630 (A) (266, P, 7) At5g06290 (B) (272, P, 7)	At3g26060 (215, P, 4)	At1g65980 (IIB) (162, C, 3) At1g65970 (IIC) (162, C, 3) At1g60740 (IID) (162, C, 3) At3g52960 (IIE) (234, P, 1) At3g06050 (IIF) (199, M, 5)
O. sativa (8)	Os07g44430 (220, C/N, 3) Os07g44440 (220, C/N, 3)	Os02g33450 (261, P, 7)	Os06g09610 (217/265, P, 3/2)	Os01g48420 (IIC) (162, C, 3) Os06g42000 (IIE1) (232, P, 1) Os02g09940 (IIE2) (225, P, 1) Os01g16152 (IIF) (198, M, 5)

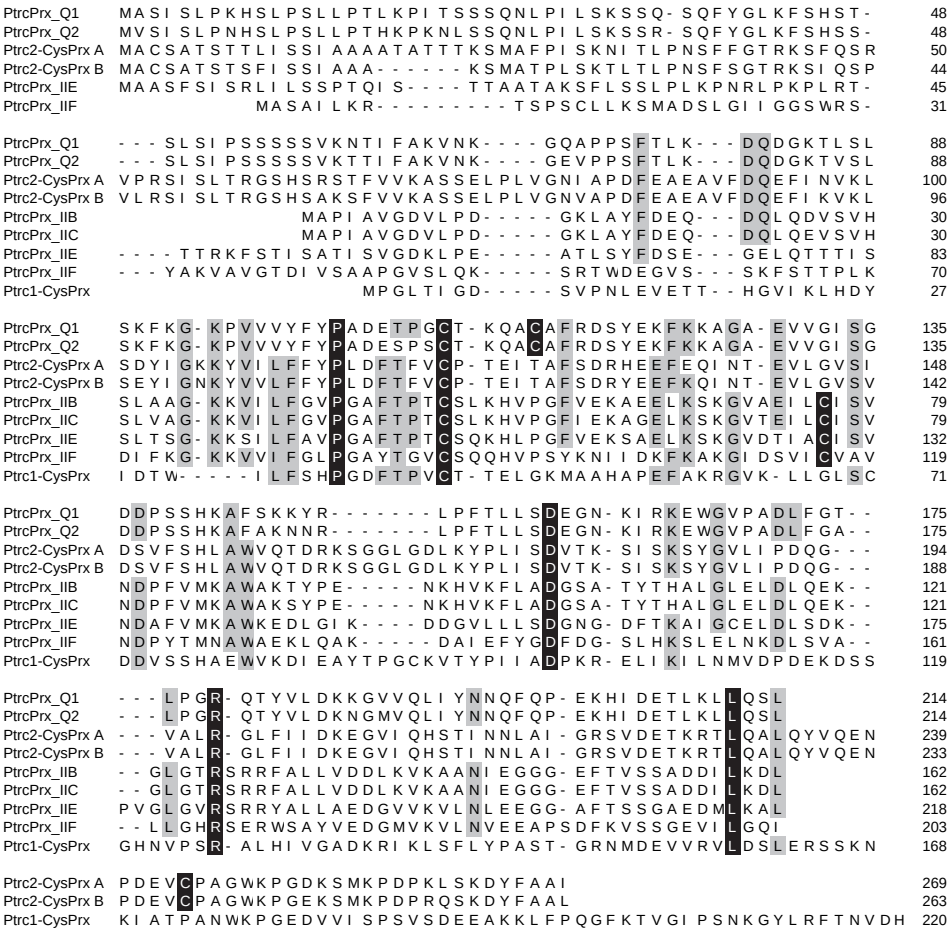

Fig. 1. Sequence comparison of peroxiredoxins (Prxs) from *Populus trichocarpa*. The alignment was performed using CLUSTALW and manually corrected for 1-Cys Prx. Gene models and expressed sequence tag (EST) accession numbers for PtrcPrx IIB, PtrcPrx IIC, PtrcPrx IIE, PtrcPrx IIF, PtrcPrx 2-CysA, PtrcPrx 2-CysB, PtrcPrx 1-Cys, PtrcPrx Q1, PtrcPrx Q2 are presented in Table 1. Ptrc stands for *P. trichocarpa*.

they are likely to be plastidial in rice. The presence of two closely related genes for the same Prx subgroup in only one organism probably reflects a duplication event.

The Prx sequences identified in the genome of *P. trichocarpa* have been compared (Fig. 1). All of them possess the strictly conserved catalytic or ''peroxidatic'' cysteine (here C55). This residue is one of the five strictly conserved amino acids in all poplar sequences (P48, C55, D106, R129 and C163, mature poplar Prx IIF numbering, see below). In the cytosolic poplar Prx IIB, the second cysteine C79 (conserved in most type II Prx) is dispensable and, when it is replaced by serine, the enzyme is still catalytically active (Rouhier et al. 2002). Likewise, we have mutated R129 and shown that, as in other Prx, it is essential for substrate binding (Rouhier et al. 2004b). This sequence comparison also suggests that D106 could be important in plant Prxs and it is certainly of interest to probe this by site-directed mutagenesis.

Expression of the recombinant Prx IIF

We next focused our attention on the poorly characterized Prx IIF. The poplar sequence coding for the full-length mitochondrial protein is 203 amino acids long, and prediction programs suggest that there is a transit sequence of approximately 30–40 amino acids in the N-terminus, which should direct the protein into the mitochondria. Because protein precursors are often insoluble, rarely stable and easily degraded (Miller et al. 2004), we decided to express the protein in a shortened version in *E. coli* cells. Surprisingly, the first two constructions coding for shortened proteins of 160 and 172 amino acids did not allow any expression at all in *E. coli*. Finally, a subsequent construction leading to a 167 amino acid long protein was successful, allowing the expression of a recombinant protein. Nevertheless, a large part of the protein is produced in inclusion bodies, suggesting that the selected cleavage site is not ideally

located to stabilize the recombinant protein. In this study, we have shortened the sequence based on the size of the cytosolic Prx IIB and checked the absence of secondary structures at the cleavage site using prediction softwares, but nevertheless this did not prevent aggregation. The molecular mass of the protein produced is 18 218 Da and its theoretical pI is 5.91.

Biochemical characterization of Prx IIF

The efficiency of the reductants and the nature of substrates used by Prx are variable between Prx isoforms. For example, the poplar cytosolic Prx IIB is reduced both by the Trx or the Grx system, but not by GSH alone (Rouhier et al. 2001). The Prx IIF of *A. thaliana* is reduced (1) by a mitochondrial Trx o but not by the cytosolic Trx h from poplar or (2) by the Grx system or (3) by GSH (Finkemeier et al. 2005, Rouhier et al. 2005). Finally, the donor to *A. thaliana* Prx IIE is not known because the two reductants tested (AtTrx h2 and AtGrx1) were unable to reduce it (Brehelin et al. 2003). We therefore decided to investigate both the substrate and donor efficiencies for the poplar Prx IIF.

Although the protein is located in mitochondria (see below) and because the specificity for the reductants in these in vitro tests is generally low, various cytosolic or mitochondrial Trx were tested. Contrary to poplar Prx IIB, which is used as a control, poplar Prx IIF is not reduced at all irrespective of which Trx was used (the cytosolic Trx h1, h3 or the mitochondrial Trx h2) (Fig. 2). The only other potential physiological reductant for Prx IIF in poplar is a Trx o. The genome of *P. trichocarpa* contains one Trx o gene but all attempts to produce the recombinant protein have failed because the protein is produced in inclusion bodies. Whether this Trx o could reduce the Prx IIF or whether poplar Prx IIF cannot be reduced by any Trx as a donor thus remain open questions. For example, the second Prx identified in mitochondria, called Gpx 3, is reduced neither by poplar Trx h2 nor by *A. thaliana* Trx o (Gelhaye et al. 2004).

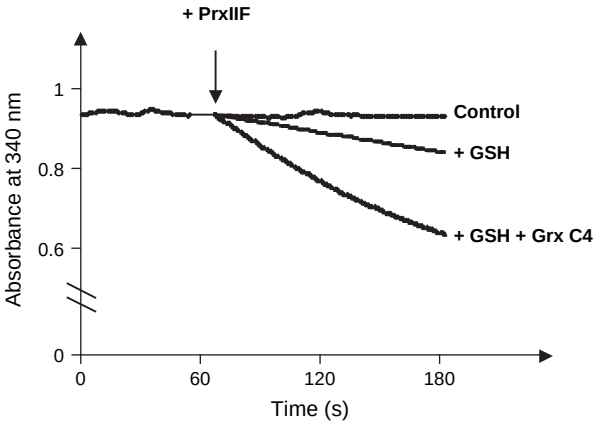

Fig. 2. Time course-dependent peroxidase activity of peroxiredoxin (Prx) IIF. The activity of poplar Prx IIF (1 μ*M*) was measured at 340 nm by following nicotinamide adenine dinucleotide phosphate oxidation, in the presence of 1 m*M tert*-butyl hydroperoxide and either 0.4 U glutathione reductase (GR) (control), or 0.4 U GR and 1 m*M* glutathione (GSH) (+GSH) or 0.4 U GR, 1 m*M* GSH and 11 μ*M* glutaredoxin (Grx) C4 (+GSH + Grx C4). The reactions were started by adding Prx IIF.

We then measured the capacity of GSH, alone or associated with a cytosolic Grx, called Grx C4, to serve as a reductant for the Prx IIF. Indeed, although at least two Grx are predicted to be located in poplar mitochondria, none of them has yet been characterized. Contrary to poplar Prx IIB, which cannot be regenerated by GSH alone (Rouhier et al. 2001), Prx IIF was able to use reduced GSH for its regeneration (Fig. 2). Nevertheless, adding the cytosolic Grx C4 greatly increased the reaction rate. This result is similar to what was observed with Prx IIF and the mitochondrial Grx S10 from *A. thaliana* (Finkemeier et al. 2005). It is also consistent with the isolation of Prx IIF from a total protein extract using a monocysteinic Grx C4 as a bait (Rouhier et al. 2005). The recycling mechanism of type II Prx remains unclear. Because the second cysteine found in position 79 (Prx IIF numbering) was not essential in Prx IIB (Rouhier et al. 2002), it is possible that the sulfenic acid formed during catalysis is reduced by GSH and either a second GSH molecule or a Grx could attack the GSH-mixed disulfide. The apparent affinity (Km) for GSH, measured under steady-state conditions, in the absence of Grx and at saturating concentration of *t*-BOOH was 260 μ*M*. These results indicate that GSH should be able to directly react with the Prx sulfenic acid. We also determined the affinity between Prx IIF and Grx C4 by measuring the rates at various Grx concentrations. The results obtained followed a typical Michaelis–Menten saturation curve, with an apparent Km for Grx C4 of 1.3 μ*M*.

Finally, the substrate specificity of Prx IIF was measured in the presence of a saturating Grx system (1 m*M* GSH

Table 2. Peroxide reduction by poplar peroxiredoxin (Prx) IIF. Catalytic parameters were determined in steady-state conditions by following nicotinamide adenine dinucleotide phosphate oxidation in the presence of various concentrations of peroxide [H_2O_2, *tert*-butyl hydroperoxide (*t*-BOOH) or cumene hydroperoxide (CuOOH)] ranging from 25 μ*M* to 3 m*M*, 1 μ*M* Prx IIF and saturating concentrations of the glutaredoxin (Grx) system (0.4 U glutathione reductase, 1 m*M* glutathione and 11 μ*M* Grx C4).

Substrate	$Km_{peroxide}$ (μ*M*)	kcat (s^{-1})	1000 kcat/$Km_{peroxide}$
H_2O_2	71 ± 21	0.38 ± 0.02	5.3 ± 2
t-BOOH	16 ± 4.5	0.51 ± 0.05	31.5 ± 5
CuOOH	326 ± 129	0.39 ± 0.02	1.2 ± 0.3

and 11 μ*M* Grx C4) and various peroxides (H_2O_2, *t*-BOOH or CuOOH). The catalytic parameters described in Table 2 indicate that the best substrate for Prx IIF is *t*-BOOH (kcat/Km of 31.5×10^3 M^{-1} s^{-1}) followed by H_2O_2 (kcat/Km of 5.3×10^3 M^{-1} s^{-1}) and CuOOH (kcat/Km of 1.2×10^3 M^{-1} s^{-1}). The turnover number (kcat around 0.4 s^{-1}) and the catalytic efficiency (kcat/Km around 10^3 M^{-1} s^{-1}) are in the same range as those determined for other plant Prxs (Finkemeier et al. 2005, Horling et al. 2003, Rouhier et al. 2004a, 2004b). This broad substrate specificity common to all Prx is crucial for their physiological functions because catalases and ascorbate peroxidases are only H_2O_2-degrading enzymes. Especially the reduction of peroxidated lipids in mitochondria is probably crucial for its integrity in stress situations. It has already been demonstrated that AtPrx IIF is able to slightly reduce phospholipid hydroperoxides (Finkemeier et al. 2005).

Expression analysis

Using Genevestigator microarray data (Zimmermann et al. 2004), we performed an in silico analysis of *A. thaliana Prx IIF* expression in various plant organs and stress conditions. The *Prx IIF* gene (At3g06050) is expressed in all organs of *Arabidopsis* plants. Concerning the various stress conditions tested, the expression of *Prx IIF* did not substantially change (i.e. less than two-fold) compared with control conditions (data not shown). Altogether, these data indicate that *Prx IIF* is likely constitutively expressed in poplar as already suggested on the basis of RT-PCR experiments in *A. thaliana* (Brehelin et al. 2003) and Western blotting experiments in pea root nodules (Groten et al. 2006).

The expression of *Prx IIF* was studied at the protein level in various plant organs, both in *A. thaliana* and *P. trichocarpa*, using an antibody raised against the *Arabidopsis* protein, which cross-reacts specifically with Prx IIF from various plants. We have checked that these antibodies do not cross-react with the recombinant Prx IIB and Prx IIE proteins from poplar (data not shown).

In *A. thaliana*, the protein is nearly equally expressed in all tissues tested with a slightly higher amount in young leaves and in roots. However, in poplar, the amount of protein is noticeably higher in leaves compared with stems, roots and petioles (Fig. 3A and B). To check the presence of Prx IIF in poplar mitochondria, we isolated CM from poplar leaves and Western blots were performed with antibodies raised against either Prx IIF or the H-subunit of the glycine decarboxylase complex (Fig. 3 C). Both signals increased in CM in comparison with total extract. Moreover, the antibodies raised against Prx IIF were tested with pure mitochondria from leaves of

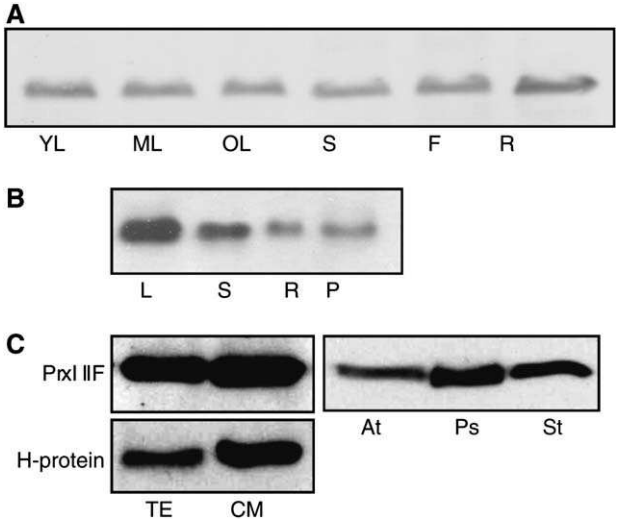

Fig. 3. Western blot analysis of peroxiredoxin (Prx) IIF expression in poplar and *Arabidopsis thaliana* organs and in mitochondrial extracts. (A) Expression of Prx IIF in *A. thaliana* organs (YL, young leaf; ML, mature leaf; OL, old leaf; S, stem; F, flower; R, root). (B) Expression of Prx IIF in *Populus trichocarpa* organs (L, leaf; S, stem; R, root; P, petiole). (C) Expression of Prx IIF in mitochondrial extracts from different species. On the left, the expression of Prx IIF was analyzed in total extracts (TE) or in crude mitochondria from poplar and compared with the H subunit of the glycine decarboxylase. On the right, the expression was detected in pure mitochondrial extracts from *A. thaliana* (At), *Pisum sativum* (Ps) and *Solanum tuberosum* (St). For Prx IIF and H-protein (C, left), 10 μg of protein was loaded. For PrxIIF on At, Ps and St (C, right), 15 μg of protein was loaded. All these experiments were repeated at least two times with identical results.

A. thaliana, *Pisum sativum* and from tubers of *Solanum tuberosum* (Fig. 3C). A single band of the expected size (18–19 kDa) was detected in all three samples. This is in line with the 79–86% homology between mature forms of Prx IIF (166 last amino acids) of the four species cited here above (according to ESTs available in Genbank database). Consequently, it indicates that Prx IIF is expressed in the mitochondria of all these species. and it confirms the good specificity of the antibodies.

The abundance of Prx IIF was finally investigated in poplar and *Arabidopsis* plants subjected to various stress conditions. As two poplar Prxs, Prx IIC and Prx Q, have previously been shown to be affected by biotic interaction with the pathogenic fungus *M. laricii populina* (Rouhier et al. 2004a), we first examined the changes in Prx IIF and 2-Cys Prx protein amounts during compatible or incompatible reactions of poplar in the presence of two different races of fungi (Fig. 4A). In the incompatible reaction, the amount of protein clearly decreased between 12 and 24 h of interaction and then increased close to the initial level at 96 h. In contrast, the levels of 2-Cys Prx, which are classically present as a monomeric and a dimeric

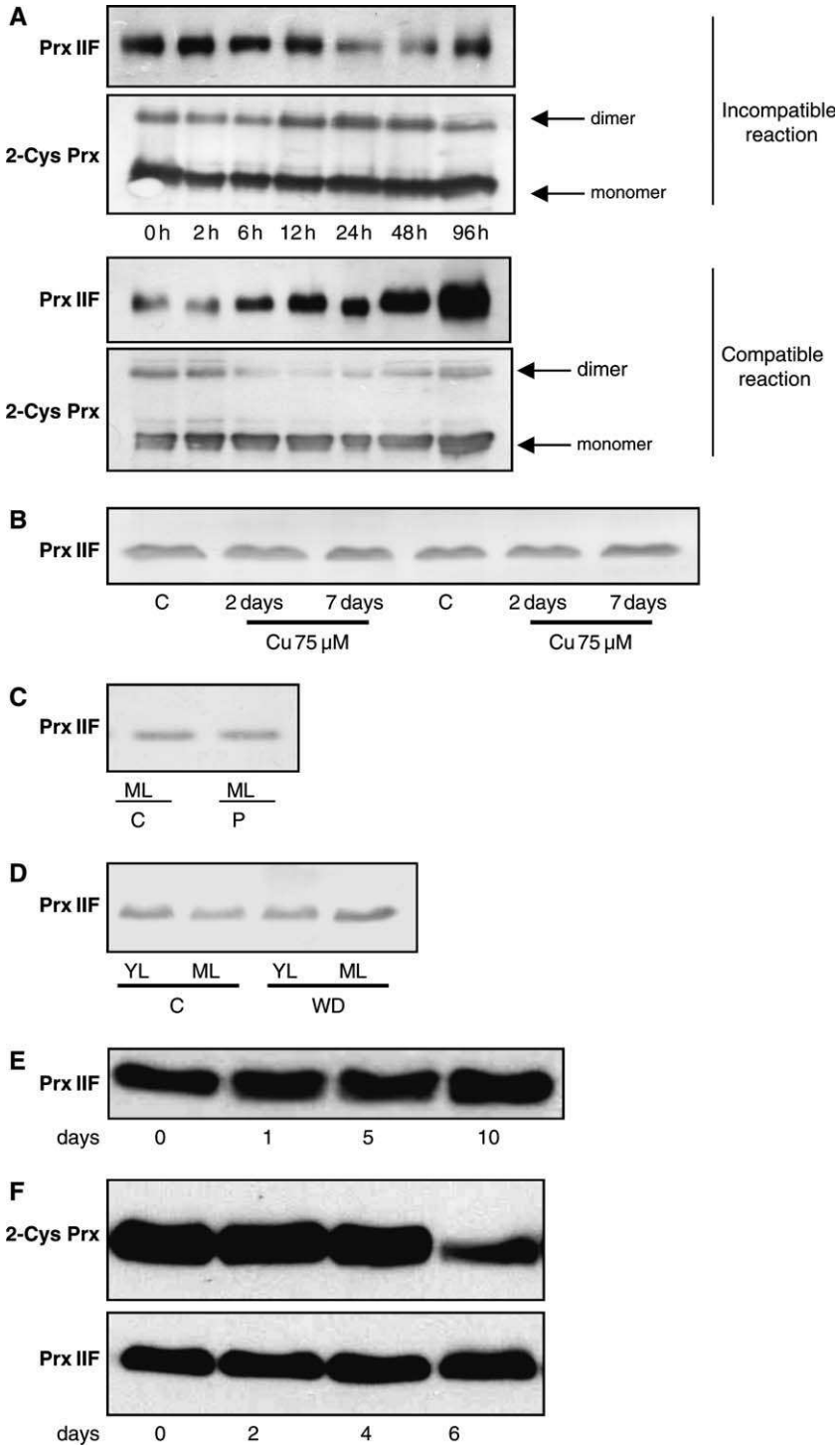

Fig. 4. Western blot analysis of peroxiredoxin (Prx) IIF expression in poplar or *Arabidopsis thaliana* in biotic or abiotic stress conditions. (A) Expression of Prx IIF was compared with 2-Cys Prx in poplar plants inoculated with two different races of *Melampsora laricii populina*, leading to a compatible or an incompatible reaction. (B) Expression of Prx IIF in leaves of *A. thaliana* after 2 or 7 days of a metallic treatment (75 μM Cu^{2+} or Cd^{2+}). (C) Expression of Prx IIF in mature leaves of *A. thaliana* under photooxidative stress [high light (1400 μmol m^{-2} s^{-1}) combined with low temperature (8°C) for 8 days] (P). (D) Expression of Prx IIF in young or mature leaves of *A. thaliana* under water deficit for 6 days (WD). (E) Expression of Prx IIF in leaves of *A. thaliana* plants subjected to 1, 5 or 10 days cold treatment (8°C/light and 5°C/dark). (F) Expression of Prx IIF and 2-Cys Prx in senescent *A. thaliana* plants. Both experiments E and F were duplicated and similar results were obtained.

form, did not vary much with time of infection, although the dimer intensity increased between 12 and 24 h. In the compatible reaction, the amount of Prx IIF increased after 6 h and reached very high levels after 96 h of infection. In the case of 2-Cys Prx, expression of the monomer did not change whereas the amount of dimer was lower between 6 and 96 h. Altogether these results indicate that expression of 2-Cys Prx, except for a slight variation of the dimeric form, does not change upon infection, in accordance with previous experiments which have shown that the levels of this protein remain remarkably constant. In contrast, the amount of Prx IIF was substantially altered during infection and varied in an opposite manner in compatible and incompatible reactions. These results are different from those described for Prx IIC and Prx Q, the expression of which are increased in an incompatible reaction and decreased in a compatible reaction (Rouhier et al. 2004). The physiological meaning of these variations is still uncertain, but it appears clear that Prxs of the type II are important players in the plant pathogenic fungus interactions.

We next examined the Prx IIF abundance in *Arabidopsis* plants subjected to various abiotic stresses. As root growth of a knockout *A. thaliana* mutant for Prx IIF is inhibited in the presence of Cd^{2+} (or by salicylhydroxamic acid, an inhibitor of cyanide insensitive respiration) (Finkemeier et al. 2005), we first analyzed Prx IIF contents during exposure to heavy metals (2 or 7 days with 75 μM Cu^{2+} or Cd^{2+}). No change was noticed in Prx IIF abundance whatever the metal or the time considered (Fig. 4B). Similarly, no change was observed in plants exposed to photooxidative treatment for 8 days compared with control plants (Fig. 4C). Other treatments led to variations of Prx IIF at the protein level. In situation of water deficit, the amount of Prx IIF slightly increased in mature leaves, but not in young leaves of the treated plants compared with the control plants (Fig. 4D). According to Prx IIF amount in *Arabidopsis* leaves, no response to cold was evidenced after 1 and 5 days, whereas a slight increase of protein was observed after 10 days of cold treatment (Fig. 4E). This in line with previous work done by Taylor et al. (2005) where they noticed that chilling treatment triggered mild oxidative stress in mitochondria.

Finally, after 6 days of dark-induced senescence, the amount of the chloroplastic 2-Cys Prx decreased drastically, whereas no change was noticed for the mitochondrial Prx IIF (Fig. 4F). A study from Groten et al. (2006) described rather similar results when they noticed that both pea nodule mitochondrial Prx IIF transcripts and protein amounts were unaffected during nodule senescence. This is in line with recent results (O. Keech, P. Gardeström, unpublished results) where an impairment of photosynthesis during dark-induced senescence in *Arabidopsis* leaves was observed, whereas mitochondrial respiration remained rather high most probably to sustain the metabolic activities for the nutrients salvation and reallocation. However, a severe decrease in number of both mitochondria and chloroplasts was observed during the process of senescence. While a lower amount of 2-Cys Prx can be explained by a decreased chloroplast number, the stable amount of mitochondrial Prx IIF does not correlate with the loss of nearly 50% of the mitochondria during senescence. Although the participation of mitochondria has not been established, an increase in ROS production has often been suggested during leaf senescence (Buchanan-Wollaston et al. 2003). Our results suggest that an increase (of ca. 50%) in Prx IIF abundance is likely to occur in mitochondria during leaf senescence. Nevertheless, further studies need to be carried out to define more clearly the redox state of mitochondria during leaf senescence.

Concluding remarks

The present study brings additional information concerning the biochemical properties, localization and expression patterns of the mitochondrial Prx IIF in plants. Using both poplar and *Arabidopsis* as plant models, we have shown that Prx IIF is constitutively expressed. Its activity is dependent on reduced GSH, with the reaction considerably amplified by Grx. Based on these observations and on the sequence similarities of all plant type II Prx, we postulate that they should all be able to use Grx as a donor and that earlier failure to observe a Grx effect may be because of the use of ineffective Grx (there are nearly 30 genes coding for these proteins in plants). We are currently testing this prediction by studying the donor requirement of the chloroplastic poplar Prx IIE. Finally, this study revealed that the Prx IIF content fluctuates widely in the course of biotic infection of the leaves by a pathogen, *M. laricii populina*. Other abiotic stresses tested did not induce so strong changes in Prx IIF abundance.

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B Les Prx II chloroplastique et mitochondriale, Prx IIE et IIF, du peuplier

Article 4 : “Biochemical and functional analysis of chloroplastic Prx IIE”

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Functional analysis and expression characteristics of chloroplastic Prx IIE

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Peroxiredoxins (Prxs) are ubiquitous thiol-dependent peroxidases capable of eliminating a variety of peroxides through reactive catalytic cysteines, which are regenerated by reducing systems. Based on amino acid sequences and their mode of catalysis, five groups of thiol peroxidases have been distinguished in plants, and type II Prx is one of them with representatives in many sub-cellular compartments. The mature form of poplar chloroplastic Prx IIE was expressed as a recombinant protein in *Escherichia coli*. The protein is able to reduce H_2O_2 and tert-butyl hydroperoxide and is regenerated by both glutaredoxin (Grx) and thioredoxin (Trx) systems. Nevertheless, compared with Trxs, Grxs, and more especially chloroplastic Grx S12, are far more efficient reductants towards Prx IIE. The expression of Prx IIE at both the mRNA and protein levels as a function of organ type and abiotic stress conditions was investigated. Western blot analysis revealed that *Prx IIE* gene is constitutively expressed in *Arabidopsis thaliana*, mostly in young and mature leaves and in flowers. Under photo-oxidative treatment and water deficit, almost no change was observed in the abundance of Prx IIE in *A. thaliana*, while the level of Prx Q (one of the two other chloroplastic Prxs with 2-Cys Prx) increased in response to both stresses, indicating that plastidic members of the Prx family exhibit specific patterns of expression under stress.

Introduction

In plants, aerobic reactions constantly generate reactive oxygen species (ROS), especially at the level of the chloroplastic and mitochondrial electron transport chains. When they accumulate at high concentrations, these ROS can damage cellular components such as nucleic acids, proteins or lipids. The ROS concentrations increase further when plants are subjected to biotic or abiotic stress conditions. In order to protect their biochemical integrity, plants rely on many non-enzymatic antioxidant compounds such as carotenoids, tocopherols, GSH and ascorbate and multiple enzymatic antioxidant and repair systems (Noctor and Foyer 1998). Enzymatic detoxifying systems include superoxide dismutases, catalases, ascorbate peroxidases and peroxiredoxins (Prxs), which are thiol-dependent peroxidases.

Prxs are found in most cell compartments and all kingdoms. Prxs display one or two conserved cysteines;

Abbreviations – Grx, glutaredoxin; GR, glutathione reductase; Gpx, glutathione peroxidase; Prx, peroxiredoxin; ROS, reactive oxygen species; Trx, thioredoxin.

the absolutely conserved cysteine is the catalytic or 'peroxidatic' cysteine. The second conserved cysteine, when involved in the regeneration of the peroxidatic cysteine, is termed 'resolving.' In plants, based on their amino acid sequences and biochemical characteristics, Prxs are classified into five subgroups, the initially identified 2-Cys Prx, 1-Cys Prx, type II Prx, Prx Q and the incorrectly named glutathione peroxidases (Gpxs) (Dietz 2003, Rouhier and Jacquot 2005). Indeed, plant Gpxs are thioredoxin (Trx)-dependent proteins (Herbette et al. 2002, Navrot et al. 2006). Gpxs are present in most sub-cellular compartments, and they are involved in the response to both biotic and abiotic stress conditions by acting as general peroxide scavengers (Navrot et al. 2006) or in some cases, as demonstrated for an *Arabidopsis* Gpx, by specifically relaying the H_2O_2 signal to other signalling molecules such as abscisic acid (Miao et al. 2006). 1-Cys Prxs are restricted to seeds and roots, and they are involved in germination and oxidative stress resistance (Haslekås et al. 2003, Requejo and Tena 2005). 2-Cys Prxs are located in the chloroplast together with Prx Q and Prx IIE. They are especially involved in the protection of the photosynthetic apparatus (Baier et al. 2000, Broin and Rey 2003, Broin et al. 2002). In addition, in accordance with the roles proposed for mammalian 2-Cys Prx, the overoxidation of plant homologues, which is also regulated by sulfiredoxins, could also be part of signalling mechanisms (Rey et al. 2007). Prxs Q are Trx-dependent enzymes involved in pathogenic response and photosystem II protection (Kiba et al. 2005, Lamkemeyer et al. 2006, Rouhier et al. 2004a).

The Prx II subgroup includes isoforms present in most sub-cellular compartments. The Prx IIB, C and D are cytosolic proteins, while Prx IIE is located in chloroplasts and Prx IIF in mitochondria (Bréhélin et al. 2003, Finkemeier et al. 2005). A remarkable finding was the discovery that Prx IIB accepts both Trx and glutaredoxin (Grx) as reductants (Finkemeier et al. 2005, Rouhier et al. 2001, 2002a). This has been confirmed later by characterizing bacterial hybrid proteins containing a Prx module linked to a Grx domain and by producing active artificial fusion proteins between poplar Prx IIB and Grx or Trx (Rouhier and Jacquot 2003, Rouhier et al. 2006a). Remarkably, the *Arabidopsis* Prx IIB and the poplar Prx IIF were only able to reduce peroxides using electrons provided by GSH and/or Grx but none of the Trxs tested (Brehelin et al. 2003, Gama et al. 2007), while the poplar Prx IIB and *Arabidopsis* Prx IIF also accept Trxs for their regeneration (Rouhier et al. 2002a, Finkemeier et al. 2005). The regeneration system used by Prx IIE is still unknown but does not involve the chloroplastic NTRc, a protein including an NADPH thioredoxin reductase (NTR) module fused to a Trx module (Moon et al. 2006).

With regard to their physiological functions, Prxs from organelles have been reported to prevent DNA damage (Dietz et al. 2006). Upon post-germination growth, Prxs are activated endogenously to provide early antioxidant protection in *Arabidopsis thaliana* (Pena-Ahumada et al. 2006). The Prx IIF is constitutively expressed and is essential for root growth under stress condition (Finkemeier et al. 2005). Its expression is modified during a biotic interaction between poplar and the rust fungus *Melampsora laricii populina* (Gama et al. 2007). On the other hand, its content does not substantially vary under abiotic stress conditions. Note that water deficit, chilling and senescence, but not photo-oxidative conditions or heavy metal treatment, led to a small increase in Prx IIF abundance in *A. thaliana* plants (Gama et al. 2007). In pea, Prx IIF accumulates in cold- and heavy-metal-treated plants (Barranco-Medina et al. 2007). The *Prx IIE* from *A. thaliana* is expressed mostly in reproductive tissues, and its product is addressed to the plastid (Bréhélin et al. 2003). The *Prx IIE* gene is upregulated after bacterial exposure, and its expression decreases upon application of ascorbate and under salt stress (Horling et al. 2002, 2003, Jones et al. 2002).

The structure as well as the catalytic and regeneration mechanisms used by most Prx II members have been extensively analysed except those employed by the chloroplastic Prx IIE (Echalier et al. 2005). We describe here the heterologous expression and biochemical characterization of poplar Prx IIE by investigating its specificity towards substrates and reductants. In addition, the mRNA and protein abundances were analysed in different organs and various abiotic stress conditions in *A. thaliana*.

Materials and methods

Heterologous expression of recombinant Prx IIE in *Escherichia coli*

The open reading frame (ORF) of poplar Prx IIE was amplified from *Populus trichocarpa* leaf reverse transcriptase polymerase chain reaction product and cloned in the *Nco*I and *Bam*HI restriction sites (underlined in the primers) of pET3d. The primers used were 5′CCCCCCATGGCTACCATTTCTGTCGGGGAC-3′ and 5′CCCCGGATCCTCAGAGAGCCTTGAGCATATC 3′. The sequence amplified encodes a protein, deprived of the first 54 amino acids corresponding to the signal peptide, starting with the N-terminus sequence MATISVGDL and ending with DMLKAL at the C-terminus. The resulting construction was termed pET3d-Prx IIE. For production, the *E. coli* BL21(DE3) strain, containing the pSBET plasmid, was transformed with the recombinant plasmid pET3d-Prx IIE (Schenk et al. 1995).

Luria Bertani (LB) cultures were successively amplified up to 2.4 l, grown at 37°C and induced in exponential phase by adding 100 μ*M* isopropyl β-D-1-thiogalactopyranoside for 4 h. The bacterial cultures were then centrifuged for 15 min at 4400 *g* (rotor JA10, Beckman, Paris, France). The pellets were resuspended in 30 ml of TE NaCl (30 m*M* Tris–HCl pH 8.0, 1 m*M* EDTA, 200 m*M* NaCl) buffer, and the suspension conserved at −20°C. Cell lysis was performed by sonication (3 × 1 min with intervals of 1 min), and the soluble and insoluble fractions were separated by centrifugation for 30 min at 27 000 *g* (JA20 Beckman). The soluble part was then fractionated with ammonium sulphate in two steps, and the protein fraction precipitating between 40 and 80% of the saturation contained the recombinant protein as estimated by 15% SDS-PAGE. The protein solubilized in TE buffer was purified by exclusion size chromatography after loading on an ACA44 (5 × 75 cm) column equilibrated in TE NaCl buffer. The fractions containing the protein were pooled, dialysed by ultrafiltration to remove NaCl and loaded onto an ion exchange column (diethylaminoethyl cellulose, Sigma, St Louis, MO) in TE (30 m*M* Tris–HCl pH 8.0, 1 m*M* EDTA) buffer. The proteins were eluted using a 0–0.4 *M* NaCl gradient. Finally, the fractions of interest were pooled, dialysed, concentrated in an Amicon cell equipped with a YM 10 membrane under nitrogen pressure and stored in TE buffer at −20°C. Purity was checked by SDS-PAGE.

Other recombinant proteins (Trx h1, Trx h3, Grx C4, Grx C4 C27S, Grx C4 C30S, Grx S12 from poplar and Grx S14 or Grx S16 from *A. thaliana*), used in this study, were produced as recombinant proteins and purified as described (Behm and Jacquot 2000, Gelhaye et al. 2003, Rouhier et al. 2002b, 2007, Bandyopadhyay et al. in press).

Peroxidase activity

The peroxidase activity was measured using spectrophotometry by following NADPH oxidation at 340 nm linked to peroxide degradation as a function of time in a coupled reaction test in the presence of the Trx or Grx system. A 500 μl reaction medium in TE buffer contained 220 μ*M* NADPH, 0.5 μ*M* NTR, 2 μ*M* Prx IIE and varying amounts of Trx h1 or Trx h3 (5–50 μ*M*) and of peroxide (15 μ*M* to 1 m*M*), either H_2O_2 or tert-butyl hydroperoxide (tBOOH) or cumene hydroperoxide (CUOOH). With the Grx reducing system, Trx reductase and Trx were replaced by 0.5 units glutathione reductase (GR), 500 μ*M* to 1 m*M* GSH and 20 μ*M* of Grx C4, Grx C4 C27S, Grx C4 C30S, Grx S12, Grx S14 or Grx S16. The background activity resulting from direct peroxide reduction by ionised GSH or by the Trx system alone was subtracted.

Plant material

Arabidopsis thaliana ecotype Columbia plants were grown in a growth chamber with an 8-h photoperiod, a photon flux density of 250 μmol m^{-2} s^{-1}, a temperature regime of 22°C/18°C (day/night) and a relative humidity of 55%. A water deficit was applied on 6-week-old plants by withholding watering for around 6 days. Barley and tomato plants were grown and subjected to water deficit as reported (Rorat et al. 2004). The leaf relative water content (RWC) was determined on leaf pieces as described by Rorat et al. (2004). The photo-oxidative treatment was carried out by exposing 6-week-old plants to a high light intensity (1400 μmol m^{-2} s^{-1}) at 8°C for 8 days (Vieira Dos Santos et al. 2005).

Protein extraction, SDS-PAGE and Western blotting experiments

Purification of intact leaf chloroplasts from *Arabidopsis* leaves, preparation of stromal proteins and extraction of soluble proteins from leaves and various organs were as reported previously (Vieira Dos Santos et al. 2005). The protein content was determined using a method based on bicinchoninic acid (BC Assay Reagent, Interchim, Montluçon, France). Proteins were separated using SDS-PAGE (13%, w/v gel) and electrotransferred onto a nitrocellulose membrane (Pall Corporation, East Hills, NY). The serum raised against anti-AtPrx IIE was used at a dilution of 1:10 000 (Brehelin et al. 2003). The sera raised against Prx Q (Rouhier et al. 2004a) and 2-Cys Prx (Broin et al. 2002) were used at dilutions of 1:1000 and 1:5000, respectively. Bound antibodies were revealed using an anti-rabbit immunoglobulin G alkaline phosphatase conjugate diluted 1:10 000 (Sigma).

Results

Sequence analysis of the Prx II family

All the Prx II sequences of the three sequenced land plants (*A. thaliana*, *Oryza sativa* and *P. trichocarpa*) have been compared (Fig. 1). Four genes are present in *P. trichocarpa* and *O. sativa* and five in *A. thaliana*. Three, two and one of them are most likely cytosolic (Prx IIB, C and D) in *A. thaliana*, *P. trichocarpa* and *O. sativa*, respectively. One Prx II in *A. thaliana* and *P. trichocarpa* and two in *O. sativa* are predicted to be chloroplastic (Prx IIE), and all three organisms possess one Prx related to the mitochondrial IIF type. Because of the transit peptide, the protein sequences of Prxs IIE and IIF display an N-terminal amino acid extension. The additional cytosolic sequence present in *A. thaliana* probably arises from

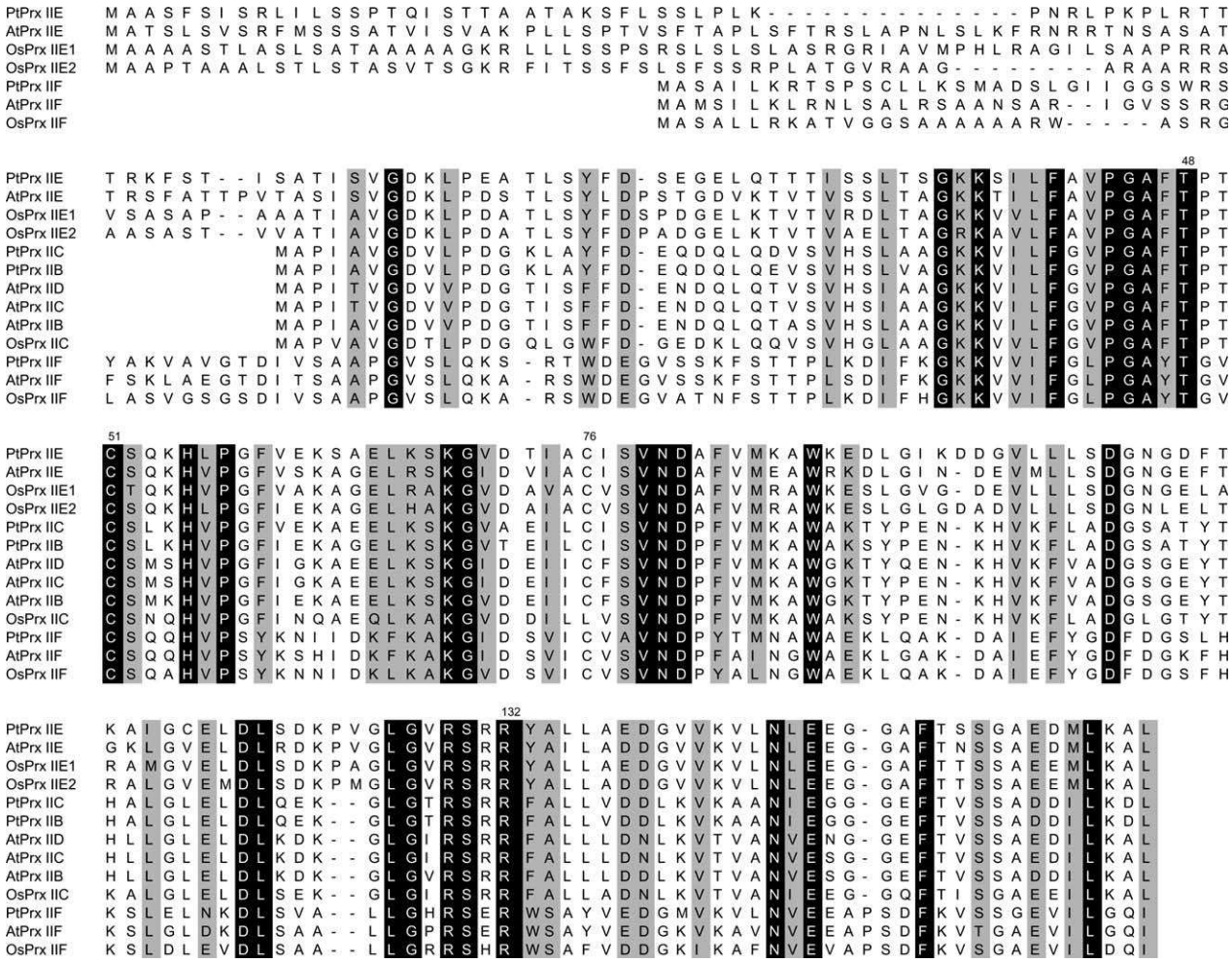

Fig. 1. Amino acid sequence alignment of plant type II Prxs. The alignment was performed using CLUSTALW. Gene models and accession numbers are as follows: PtPrx IIB (ESTEXT_FGENESH4_KG.C_LG_XIII0043), PtPrx IIC (ESTEXT_FGENESH4_PG.C_LG_XVIII0691), PtPrx IIE (GW1.41.572.1) and PtPrx IIF (EUGENE3.00190240), Pt stands for *P. trichocarpa*; AtPrx IIB (At1g65980), AtPrx IIC (At1g65970), AtPrx IID (At1g60740), AtPrx IIE (At3g52960) and AtPrx IIF (At3g06050), At stands for *Arabidopsis thaliana*; and OsPrx IIC (Os01g48420), OsPrx IIE1 (Os06g42000), OsPrx IIE2 (Os02g09940) and OsPrx IIF (Os01g16152), Os stands for *Oryza sativa*. Gene models for *P. trichocarpa* sequences are available at http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html.

a supplementary duplication event in *A. thaliana* genome compared with rice and poplar. Prxs II are also found in other sequenced genomes from green lineage organisms and are present in green algae (*Ostreoccocus tauri, Chlamydomonas reinhardtii*) and mosses (*Physcomitrella patens* ssp. *patens*), and close homologues are found in most living kingdoms.

All of them possess the strictly conserved peroxidatic cysteine (Cys51 in the mature recombinant poplar Prx IIE), while the second cysteine (Cys76) is also present in all Prx IIE isoforms. It is worth mentioning that this cysteine, which is not absolutely necessary for activity, is absent in rice Prx IIC (Fig. 1) (Rouhier et al. 2002a). This cysteine is also absent in cytosolic isoforms from other plant species such as *Nelumbo nucifera* and *Vitis vinifera* (respective GenBank accession numbers ABN46981 and CAN73709) and also in an isoform from *P. patens* (JGI gene model: estExt_gwp_gw1.C_430017). In addition, the second cysteine is also not present in the bacterial Prx–Grx fusion proteins from *Haemophilus influenzae* and *Actinobacillus actinomycetemcomitans* (respective GenBank accession numbers P44758 and BAD02311), supporting the fact that it is not essential for catalysis (Cha et al. 2004, Kim et al. 2003). A third cysteine, Cys115, is also present in poplar Prx IIE but absent in all other Prx IIE sequences, so unlikely to play a role in catalysis. Besides the peroxidatic cysteine, the two other amino acids (Thr 48 and Arg 132 in Prx IIE) composing the catalytic triad are present in all Prx II sequences (Rouhier et al. 2004b).

Expression of Prx IIE as a recombinant protein

The sequence for the precursor of chloroplastic poplar Prx IIE (JGI gene model: GW1.41.572.1) codes for a protein of 218 amino acids, and prediction programs suggest that the protein is directed to chloroplasts via a transit sequence of nearly 54 amino acids situated at the N-terminus. As protein precursors are often insoluble, rarely stable and easily degraded (Miller et al. 2004), we expressed the protein in a shortened version in *E. coli* cells. The plasmid construction pET-Prx IIE, generated as indicated in the Methods section, codes for a shortened protein of 165 amino acids starting with the N-terminus sequence MATIS and allows expression of a soluble recombinant protein in *E. coli*. The theoretical molecular mass and pI of the recombinant protein are 17 380 Da and 4.7, respectively. By addition of ammonium sulphate, we separated the Prx IIE which precipitates between 40 and 80% of the saturation from the generally more poorly soluble proteins present in the 0–40% fraction. A combination of gel filtration and ion exchange chromatography resulted in a protein preparation more than 95% homogeneous when analysed by SDS-PAGE (see inset in Fig. 2).

Biochemical characterization

The capacity of various reductants (Trx and GSH in the presence or absence of Grx) to support poplar Prx IIE activity was first measured. While poplar Prx IIB is not regenerated by GSH alone (Rouhier et al. 2001), Prx IIE is very slightly able to use reduced GSH alone for its regeneration (Fig. 2). However, when GSH was complemented with either dithiol cytosolic Grx C4 (CPYC active site) or monothiol chloroplastic Grx S12 (CSYS active site), a very high Prx IIE activity was recorded (Fig. 2). We then tested two other *Arabidopsis* chloroplastic Grxs termed Grx S14 and S16, which possess a CGFS active site. Both proteins were unable to serve as reductants for Prx IIE in the presence of GSH. In order to get additional information concerning the regeneration mechanism of Prx II proteins, we have also investigated the ability of cysteine-mutated Grxs to support Prx IIE activity. The removal of the first active site cysteine (mutant C27S) of the well-characterized Grx C4 completely abolished the Grx-dependent activity of Prx IIE, while the suppression of the second 'backup' cysteine (mutant C30S) did not affect the regeneration and catalysis of Prx IIE (Fig. 2). Trxs were also found to be potential donors to Prx IIE, but the rates recorded in the presence of the non-physiological tandem constituted by the cytosolic Trx h1 and NTR in the presence of NADPH are 7- to 10-fold lower than those obtained with the GSH/Grx system under the experimental conditions used (Fig. 2).

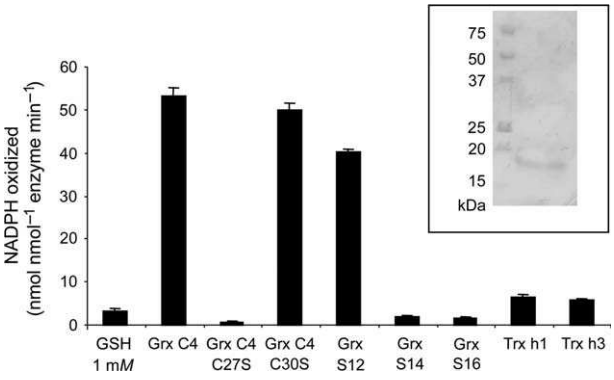

Fig. 2. GSH-, Grx- and Trx-dependent activity of poplar Prx IIE. Activities were recorded at 25°C with 50 μ*M* tBOOH as accepting substrate and 1 m*M* GSH or 20 μ*M* of each Grx and Trx tested by following NADPH oxidation at 340 nm. The measurements have been performed in triplicate. The values expressed in mean ± SE. In the inset, the recombinant poplar Prx IIE protein was separated on SDS-PAGE.

The catalytic parameters and substrate specificity of Prx IIE in the presence of a saturating Grx system (0.5 units GR, 20 μ*M* Grx C4 and 2 μ*M* Prx IIE) and various peroxides (H_2O_2, tBOOH or CUOOH) have been determined. The kinetic parameters are listed in Table 1. The apparent affinity of Prx IIE for tBOOH and H_2O_2 is in the 10 μ*M* range, which is quite low for a thiol peroxidase compared with Prx Q or Gpx for example (Navrot et al. 2006, Rouhier et al. 2004a). The best catalytic efficiency (k_{cat}/K_m) for Prx IIE was found with tBOOH (10.4×10^4 M^{-1} s^{-1}) followed by H_2O_2 (2.6×10^4 M^{-1} s^{-1}), whereas CUOOH is not reduced by the enzyme. Moreover, we observed that CUOOH completely inhibits Prx IIE activity. Indeed, adding CUOOH to a reaction medium containing H_2O_2 almost instantaneously abolished the activity of the Prx IIE (data not shown). When the protein was pre-treated with CUOOH, its H_2O_2-linked activity was also strongly inhibited. We also determined the affinity between Prx IIE and Grx C4 by measuring the rates at various Grx concentrations. The results obtained followed a typical Michaelis–Menten saturation curve, with an apparent K_m for Grx C4 of 0.51 ± 0.05 μ*M*.

Table 1. Kinetic parameters of poplar Prx IIE. These parameters were determined in steady-state conditions by following NADPH oxidation in the presence of various concentrations of peroxide (H_2O_2 and tBOOH) ranging from 10 to 200 μ*M*, 2 μ*M* Prx IIE and saturating concentrations of the Grx system (0.4 units GR, 1 m*M* GSH and 20 μ*M* Grx C4).

Substrate	$K_{peroxide}$ (μ*M*)	k_{cat} (s^{-1})	$k_{cat}/K_{peroxide}$ ($\times 10^4$ M^{-1} s^{-1})
H_2O_2	21.7 ± 5.8	0.57 ± 0.04	2.6 ± 0.9
tBOOH	8.6 ± 5.3	0.90 ± 0.10	10.4 ± 0.1

Expression analysis

Abundance of plastidial Prx IIE in Arabidopsis *organs*

The Prx II and Grx S12 (a putative physiological reductant of Prx IIE) transcripts were analysed using microarray data available through Genevestigator (Zimmermann et al. 2004). Besides *Prx IIB*, *Prx IIE* is the most expressed Prx II in green tissues (rosettes and seedlings), and it is less expressed in roots (Fig. 3A). In addition, the expression detected in flowers is consistent with the very strong promoter activity observed in stamens of *Arabidopsis* plants carrying the *AtPrx IIE*–promoter-β-glucuronidase reporter gene fusion (Brehelin et al. 2003). *Grx S12* is found in all organs analysed, generally at quite low levels, and does not show huge variation between organs although smaller levels are found in roots and seeds. This transcript analysis has been extended to other members of the *Arabidopsis* Prx II family. *Prx IIB* is the most expressed whatever the organ considered; *Prx IIC* and *Prx IID* cannot be differentiated from microarray data, but their expression is very high in flowers (especially huge in stamen and pollen, data not shown) and roots and very low in rosettes. In agreement with previous work, *Prx IIF* is also constitutively expressed (Finkemeier et al. 2005, Gama et al. 2007, Navrot et al. 2006).

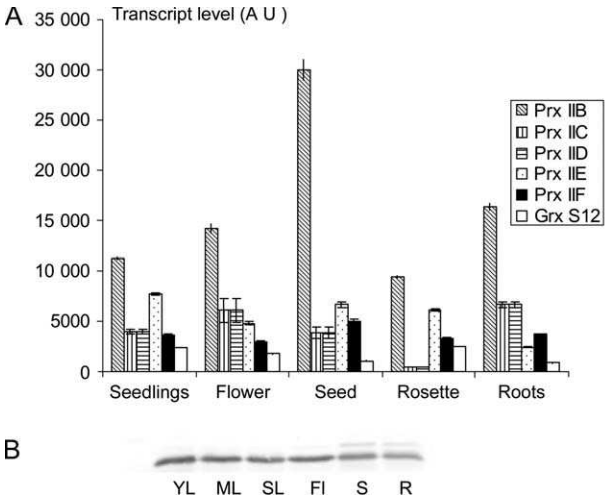

Fig. 3. Transcript and protein amount analysis of Prx IIE in various organs of *Arabidopsis thaliana*. (A) Transcript profile analysis of *Arabidopsis* Grx S12 and Prx II members from microarray data available using Genevestigator (http://www.genevestigator.ethz.ch/at/) (Zimmermann et al. 2004). The diagrams show the means of transcript levels of *Grx S12* and *Prx II* genes in seedlings and different organs of *A. thaliana*. Only experiments using *Arabidopsis* ATH1 chips (Affymetrix) and *A. thaliana* ecotype Columbia-0 were analysed. AtPrx IIC and AtPrx IID transcript levels are represented by the same bar, the two mRNAs being recognized by the same probe on ATH1 chips. Standard error is represented by error bar. (B) Western analyses of Prx IIE abundance in *Arabidopsis*. Soluble proteins (25 μg lane^{-1}) were separated by SDS-PAGE. YL, young leaf; ML, mature leaf; SL, senescent leaf; Fl, flower; S, stem; R, root; AU, arbitrary unit. The band corresponding to Prx IIE was revealed at 17 kDa.

Using a serum raised against the *A. thaliana* recombinant Prx IIE, the putative sub-cellular localization of Prx IIE was confirmed. *Arabidopsis* leaf chloroplasts were prepared, and as a control, we used a serum raised against the cytosolic Prx IIB, which did not react with the liberated stromal proteins (data not shown). With regard to Prx IIE, Western blot data showed a higher protein abundance in plastidial proteins in agreement with the results of Brehelin et al. (2003) (data not shown). Using Western analysis, the abundance of Prx IIE was investigated in various *A. thaliana* plant tissues (Fig. 3B). Unfortunately, this antibody did not cross-react with poplar extracts, preventing further characterization in this species. The protein was found at an apparent molecular mass of around 17 kDa in all organs analysed, the lowest level being noticed in roots and the highest in flowers and young leaves. A slight decrease in protein abundance was observed with leaf age. The protein distribution pattern of *A. thaliana* Prx IIE is in agreement with transcript data. A faint band around 18 kDa corresponding to Prx IIB, C or D, which are not distinguishable, was also visible in some organs (Brehelin et al. 2003).

Abundance of plastidic Prx IIE under stress conditions

The abundance of Prx IIE was investigated under various stress conditions and compared with the levels of the two other Prxs present in plastids, namely 2-Cys Prx and Prx Q, and of the cytosolic Prx IIB, C and D as the antibody cannot differentiate these isoforms. *Arabidopsis* plants were subjected to water deficit. After 6 days, they displayed severe wilting symptoms, mainly in mature leaves where the leaf RWC dropped to approximately 65% (data not shown). Almost no change in leaf Prx IIE amount was observed in water-stressed plants (Fig. 4A). Interestingly, the BAS1 2-Cys Prx protein abundance was also not noticeably modified upon water deficit, whereas in well-expanded leaves, water shortage resulted in substantial increases in cytosolic Prx IIB and plastidial Prx Q abundances. In leaf protein extracts from tomato and barley, the serum specifically revealed bands at around 16 and 17 kDa, respectively, likely corresponding to orthologs of Prx IIE in these species. The abundance of these proteins is not varying so much in both species exposed to water deficit when well-expanded leaves displayed an RWC of around 60% (Fig. 4B).

Arabidopsis plants were exposed to a photo-oxidative treatment under high light (1400 μmol m^{-2} s^{-1}) and low temperature (8°C) for 8 days. After 3 days of treatment, old leaves exhibited symptoms as bleaching and necrosis

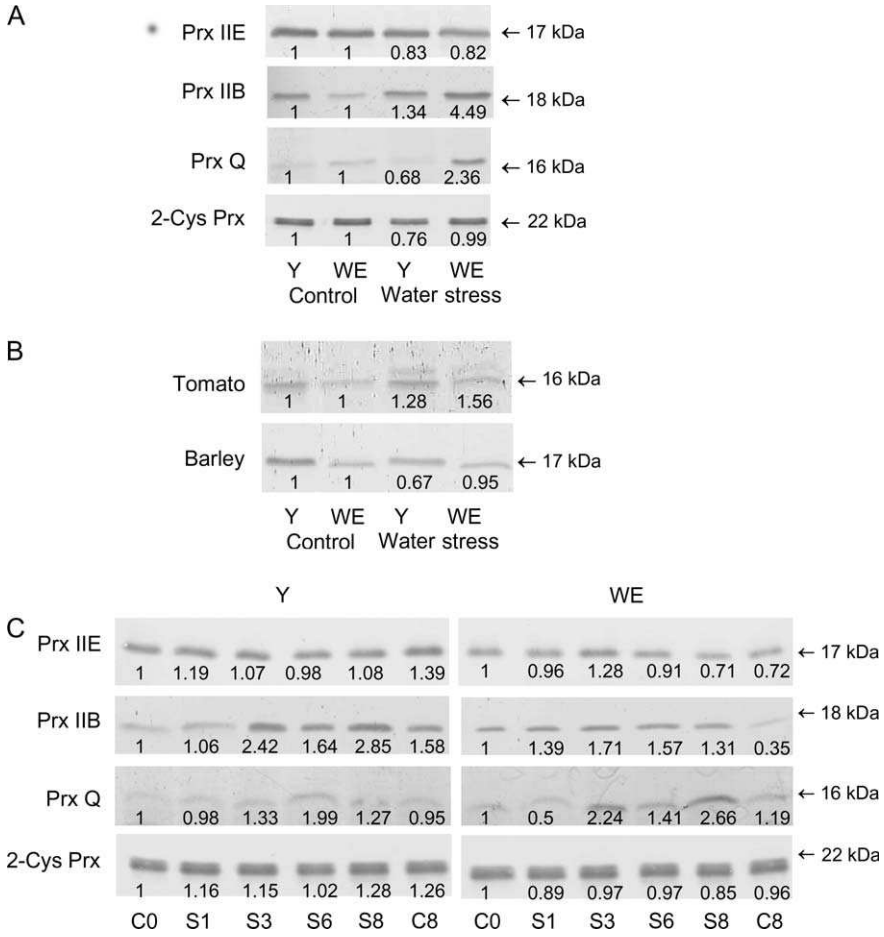

Fig. 4. Western analyses of Prx IIE abundance in plants subjected to abiotic stress conditions. Each experiment has been done on two independent biological samples. The protein bands have been quantified using the Quantity one software, and the number indicated represents the ratio of the band intensity between the stress and control experiments. (A) Prx IIE abundance in young and well-expanded leaves of *Arabidopsis thaliana* in water deficit conditions. (B) Abundance of plastidic Prx IIE in young and well-expanded leaves of tomato and barley plants subjected to water deficit (RWC around 65%). (C) Prx IIE abundance in young and well-expanded leaves of *A. thaliana* during photo-oxidative treatment (high light 1400 μmol m^{-2} s^{-1} and low temperature 8°C) for 8 days. C, control conditions for 0 or 8 days; S, photo-oxidative treatment for 1, 3, 6 or 8 days. Immunoblotting was performed on 15 μg protein per lane using the Prx IIE antibody diluted 1:10 000. Y, young developing green leaves; WE, well-expanded leaves; Control, well-watered plants; Water stress, plants subjected to water shortage for 6 days (RWC around 70%).

and were much more damaged than young leaves, which accumulated anthocyanins earlier (data not shown). In both young and well-expanded leaves, no noticeable change was observed in Prx IIE abundance during the treatment (Fig. 4C). With regard to the two other plastidial Prxs, almost no change in BAS1 2-Cys Prx amount was recorded during the stress period either in young or in well-expanded leaves, whereas an increase in Prx Q level was noticed in well-expanded leaves of plants exposed to high light under low temperature (Fig. 4C). In other respects, the treatment induced an increase in Prx IIB abundance in young leaves.

Discussion

The catalytic and regeneration mechanisms used by most Prx II members have been extensively analysed, but the reductant employed by the chloroplastic Prx IIE to support its peroxidase activity has remained so far elusive. As an example, *Arabidopsis* Prx IIE was reported not to accept electrons from AtTrx h2 or AtGrx1 (Brehelin et al. 2003). Likewise, a Prx II from *Synechocystis* was shown to use GSH for its activity with a very weak effect of Grx and almost no activity with a Trx reducing system (Hosoya-Matsuda et al. 2005). As some differences have already been observed for Prx IIB and Prx IIF between ortholog

proteins from different species, it was of interest to analyse the regeneration mechanism of poplar Prx IIE and its specificity for a given reductant. Unlike the mitochondrial Prx IIF, which is quite efficiently regenerated via GSH alone although with a lower efficiency than with a GSH–Grx system (Gama et al. 2007), poplar Prx IIE is almost not reduced by GSH alone but is very efficiently regenerated by the GSH–Grx system. The Prx IIE is thus closer to the cytosolic Prx IIB, which is reduced by either the Trx or the Grx system, the GSH–Grx system being more efficient. In terms of substrate specificity, Prx IIE can reduce both H_2O_2 and more complex peroxides such as tBOOH but is unable to reduce CUOOH, which even inactivated the protein. The broad substrate specificity common to most Prx is crucial for their physiological functions because catalases and ascorbate peroxidases degrade only H_2O_2.

Based on results obtained with the Grx reducing system, a regeneration scheme can be proposed, based on the absence of resolving cysteines in several Prxs II and on the full activity observed with a Grx mutated on the second active site cysteine. The sulfenic acid formed on the peroxidatic cysteine would be first attacked by GSH (Fig. 5). As GSH alone cannot support Prx IIE activity, the catalytic cysteine of Grx (in position 27 in Grx C4) is probably attacking then the glutathionylated Prx, leading to the formation of a GSH adduct on the Grx catalytic cysteine, which is then resolved by another GSH molecule. These data are also consistent with the description of glutathionylated forms of poplar Prx IIB (Noguera-Mazon et al. 2006). This mechanism would be compatible with both dithiol and monothiol Grxs. Nevertheless, we cannot exclude that an intramolecular disulfide bond can be formed in poplar Prx IIE between the peroxidatic and the resolving cysteines. In this case, the reduction of this disulfide by GSH, Grx or Trx could occur using several alternative mechanisms, not presented in Fig. 5.

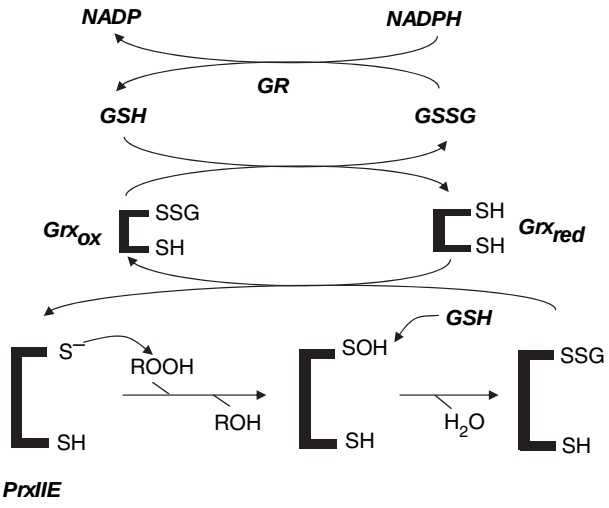

Fig. 5. Proposed regeneration mechanism of Prx IIE using the GSH/Grx system.

The number of Grxs in the chloroplast is dependent on species type. While six are predicted to be present in this compartment in *A. thaliana*, there are only three in *P. trichocarpa*, Grx S12 with a CSYS active site belonging to subgroup I and Grx S14 and S16 with CGFS active sites belonging to the subgroup II. We have experimentally confirmed the chloroplastic localization for Grx S12, S14 and S16 (Rouhier N, Gualberto JM, Couturier J, in preparation; Bandyopadhyay et al. in press). At this point, it cannot be ruled out that other Grxs, especially those belonging to the large subgroup III with CCxC active sites, can be imported into plastids (Rouhier et al. 2004c, 2006b). Grx S12, S14 and S16 have been tested in this study, but only Grx S12 was able to support regeneration of Prx IIE activity, suggesting that it could be the physiological reductant used by this Prx in vivo. Moreover, microarray data indicate that Grx S12 is coexpressed with Prx IIE in the five organs tested, although the Grx S12 transcript levels are weak in seeds and roots (Fig. 3). This is consistent with the isolation of a Prx IIE on a Grx affinity column (Rouhier et al. 2005). The absence of regeneration observed with the two CGFS Grxs is not unexpected because no reductase activity with other conventional substrate such as hydroethyldisulfide or dehydroascorbate has been observed with those Grxs in the presence of reduced GSH (data not shown).

In other respects, two cytosolic poplar Trxs (Trx h1 and h3) were shown to poorly reduce Prx IIE. As Trx h1 was not very efficient for Prx IIE regeneration, but was always as active as the best chloroplastic Trxs for the regeneration of other chloroplastic thiol peroxidases (poplar Prx Q and Gpx 1 and 3.2), we assume that the chloroplastic Trxs are not good physiological partners, although we cannot completely exclude the possibility that some untested chloroplastic Trxs might be better catalysts. For instance, CDSP32, a specific Trx present in the chloroplast which is able to interact with other chloroplastic Prxs (Prx Q and 2-Cys Prx), could use a mechanism similar to Grx (Rey et al. 2005, Vieira Dos Santos et al. 2007). As the chloroplastic Trxs are not reduced by NTR, the activity test based on NADPH oxidation cannot be used. An alternative would have been to use the ferrous oxidation in xylenol orange (FOX) colorimetric assay, but contrary to 2-Cys Prx, Prx Q or Gpxs, Prx IIE is directly reduced by dithiothreitol present in the reaction media, preventing the measurement of chloroplastic Trx-dependent activity through this experimental design.

From a physiological point of view, the preferential reduction of Prx IIE in the chloroplast by an NADPH/GR/

GSH/Grx-dependent system is important as this antioxidant couple could function in the dark contrary to the light-dependent reduction of some other thiol peroxidases when they used FTR and chloroplastic Trxs for their reduction. Nevertheless, this is also true for some other chloroplastic Prxs (2-Cys Prx and Prx Q), which are possibly reduced by both the light and the NADPH/NTRc-dependent pathways (Moon et al. 2006). The hypothesis would be that these NADPH-dependent pathways are used in non-chlorophyll tissues, while light-dependent pathways are used in photosynthetic tissues.

This biochemical analysis was completed by studying the abundance of Prx IIE in *Arabidopsis* organs and plants subjected to various abiotic stresses and comparing it with the one of the two other plastidial Prxs (Prx Q and 2-Cys Prx) and of the three cytosolic Prx (Prx IIB, C and D). The expression pattern of Prx IIF under these conditions was previously investigated (Gama et al. 2007). The Prx IIE is constitutively present in all tissues analysed in *A. thaliana,* with a lower level in roots. Under the stress conditions examined (water deficit and photo-oxidative treatment) and regardless of the species considered (*A. thaliana,* tomato and barley), there is almost no variation in Prx IIE amount. This is in agreement with other data in *Arabidopsis* plants exposed to heavy metals (copper or cadmium; Collin et al. 2007). Concerning the cytosolic compartment, the abundance of Prx IIB, C and D members, which cannot be distinguished at the protein level because the antibody is not specific enough, increases in response to photo-oxidative stress and water deficit (this study) and is modified in response to a biotic stress in poplar (infection by the rust fungus *Melampsora laricii populina*) (Rouhier et al. 2004a). At the transcript level, the expression of *A. thaliana Prx IIB* and *IIC* is induced by oxidative stress conditions (peroxide and diamide treatments) and salt stress, whereas the expression of *AtPrx IIE* was not affected by these oxidative stress conditions but was decreased in response to ascorbate application and a salt stress and after a transfer of plants from adequate light to low light (Horling et al. 2002, 2003). On the contrary, *AtPrx IIE* expression increased after a transfer from adequate light to high light (Horling et al. 2003).

If the expression patterns of the different plastidial Prxs under stress conditions are compared, two types of behaviour can be delineated: the abundances of 2-Cys Prx and Prx IIE do not generally vary in leaves of whole plants subjected to biotic or abiotic stresses (Collin et al. 2007, Gama et al. 2007, Havaux et al. 2005), while Prx Q and 'Gpx' abundances and expressions are modified by environmental stress conditions (Fig. 4) and also by biotic stress conditions (Kiba et al. 2005, Lamkemeyer et al. 2006, Miao et al. 2006, Navrot et al. 2006, Rodriguez Milla et al. 2003, Rouhier et al. 2004a). Altogether, these data would suggest that among plastidial Prxs, Prx Q and Gpxs, but not 2-Cys Prx and Prx IIE, are mostly involved in the responses to environmental constraints. In some circumstances, the expression of 2-Cys Prx and Prx IIE could nevertheless respond to oxidative stress conditions as in the case of salt stress where the transcript amount for the two *Arabidopsis* genes strongly decreases (Horling et al. 2002). However, we cannot exclude that post-translational modifications could control the activity of Prxs during stress conditions, without changing protein amount. Indeed, overoxidation of the catalytic cysteine to sulfinic or sulfonic acid form in 2-Cys Prxs results in inactivation of the enzyme, and the proportion of overoxidized Prx has been reported to vary as a function of environmental conditions independent of the protein abundance (Rey et al. 2007). Furthermore, Fang et al. (2007) very recently showed that the two catalytic cysteines in mammal Prxs are prone to nitrosylation and that this post-translational modification prevents the reaction with organic peroxides. Similar results have been described for *A. thaliana* Prx IIE, whose nitrosylation of the peroxidatic cysteine prevents its peroxide and peroxynitrite detoxifying activity (Romero-Puertas et al. 2007). In knock-out mutants treated with peroxynitrite, the authors observed increased nitrotyrosine residue formation, supporting the fact that Prx IIE could regulate both NO and ROS signalling pathways.

Conclusions

To date, the reducing partners of the chloroplastic Prx IIE were unknown. Here, we have demonstrated that both Trx and Grx were able to serve as electron donors for the poplar Prx IIE, although Grxs are by far more efficient. The physiological regeneration of poplar Prx IIE is likely to be dependent on Grx S12, as (1) among the three different established poplar chloroplastic Grxs (Grx S12, S14 and S16), this is the only one able to support Prx IIE activity in vitro and (2) *Grx S12* and *Prx IIE* transcripts are coexpressed in the same tissues analysed.

On the other hand, although the expression analyses have been done essentially in *A. thaliana* and it is sometimes difficult to extend the findings to other organisms, we can conclude from this study and a lot of previous reports that among the plastidial Prxs, Prx IIE and 2-Cys Prx are essentially not involved in the responses to environmental constraints, while the Gpx and Prx Q are.

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II Etudes du rôle des Grxs dans les machineries d'assemblage des centres fer-soufre

Les connaissances sur les machineries d'assemblage des centres Fe-S chloroplastiques et mitochondriaux restent relativement pauvres notamment chez les plantes, sans parler du système cytosolique qui est quasiment inexploré (Balk and Lobreaux, 2005). La plupart des données proviennent de la comparaison avec les systèmes bactériens et de levure, beaucoup plus détaillées (Lill and Mühlenhoff, 2005).

Les études effectuées chez *S. cerevisiae* ont démontré l'implication d'une Grx appelée Grx5 et possédant un site actif CGFS dans la biogenèse mitochondriale des centres Fe-S (Muhlenhoff *et al*., 2003; Rodriguez-Manzaneque *et al*., 2002). Des cellules de levures où le gène codant la Grx5 est éliminé sont plus sensibles au stress oxydant, sans doute en réponse à l'accumulation de fer libre issu de la dégradation des centres fer-soufre de certaines enzymes, en particulier l'aconitase et la succinate déshydrogénase (Rodriguez-Manzaneque *et al*., 2002). Des études ultérieures ont suggéré que la Grx5 pourrait servir à transférer des clusters préformés sur la protéine scaffod Isu1 vers des protéines receveuses (Mühlenhoff *et al*., 2003). En effet, ce mutant accumule des grandes quantités de protéine Isu1 possédant soit du fer, soit un cluster fer-soufre. La plupart des Grxs CGFS, qu'elles soient d'origine procaryote ou eucaryote, sont capables de complémenter l'essentiel des défauts du mutant *Grx5* de levure, suggérant que ces protéines pourraient jouer un rôle similaire dans l'organisme dont est issu la protéine (Cheng *et al*., 2006; Molina-Navarro *et al*., 2006). Le fait que certaines Grxs (Grx C1 de peuplier (CGYC) et Grx2 humaine (CSYS)) puissent assembler de façon

naturelle des centres Fe-S laissait à penser que le rôle de ces Grxs est directement lié à cette propriété.

Dans le but de caractériser les Grxs de classe II de plantes, les séquences matures des Grxs CGFS de peuplier et d'*Arabidopsis* ont été produites sous forme de protéines recombinantes dans *E. coli*. Cela a permis de mettre en évidence la présence de clusters Fe-S dans Grx S14 et S16 mais pas dans Grx S15. Cependant, des expériences de reconstitution de centres fer-soufre *in vitro* ont maintenant établi que l'on pouvait également incorporer un centre [2Fe-2S] dans GrxS15. La publication présente la caractérisation analytique et spectroscopique du centre [2Fe-2S] incorporé dans les Grx S14 et S16 de plantes.

Outre les localisations subcellulaires de chaque Grx CGFS de peuplier, ce travail présente également la capacité des Grx S14 et S16, mais pas Grx S15, à complémenter le mutant *Grx5* de levure.

Ces études sont représentées dans l'article 5: "Chloroplast monothiol glutaredoxins act as scaffold proteins for the assembly and delivery of [2Fe-2S] clusters"

II Etudes du rôle des Grx dans l'assemblage des centres fer-soufre

Article 5 : "Chloroplast monothiol glutaredoxins act as scaffold proteins for the assembly and delivery of [2Fe-2S] clusters"

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Chloroplast monothiol glutaredoxins as scaffold proteins for the assembly and delivery of [2Fe–2S] clusters

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Glutaredoxins (Grxs) are small oxidoreductases that reduce disulphide bonds or protein-glutathione mixed disulphides. More than 30 distinct grx genes are expressed in higher plants, but little is currently known concerning their functional diversity. This study presents biochemical and spectroscopic evidence for incorporation of a [2Fe–2S] cluster in two heterologously expressed chloroplastic Grxs, GrxS14 and GrxS16, and *in vitro* cysteine desulphurase-mediated assembly of an identical [2Fe–2S] cluster in apo-GrxS14. These Grxs possess the same monothiol CGFS active site as yeast Grx5 and both were able to complement a yeast *grx5* mutant defective in Fe–S cluster assembly. *In vitro* kinetic studies monitored by CD spectroscopy indicate that [2Fe–2S] clusters on GrxS14 are rapidly and quantitatively transferred to apo chloroplast ferredoxin. These data demonstrate that chloroplast CGFS Grxs have the potential to function as scaffold proteins for the assembly of [2Fe–2S] clusters that can be transferred intact to physiologically relevant acceptor proteins. Alternatively, they may function in the storage and/or delivery of preformed Fe–S clusters or in the regulation of the chloroplastic Fe–S cluster assembly machinery.

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Introduction

Iron–sulphur (Fe–S) proteins are intimately involved in numerous essential biological processes, such as photosynthesis, respiration and the metabolism of carbon, oxygen, hydrogen, nitrogen and sulphur (Johnson and Smith, 2005). However, little is known concerning the mechanism of Fe–S cluster biogenesis in plants. Much of the current understanding of Fe–S cluster biogenesis stems from investigation of components of the bacterial *isc* (iron–sulphur cluster assembly), *suf* (sulfur mobilization) and *nif* (nitrogen fixation) operons (Johnson *et al*, 2005) and identification and characterization of homologous ISC-type proteins in yeast and mammalian mitochondria (Lill and Mühlenhoff, 2005). The ISC, SUF and NIF Fe–S cluster assembly machineries share a common basic mechanism involving cysteine desulphurase (IscS, SufS and NifS)-mediated assembly of [2Fe–2S] or [4Fe–4S] clusters on U-type (IscU, SufU and N-terminal domain of NifU), A-type (IscA, SufA and ^{Nif}IscA) and Nfu-type (corresponding to the C-terminal domain of NifU) scaffold proteins, and subsequent intact cluster transfer into acceptor apo-proteins. In the case of the ISC machinery, [2Fe–2S] cluster transfer from IscU is facilitated by specific molecular co-chaperones (HscA and HscB) in an ATP-dependent reaction (Chandramouli and Johnson, 2006) and [4Fe–4S] cluster assembly on dimeric IscU occurs at the subunit interface via reductive coupling of two [2Fe–2S] clusters (Chandramouli *et al*, 2007). However, little is currently known concerning the detailed mechanism of Fe–S cluster assembly and transfer involving scaffold proteins. In plants, Fe–S cluster biosynthesis primarily occurs in mitochondria, using the ISC machinery with Isu, IscA and Nfu as potential scaffold proteins, and in chloroplasts using the SUF machinery with SufA, SufB and Nfu proteins as potential scaffold proteins (Balk and Lobreaux, 2005; Ye *et al*, 2006b; Layer *et al*, 2007).

Glutaredoxins (Grxs) are small proteins that normally function in the reduction of disulphide bridges or glutathionylated proteins. However, recent studies in *Saccharomyces cerevisiae* and *Escherichia coli* have indicated specific roles for some Grxs in facilitating Fe–S cluster biosynthesis (Rodríguez-Manzaneque *et al*, 2002; Mühlenhoff *et al*, 2003; Achebach *et al*, 2004). Yeast cells deleted for the *GRX5* gene were found to be more sensitive to oxidative stress, to accumulate free iron and to have impaired mitochondrial Fe–S cluster biogenesis and respiratory growth (Rodríguez-Manzaneque *et al*, 1999, 2002). Other prokaryotic and eukaryotic CGFS Grxs have been shown to be efficient functional substitutes for yeast Grx5 (Cheng *et al*, 2006; Molina-Navarro *et al*, 2006). Although the specific function of yeast Grx5 in Fe–S cluster biogenesis remains to be elucidated, ^{55}Fe radiolabelling studies of knockout mutants implicate a role in mediating transfer of clusters preassembled on the Iscu1p scaffold protein into acceptor

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proteins (Mühlenhoff *et al*, 2003). The discovery that Grx5 is also required for vertebrate haem synthesis raises the possibility that Grx5 is a key determinant for channelling Fe into haem and Fe–S cluster biosynthesis in mammals (Wingert *et al*, 2005).

The most obvious role for Grxs in Fe–S cluster biogenesis lies in facilitating Fe–S cluster assembly or transfer by reducing disulphides on scaffold or apo forms of Fe–S proteins. However, the discovery that some Grxs can assemble Fe–S clusters suggests the possibility of alternative roles in Fe–S cluster assembly or transfer. Poplar GrxC1 (CGYC active site) and human Grx2 (CSYS active site) are homodimers with a subunit-bridging [2Fe–2S] cluster ligated by one active site cysteine of each monomer and the cysteines of two external glutathione (GSH) molecules (Feng *et al*, 2006; Johansson *et al*, 2007; Rouhier *et al*, 2007). The cluster-containing dimeric form of human Grx2 was proposed to function as a redox sensor for the activation of Grx2 in case of oxidative stress (Lillig *et al*, 2005). Although this is a viable hypothesis, mutagenesis studies on poplar GrxC1 indicate that incorporation of a [2Fe–2S] cluster is likely to be a general feature of plant Grxs possessing a glycine adjacent to the catalytic cysteine (Rouhier *et al*, 2007). Hence CGFS Grxs, such as yeast Grx5, might have the capacity to incorporate a Fe–S cluster. Moreover, the requirement of GSH for the export of a Fe–S cluster (or a precursor thereof) from mitochondria to facilitate the assembly of cytosolic Fe–S proteins in *S. cerevisiae* (Lill and Mühlenhoff, 2005) provides further circumstantial evidence in support of a role for GSH- and Grx-ligated [2Fe–2S] clusters in Fe–S biogenesis.

In higher plants, around 30 different Grx isoforms can be classified into three distinct subgroups depending on their active site sequences (Rouhier *et al*, 2004). The first class, which contains Grxs with C[P/G/S][Y/F][C/S] motifs other than CGFS, is homologous to the classical dithiol Grxs such as *E. coli* Grx1 and 3, yeast Grx1 and 2 and mammalian Grx1 and 2. The second class has a strictly conserved CGFS active site sequence and includes Grxs homologous to yeast Grx3, 4 and 5 or *E. coli* Grx4. Plants have generally four members in this group (GrxS14 to S17). The properties of proteins of the third class, which is specific to higher plants and involves a CC[M/L][C/S] active site, are largely unknown.

This study presents biochemical, spectroscopic and analytical evidence for the incorporation of [2Fe–2S] clusters in two plant chloroplast CGFS Grxs, GrxS14 and S16, and both *in vivo* and *in vitro* evidence for their involvement in the maturation of Fe–S proteins. The results demonstrate that monothiol Grxs have the potential to function as scaffold proteins for *de novo* synthesis and efficient delivery of [2Fe–2S] clusters, as Fe–S cluster delivery or storage proteins for mediating the transfer of Fe–S clusters from ISC or SUF scaffold proteins to acceptor proteins, or as sensors of the cellular Fe–S cluster status in Fe homoeostasis.

Results

The plant CGFS Grx subgroup

In silico analysis of Grxs from different kingdoms reveals that four or five Grxs with CGFS active site are generally present in higher plants and in *Chlamydomonas reinhardtii*, whereas only three are present in *S. cerevisiae*, two in most other fungi and in mammals, one in *Synechocystis* and in *E. coli* (Rouhier *et al*, 2004). In *Populus trichocarpa*, GrxS14 and S15 are small proteins (171 and 172 amino acids, respectively, including the transit peptide sequence) with a single repeat of the Grx module. GrxS16 is larger (296 amino acids including the transit peptide sequence) with an N-terminal extension linked to the Grx module. GrxS17 is larger (492 amino acids) and displays an N-terminal Trx-like domain with a WCDAS active site followed by three successive Grx modules. A careful examination of amino-acid sequence alignments (Supplementary Figure 1) indicates that although not present in all CGFS Grxs, a second cysteine is found in 60% of the 250 CGFS Grxs found in GenBank at a conserved position closer to the C terminus. It is conserved in GrxS14 and S16 (Cys 87 in GrxS14 and Cys 221 in GrxS16, numbering based on the recombinant mature protein sequences), in the third module of GrxS17 and in GrxC1, whereas it is absent in GrxS15, in the second Grx domain of GrxS17 and in GrxC4 and only partly conserved in the first Grx domain of plant GrxS17. In *Sc*Grx5, these two cysteines are able to form a disulphide bridge in the presence of oxidized GSH (Tamarit *et al*, 2003).

Subcellular localization of the CGFS Grxs

We have determined the localization of all the CGFS Grxs that possess an N-terminal transit sequence. GrxS17, predicted to be cytosolic, does not seem to possess such an extension and its localization has not been characterized further. The full-length sequences of the three other Grxs devoid of the stop codon were introduced in frame before the GFP sequence and the construction was used to bombard tobacco leaves. As shown in Figure 1, the fluorescence of GrxS14 and S16 strictly coincides with the one of the chlorophyll, whereas the fluorescence of GrxS15 superimposed well with one of the mitochondrial marker. Therefore, GrxS14 and S16 are chloroplastic and GrxS15 is mitochondrial.

Some poplar monothiol but not dithiol Grxs rescue the defects of a yeast mutant lacking Grx5

To determine whether the four poplar monothiol Grxs rescue the defects of a *yeast* Δ*grx5* mutant, we targeted the proteins at the mitochondrial matrix (Molina *et al*, 2004). All the proteins were adequately compartmentalized in the mitochondrial matrix (Figure 2A). Of the two poplar Grxs with a single Grx domain, GrxS14 and S15, only the first one rescued the *grx5* mutant defects in respiratory growth (Figure 2B) and its sensitivity to oxidants (Figure 2C). These defects were also efficiently rescued by GrxS16 and S17. To test whether a single Grx domain is sufficient for the function of GrxS17, we fused only the most C-terminal domain of S17 (from amino acid 398 to 492) to the mitochondrial targeting sequence of Grx5 ($S17_{398-492}$). This protein was also compartmentalized at the mitochondrial matrix (Figure 2A), although a double band appeared. The band with lower mobility probably corresponds to unprocessed precursor still compartmentalizing at yeast mitochondria. $S17_{398-492}$ suppressed partially the growth phenotypes of the *grx5* mutant, in particular growth in respiratory conditions (Figure 2B). The ratio of activities of the mitochondrial enzymes aconitase (containing Fe–S clusters) and malate dehydrogenase (without Fe–S clusters) was used as a measure of the efficiency of the Fe–S cluster assembly in mitochondrial proteins (Molina *et al*, 2004). This ratio was measured in strains carrying all these constructions in a chromosomal *grx5* back-

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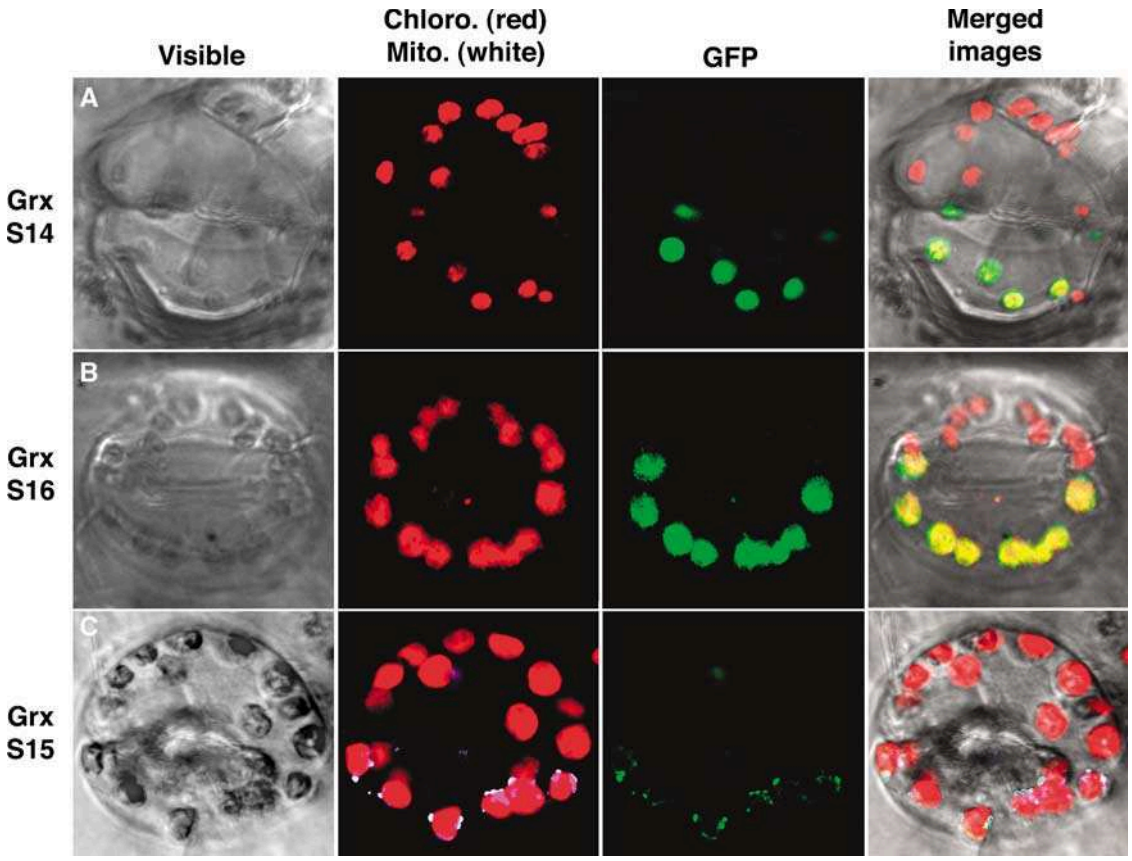

Figure 1 Subcellular localization of CGFS Grxs by GFP fusion. (**A**) GrxS14, (**B**) GrxS16 and (**C**) GrxS15. From left to right: visible light, autofluorescence of chlorophyll (red) or mitochondrial marker (white); fluorescence of the constructions and merged images. Only one of the guard cells shows chloroplast-localized GFP, because a small numbers of cells were transfected. As the mitochondrial marker (DsRed) is co-transfected with the GFP construction, it is only visible in the cell that expresses GFP.

ground (Figure 2D). Ratios were comparable with wild type in strains expressing the mitochondrial forms of S14, S16 and S17, in accordance with the growth phenotypes. In contrast, both S15 and the truncated form of S17 exhibited much lower enzyme ratios (Figure 2D). For all the strains tested, absolute malate dehydrogenase levels were basically similar, and doxycycline addition to growth media lowered aconitase activity to basal levels (data not shown). For the monothiol Grxs analysed, there appears to be a correlation between efficiency to express active aconitase and growth phenotypes, except that poplar mitochondrial S15 did not rescue at all the growth defects of the yeast mutant, although still being able to synthesize low levels of mature aconitase. The anomalous results with S15 and $S17_{398-492}$ are puzzling and may indicate functional diversity within the general class of monothiol Grxs (see below) or that aconitase maturation does not provide a good measure of the efficiency of general Fe–S cluster biosynthesis in mitochondria. The latter is supported by a very recent study of the requirements for mitochondrial aconitase Fe–S cluster maturation in *S. cerevisiae*, which indicated a specific requirement for Isa1p, Isa2p and the Iba57, proteins that are not required for general Fe–S cluster biogenesis in mitochondria (Gelling *et al*, 2008).

To determine whether the capacity to bind a Fe–S cluster is sufficient for *grx5* complementation, we then used poplar GrxC1 (CGYC), which incorporates a [2Fe–2S] centre and GrxC1 G32P (CPYC) and GrxC4 (CPYC) which do not (Rouhier *et al*, 2007). Although all these Grxs were targeted to the matrix (Figure 3A), none of these proteins rescued (i) the inability of a *grx5* mutant for respiratory growth, (ii) the sensitivity to oxidants and (iii) the capacity to assembly a Fe–S cluster in aconitase (Figure 3B–D). None of the three dithiol Grxs, even the one binding a Fe–S cluster, is functional in yeast mitochondria for the maturation of Fe–S proteins. To determine whether this is caused by structural incompatibility of the dithiol Grxs with the Fe–S cluster biosynthetic machinery or more specifically by the different active site sequences with either dithiol or monothiol motifs, we modified the CGYC and CPYC active sites of GrxC1 and GrxC4 into CGFS to mimic the active site sequence of Grx5. The resulting GrxC1 CGFS fully substituted for yeast Grx5 with respect to all phenotypes analysed (Figure 3), whereas GrxC4 CGFS did not (data not shown). The GrxC1 CGFS rescuing effects did not occur when its expression from the *tet* promoter was switched off by doxycycline addition to the growth medium (data not shown). We therefore conclude that the requirement for a monothiol Grx active site could preclude poplar dithiol Grxs from functionally rescuing a *grx5* mutant, but in some cases, exemplified by the GrxC4 CGFS derivatives, other sequence or structural requirements are needed.

Purification and spectroscopic characterization of Fe–S cluster-containing poplar GrxS14 and AtGrxS16

The mature form of the three organellar poplar CGFS Grxs was expressed in *E. coli* to check their ability to incorporate

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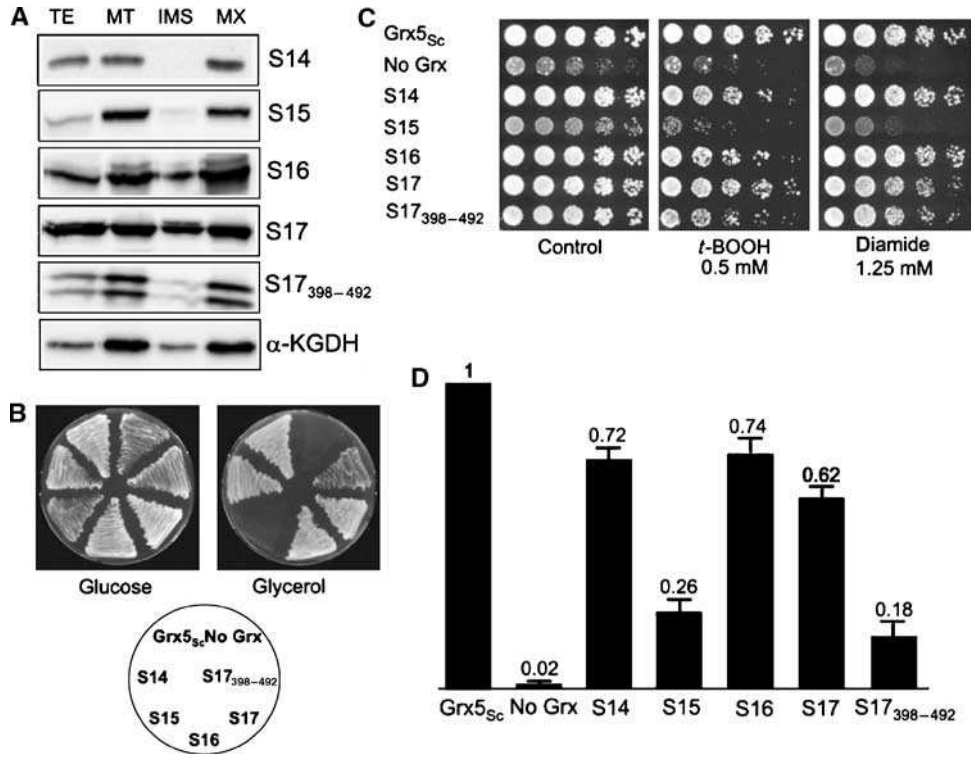

Figure 2 Rescue of the *S. cerevisiae grx5* mutant defects by poplar monothiol glutaredoxins. (**A**) Compartmentalization of GrxS14, S15, S16, S17 and $S17_{398-492}$ in the mitochondrial matrix of *S. cerevisiae* cells. Cultures were grown exponentially in YPLactate medium at 30°C to about 3×10^7 cells ml^{-1}, before mitochondrial isolation and subfractionation. TE, total cell extract; MT, mitochondrial fraction; IMS, intermembrane space; MX, matrix. Proteins (20 μg) were loaded in the TE lanes, and 5 μg was loaded in the other lanes. Anti-HA anti-lipoic acid antibodies were used in the western blot to detect the HA-tagged proteins, and the matrix marker α-ketoglutarate dehydrogenase (α-KGDH). (**B**) Growth on glucose (YPD plates) or glycerol (YPGly plates), after 3 days at 30°C. (**C**) Sensitivity to *t*-BOOH or diamide of the strains after 3 days at 30°C on YPD plates. (**D**) Ratio between aconitase and malate dehydrogenase activities in exponential cultures at 30°C in YPGalactose medium.

Fe–S clusters. On the basis of our previous experience with GrxC1, GSH, which stabilize and ligate the [2Fe–2S] cluster, was added during the first steps of the purification (Rouhier *et al*, 2007). Although the presence of a brownish colouration typical of a Fe–S cluster was clearly evident in cells overexpressing poplar and *Arabidopsis thaliana* (At) GrxS14 and S16, almost no holoprotein was obtained at the end of an aerobic purification, even in the presence of GSH, suggesting that the cluster degrades quickly in air. In contrast, there was no indication of a Fe–S cluster prosthetic group in recombinant poplar or *A. thaliana* GrxS15.

Purification of poplar GrxS14 under strictly anaerobic conditions was undertaken to address the type, stoichiometry and properties of the putative Fe–S centre. The reddish-brown purified samples contained 0.80 ± 0.10 Fe per monomer. The UV–visible absorption and CD spectra of anaerobically purified poplar GrxS14 are shown in Figure 4 and both are characteristic of a $[2Fe–2S]^{2+}$ centre (Stephens *et al*, 1978; Dailey *et al*, 1994). On the basis of the theoretical and experimental ε_{280} values for the apo protein ($9.9\,mM^{-1}\,cm^{-1}$), the ε_{280} and ε_{411} values for the $[2Fe–2S]^{2+}$ centre are estimated to be 3.9 and $4.4\,mM^{-1}\,cm^{-1}$, respectively, and the A_{411}/A_{280} was found to be 0.31 ± 0.04. In accord with the analytical data, these extinction coefficients are indicative of 0.4–0.5 $[2Fe–2S]^{2+}$ clusters per monomer. Hence the analytical, absorption and CD data are consistent with approximately one $[2Fe–2S]^{2+}$ per dimeric GrxS14. Anaerobically purified At GrxS16 contained an analogous $[2Fe–2S]^{2+}$ centre as judged by very similar UV–visible absorption and CD spectra (Figure 4).

In vitro reconstitution of aerobically purified apo GrxS14 was attempted under strictly anaerobic conditions in the presence of 5 mM GSH and 2 mM DTT, using Fe(II), L-cysteine and catalytic amounts of *E. coli* IscS. After chromatographic removal of excess reagents, the resulting cluster-loaded form of GrxS14 was essentially identical to anaerobically purified [2Fe–2S] GrxS14, as judged by Fe analyses and UV–visible absorption and CD spectra (data not shown). GSH was required for successful reconstitution of a [2Fe–2S] cluster on GrxS14. Samples of apo GrxS14 reconstituted using the same procedure in a reaction mixture containing 2 mM DTT, but no GSH, showed no evidence of the presence on a bound Fe–S cluster following repurification. Hence, *in vitro* Fe–S cluster reconstitution studies confirm the potential of poplar GrxS14 to act as a scaffold for the assembly of [2Fe–2S] clusters in a cysteine desulphurase-mediated reaction and indicate that GSH is required for cluster assembly.

Resonance Raman and Mössbauer studies of anaerobically purified poplar GrxS14 confirm the presence of a $[2Fe–2S]^{2+}$ centre and provide insight into the cluster ligation. Resonance Raman spectra obtained using 457- and 514-nm excitation reveal Fe–S stretching modes at 288, 332, 347, 365, 402 and $424\,cm^{-1}$ (Figure 5). The vibrational frequencies are generally similar to those of structurally characterized [2Fe–2S]

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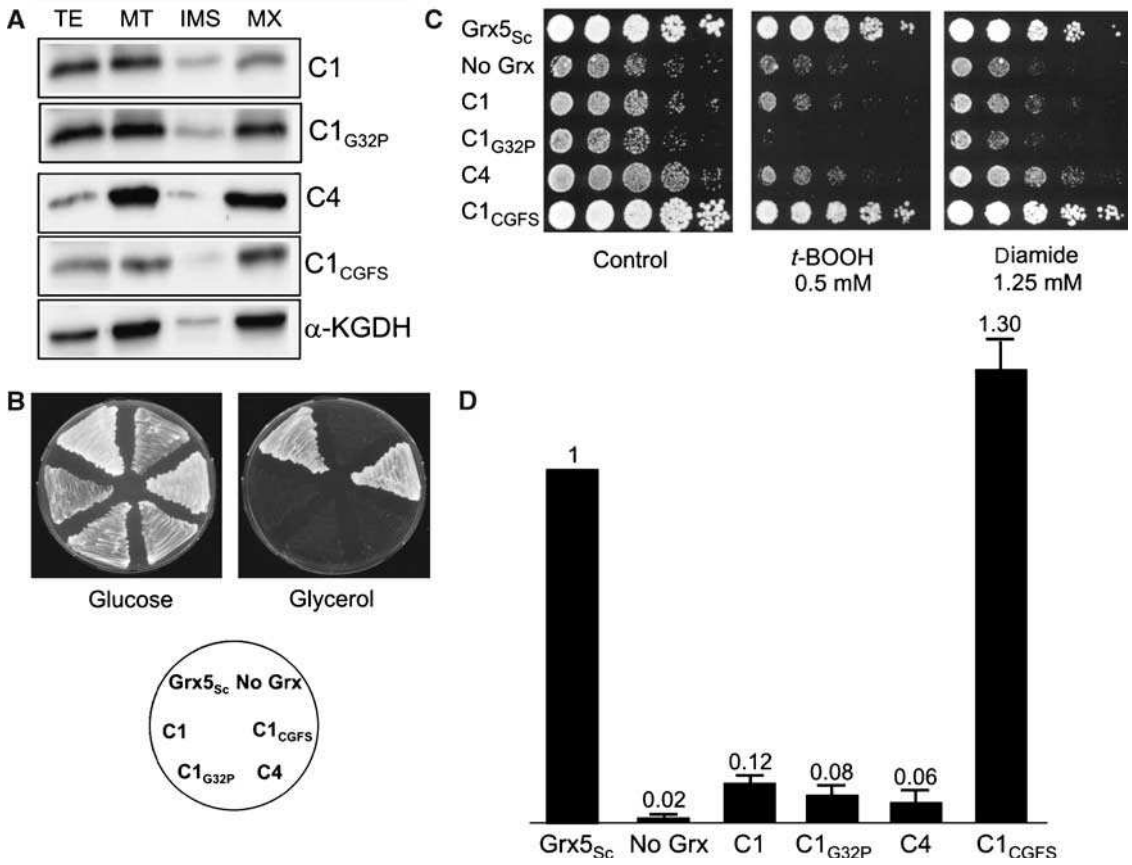

Figure 3 Rescue of the *S. cerevisiae grx5* mutant defects by poplar dithiol glutaredoxins. (**A**) Compartmentalization of GrxC1, $C1_{G32P}$, C4 and $C1_{CGFS}$ in the mitochondrial matrix of *S. cerevisiae* cells. Growth conditions and western blot analyses are similar to those described in Figure 2. TE, total cell extract; MT, mitochondrial fraction; IMS, intermembrane space; MX, matrix. (**B**) Growth on glucose (YPD plates) or glycerol (YPGly plates), after 3 days at 30°C. (**C**) Sensitivity to *t*-BOOH or diamide of the strains after 3 days at 30°C on YPD plates. (**D**) Ratio between aconitase and malate dehydrogenase activities in exponential cultures at 30°C in YPGalactose medium.

ferredoxins with complete cysteinyl cluster ligation and are readily assigned to vibrational modes of the $Fe_2S_2^bS_4^t$ unit (S^b = bridging S and S^t = terminal or cysteinyl) by direct analogy with published data (Han *et al*, 1989; Fu *et al*, 1992). Figure 6 compares the Mössbauer spectra of poplar GrxS14 with those of the all cysteinyl-ligated $[2Fe–2S]^{2+}$ cluster in poplar GrxC1 and the IscU $[2Fe–2S]^{2+}$ cluster which has one non-cysteinyl ligand (Agar *et al*, 2000). Each spectrum is indicative of a $S=0$ $[2Fe–2S]^{2+}$ centre that results from antiferromagnetic coupling of two high-spin Fe(III) ions and is simulated as the sum of quadrupole doublets from each Fe site using the parameters listed in the figure legend. The similarity and values of the isomer shift (δ) and quadrupole splitting (ΔE_Q) parameters for each Fe site of the $[2Fe–2S]^{2+}$ clusters in GrxC1 and GrxS14 are consistent with approximately tetrahedral S ligation at each Fe site. Non-cysteinyl ligation is generally manifested by anomalous isomer shifts and quadrupole splittings for the unique Fe site, which results in marked asymmetry in the observed spectrum, as is apparent in the spectrum of $[2Fe–2S]^{2+}$ centre in IscU (Agar *et al*, 2000). Hence, the Mössbauer data indicate a $[2Fe–2S]^{2+}$ cluster as the sole Fe-containing prosthetic group in anaerobically purified poplar GrxS14, and the Mössbauer and resonance Raman data taken together provide support for complete cysteinyl ligation.

Cluster ligation in GrxS14 and S16

The two structurally characterized $[2Fe–2S]^{2+}$ centres in dithiol Grxs, human Grx2 (CSYC active site) and poplar GrxC1 (CGYC active site) have very similar absorption and CD spectra and have analogous coordination environments, involving the catalytic cysteine of two Grxs and the cysteines of two GSH (Johansson *et al*, 2007; Rouhier *et al*, 2007). On the basis of UV–visible absorption and CD spectra shown in Figure 4, a distinct type of $[2Fe–2S]^{2+}$ centre may be present in GrxS14 and S16. Marked differences in the excited-state electronic properties and ground-state vibrational properties of the $[2Fe–2S]^{2+}$ centres in poplar GrxC1 and GrxS14 are evident in comparing the UV–visible absorption/CD and resonance Raman spectra shown in Figures 4 and 5, respectively. Differences in the relative intensities of corresponding Raman bands reflect changes in excitation profiles resulting from perturbation of the excited-state electronic structure. Nevertheless, it is clear that corresponding Fe–S stretching frequencies are upshifted by 2–8 cm^{-1} in GrxS14 compared with GrxC1, suggesting stronger Fe–S bonds for both terminal and bridging S.

As the spectroscopic properties of the $[2Fe–2S]^{2+}$ centres in monothiol Grxs indicate complete cysteinyl ligation, mutagenesis studies were undertaken to address the possibility that GSH is replaced as a ligand by an intrinsic cysteine

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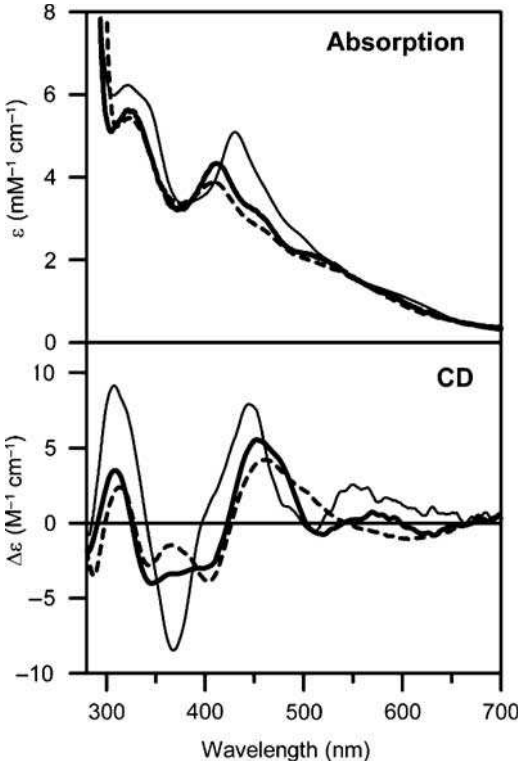

Figure 4 Comparison of the UV–visible absorption and CD spectra of [2Fe–2S] cluster-bound forms of poplar GrxS14 (thick line), At GrxS16 (broken line) and poplar GrxC1 (thin line).

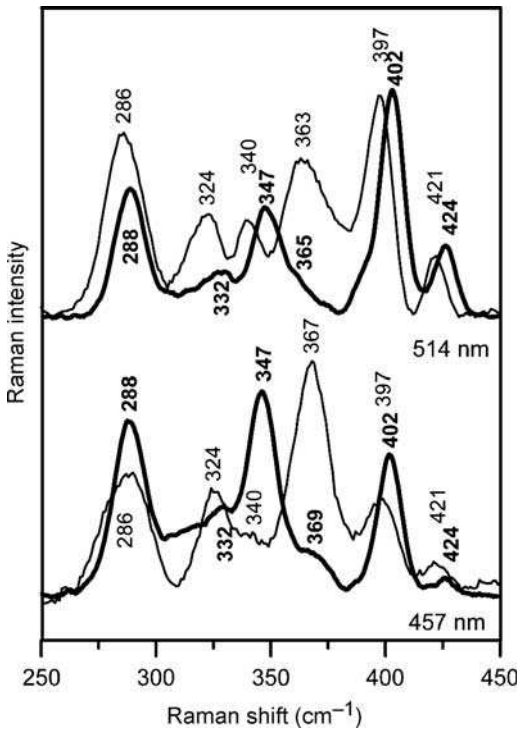

Figure 5 Comparison of the resonance Raman spectra of [2Fe–2S] cluster-bound forms of poplar GrxS14 (thick line) and GrxC1 (thin line) with 514- and 457-nm laser excitation. Samples were ~4 mM in Grx and were in the form of a frozen droplet at 17 K. Each spectrum is the sum of 100 scans, with each scan involving counting photons for 1 s each 0.5 cm^{-1} with 6 cm^{-1} spectral resolution. Lattice modes of ice have been subtracted.

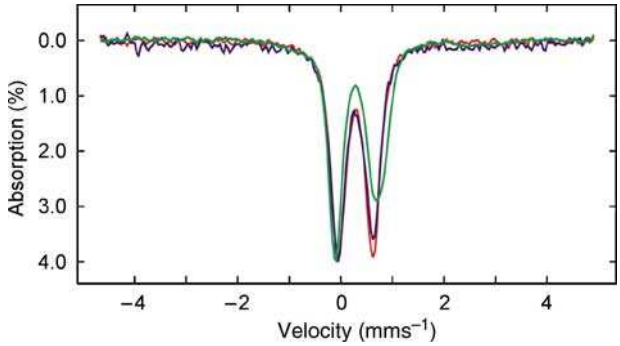

Figure 6 Comparison of the Mössbauer spectra of [2Fe–2S] cluster-bound forms of poplar GrxS14 (blue), poplar GrxC1 (red) and *A. vinelandii* IscU (green). The GrxS14 and C1 Mössbauer samples were prepared by growing cells on ^{57}Fe-enriched media and the IscU sample was prepared by IscS-mediated reconstitution using ^{57}Fe(II) (Agar *et al*, 2000). The Mössbauer spectra were recorded at 4.2 K with a magnetic field of 50 mT applied parallel to the γ-beam. Each spectrum is best simulated as the sum of two overlapping quadrupole doublets with the following parameters: $\Delta E_Q = 0.56$ and $\delta = 0.26\,mm\,s^{-1}$ for doublet 1, and $\Delta E_Q = 0.76$ and $\delta = 0.28\,mm\,s^{-1}$ for doublet 2 of GrxS14; $\Delta E_Q = 0.54$ and $\delta = 0.27\,mm\,s^{-1}$ for doublet 1, and $\Delta E_Q = 0.76$ and $\delta = 0.28\,mm\,s^{-1}$ for doublet 2 of GrxC1; $\Delta E_Q = 0.66$ and $\delta = 0.27\,mm\,s^{-1}$ for doublet 1, and $\Delta E_Q = 0.94$ and $\delta = 0.32\,mm\,s^{-1}$ for doublet 2 of IscU.

residue in GrxS14 and S16. Three cysteine residues are present in GrxS14 at positions 33, 87 and 108 (recombinant poplar GrxS14 numbering). The active site cysteine (Cys33) is conserved in all CGFS Grxs. Cys87 is present in all S14- and S16-type plant Grxs, but not in S15-type plant Grxs, whereas Cys108 is even not conserved in all S14-type plant Grxs (Supplementary Figure 1). Hence, to address the cluster ligation in GrxS14 and check whether the inability for GrxS15 to incorporate a Fe–S cluster is a consequence of the absence of the second cysteine residue, cysteine mutants both on poplar and AtGrxS14 and on poplar GrxS15 have been generated. AtGrxS14 was used for these mutation studies because introducing these mutations in poplar GrxS14 led mostly to insoluble proteins. On the basis of the colour of the cells and supernatant following sonication and centrifugation, it was clear that AtGrxS14 C33S was no longer able to incorporate the cluster, whereas AtGrxS14 C87S, AtGrxS14 C108S and AtGrxS14 C87/108S were still able to incorporate it. Further confirmation that the second cysteine residue is not a ligand came from the observation that poplar GrxS15 S87C was still unable to accommodate a Fe–S cluster. Taken together, the mutagenesis and spectroscopic data, coupled with the requirement for GSH in cluster reconstitution experiments, strongly support similar cluster ligation in monothiol and dithiol Grxs (as typified by GrxS14 and GrxC1, respectively), involving the catalytic cysteine of two monomers and two external GSH molecules. The structural origin of the observed differences in spectroscopic properties of the $[2Fe–2S]^{2+}$ clusters in monothiol and dithiol Grxs is therefore likely to result from differences in ligand arrangement or cluster environment.

In vitro cluster transfer from [2Fe–2S] GrxS14 to apo chloroplast ferredoxin

The results presented above provide *in vitro* evidence in support of a role for monothiol Grxs as scaffolds for the assembly of [2Fe–2S] clusters. However, functional Fe–S

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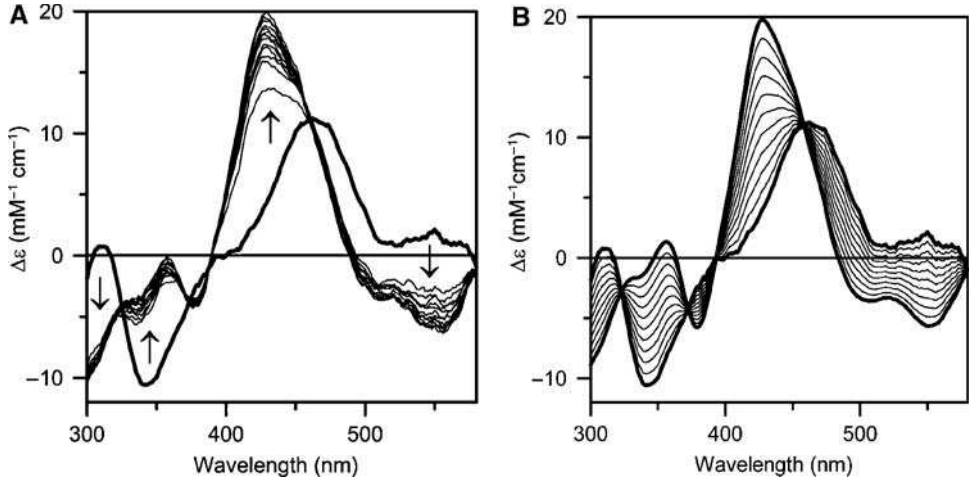

Figure 7 Time course of cluster transfer from poplar GrxS14 to apo *Synechocystis* Fd monitored by UV–visible CD spectroscopy at 23°C in 1 cm cuvettes. (**A**) CD spectra were recorded at 5-min intervals for a period of 60 min for a reaction mixture that was initially 15 μM in GrxS14 [2Fe–2S] clusters and 15 μM apo Fd. The spectrum at zero time (thick line) corresponds to [2Fe–2S] GrxS14 in the same reaction mixture in the absence of apo Fd. The arrows indicate the direction of intensity change with time at selected wavelengths. (**B**) Predicted changes in the CD spectra for quantitative cluster transfer. Thick lines correspond to holo forms of *Synechocystis* [2Fe–2S] Fd and [2Fe–2S] GrxS14 and thin lines correspond to simulated CD spectra corresponding to 10–90% [2Fe–2S] cluster transfer from GrxS14 to Fd in 10% increments. In both panels, Δε values are expressed per $[2Fe–2S]^{2+}$ cluster.

cluster scaffold proteins need to be capable both of assembling clusters and transferring them to apo forms of physiologically relevant acceptor proteins. We have therefore investigated the ability of [2Fe–2S] GrxS14, which is localized in chloroplasts, to transfer its cluster to an apo form of *Synechocystis* [2Fe–2S] ferredoxin, one of the most abundant and highly conserved of all chloroplastic Fe–S proteins.

Direct evidence for rapid and quantitative cluster transfer from [2Fe–2S] GrxS14 to apo Fd was provided by anaerobic CD studies as a function of time using a reaction mixture involving stoichiometric [2Fe–2S] GrxS14 and apo Fd (Figure 7). The marked difference in the CD spectra of $[2Fe–2S]^{2+}$ centres in GrxS14 and holo Fd in the reaction mixture facilitates direct monitoring of cluster transfer and assessment of the extent of intact cluster transfer via concomitant decrease and increase of the CD spectra of the cluster donor and acceptor, respectively. Comparison of the time course of CD changes in the reaction mixture (Figure 7A) with simulated data for 0–100% intact cluster transfer (Figure 7B) indicates quantitative cluster transfer from [2Fe–2S] GrxS14 to apo Fd that is 60% complete after 5 min and 100% complete after approximately 60 min. The agreement between the observed and simulated data and the rapid rate of Fd reconstitution suggests quantitative and intact cluster transfer from [2Fe–2S] GrxS14 to apo Fd and argues strongly against cluster degradation and reassembly of apo Fd. This conclusion is further supported by three additional pieces of evidence. First, absorption and CD studies of the reaction mixture in the absence of the apo Fd showed $<10\%$ degradation of the [2Fe–2S] cluster on GrxS14 after 60 min. Second, parallel CD studies of apo *Synechocystis* Fd reconstitution with equivalent amounts of S^{2-} and Fe^{3+} or Fe^{2+} under identical conditions resulted in $\sim 5\%$ cluster assembly over a 60-min period. Third, the addition of 1 mM EDTA to the reaction mixture completely inhibited cluster reconstitution using S^{2-} and Fe^{3+} or Fe^{2+}, but had no significant effect on the time course of [2Fe–2S] GrxS14-mediated cluster assembly on apo Fd. Taken together, these observations indicate rapid, quantitative and intact cluster transfer from [2Fe–2S] GrxS14 to apo Fd. In contrast, parallel cluster transfer studies using the poplar dithiol [2Fe–2S] GrxC1 showed no indication of cluster transfer to apo *Synechocystis* Fd after 120 min (data not shown). Hence, the ability to transfer [2Fe–2S] clusters to acceptor proteins appears to be limited to monothiol Grxs. Comparative stability studies of GrxS14 and GrxC1 indicate that this is likely to be a consequence of increased accessibility and lability for the [2Fe–2S] clusters in monothiol Grxs compared with dithiol Grxs, see Supplementary Figure 2.

Quantitative assessment of the rate of cluster transfer as a function of [2Fe–2S] GrxS14 to apo Fd stoichiometry (0.22:1–1.5:1 based on cluster content of GrxS14) was obtained by continuous monitoring of the CD changes at 423 nm (Figure 8). On the basis of the initial concentrations of GrxS14 [2Fe–2S] clusters and apo Fd, the data in each case are well fit by second-order kinetics corresponding to stoichiometric cluster transfer with a rate constant of $20\,000\ M^{-1}\,min^{-1}$. To put this into context, the fastest intact [2Fe–2S] cluster transfer reported so far is for HscA/HscB/ATP-mediated [2Fe–2S] cluster transfer from [2Fe–2S] IscU to apo-IscFdx, which had a second-order rate constant of $800\ M^{-1}\,min^{-1}$ (Chandramouli and Johnson, 2006). Hence, the results demonstrate that [2Fe–2S] cluster transfer from [2Fe–2S] GrxS14 to apo Fd is stoichiometric, quantitative and occurs at a rate that is 25 × faster than *in vitro* cluster transfer studies using the IscU scaffold protein. Clearly these *in vitro* studies demonstrate that CGFS Grxs have the potential to function as scaffold proteins for the assembly and efficient delivery of [2Fe–2S] clusters.

Discussion

Involvement of chloroplastic CGFS Grxs in iron–sulphur cluster assembly

Grxs with a CGFS active site constitutes a recently described subgroup of the Grx family, whose functions are largely

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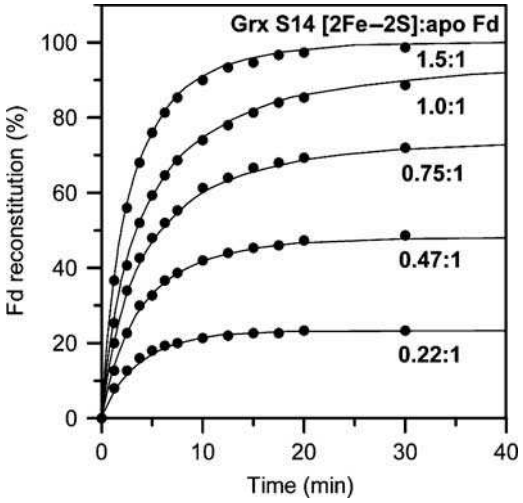

Figure 8 Kinetics of cluster transfer from poplar GrxS14 to apo *Synechocystis* Fd at 23°C as a function of the stoichiometry of GrxS14 [2Fe–2S] clusters to apo Fd. The experimental conditions are as described in Figure 7, except that the concentration of GrxS14 [2Fe–2S] clusters was varied to give the indicated GrxS14 [2Fe–2S] to apo Fd ratios. Reactions were continuously monitored using the CD intensity at 423 nm and converted to percent Fd reconstitution based on simulated data (as illustrated in Figure 7 for a 1:1 stoichiometry). Solid lines correspond to second-order kinetics with $k = 20\,000\,M^{-1}\,min^{-1}$ based on the initial concentrations of GrxS14 [2Fe–2S] clusters and apo Fd.

unknown at the present time (Herrero and de la Torre-Ruiz, 2007). In yeast, Grx5 was originally identified as playing a central role in protecting against oxidative damage and subsequent investigations refined a role in mitochondrial Fe–S cluster biogenesis (Rodríguez-Manzaneque *et al*, 1999, 2002). Immunoprecipitation studies suggested that Grx5 facilitates transfer of clusters assembled on the Isu scaffold protein to acceptor proteins (Mühlenhoff *et al*, 2003). In contrast, the other two CGFS Grxs in yeast, Grx3 and Grx4, are nuclear proteins involved in the nuclear localization regulation of the transcriptional iron regulators Aft1 and Aft2 (Ojeda *et al*, 2006). In plants, the CGFS Grx subgroup is expanded to four members, but the only functional information stems from *in vivo* analysis of *A. thaliana* GrxS14 (aka AtGRXcp), which was shown to be a chloroplastic protein probably involved in the oxidative stress response (Cheng *et al*, 2006).

In this study, the subcellular locations of each of the poplar CGFS Grxs were determined and yeast Δ*grx5* complementation studies of mitochondrial-targeted forms were used to assess the possibility that plant CGFS Grxs are also involved in Fe–S cluster assembly. All the CGFS Grxs, except GrxS15, but not dithiol Grxs (even GrxC1, which contains a [2Fe–2S] cluster), were able to complement the defects of the *yeast* Δ*grx5* mutant, although not always to a similar extent. These results were somewhat surprising because the two chloroplastic Grxs (GrxS14 and S16) are the most efficient proteins, whereas the mitochondrial GrxS15 is essentially not effective. In addition, the proteins involved in plant and yeast mitochondrial Fe–S cluster assembly belong to the ISC type of machinery, whereas plant chloroplasts contain essentially the SUF system. Nevertheless, it is evident that both GrxS14 and S16 have the ability to assume a role analogous to that of Grx5 in mitochondrial Fe–S cluster biosynthesis. Whether GrxS15 is involved in plant mitochondria Fe–S cluster assembly is still uncertain, but only one or two other Grxs, displaying a CCMS active site, are predicted to be present in mitochondria and could fulfil an analogous role. As discussed below, a major difference between GrxS14 or S16 and S15, that could explain their different behaviour, is the capacity of the two chloroplastic Grxs to incorporate a Fe–S cluster when expressed in *E. coli*.

GrxS14 and S16 as Fe–S cluster scaffold proteins

The observation that both plant GrxS14 and S16 contain analogous $[2Fe–2S]^{2+}$ centres when heterologously expressed in *E. coli* raises the possibility of a role for CGFS Grxs as scaffolds for the assembly of chloroplastic Fe–S clusters. Additional support for a scaffolding role comes from the ability to assemble spectroscopically identical clusters on apo GrxS14 in a cysteine desulphurase-mediated reaction in the presence of L-cysteine, Fe^{2+} ion and GSH. Cysteine mutagenesis studies, analytical and spectroscopic data, and the requirement of GSH to effect cluster assembly on apo GrxS14, indicate the presence of a subunit-bridging $[2Fe–2S]^{2+}$ cluster ligated by the first active site cysteine of two Grxs and the cysteines of two GSH molecules. A similar ligation has also been structurally established in the dithiol poplar GrxC1 (Rouhier *et al*, 2007). However, pronounced differences in the lability or accessibility and the ground-state vibrational and excited-state electronic properties of the [2Fe–2S] centres in monothiol and dithiol plant Grxs indicate differences in the cluster environment or the arrangement of coordinating cysteine residues. Possible differences could involve the extent and arrangement of aromatic residues in the vicinity of the cluster or *cis* rather than *trans* GSH cluster ligation (Rouhier *et al*, 2007). Crystallographic studies of cluster-bound forms of monothiol Grxs will be required to address these differences and to assess the cluster release mechanism in monothiol Grxs.

A functional Fe–S cluster scaffold protein must also be effective in transferring clusters to physiologically relevant acceptor proteins. Hence, the observation of rapid, stoichiometric and intact cluster transfer from [2Fe–2S] GrxS14 to apo Fd provides *in vitro* evidence for a role of CGFS Grxs as [2Fe–2S] cluster donors for maturation of chloroplast Fe–S proteins. In contrast, analogous cluster transfer experiments using the structurally characterized [2Fe–2S] cluster-bound dithiol GrxC1 failed to show any evidence of cluster transfer to apo Fd. Clearly, there is a pressing need to investigate the ability of [2Fe–2S] GrxS14 and [2Fe–2S] GrxS16 to effect maturation of the apo forms of a variety of [2Fe–2S] cluster-containing chloroplastic proteins, for example, sirohydrochlorin ferrochelatase, dihydroxyacid dehydratase and Rieske-type centres in oxygenases and the cytochrome b_6f, complex, and to assess the possibility that these proteins also participate in the maturation of [3Fe–4S] and [4Fe–4S] clusters in chloroplastic proteins. Such studies will address the specificity of these Grxs in chloroplastic Fe–S cluster biogenesis and are currently in progress in our laboratories. The proposed role for CGFS Grxs as Fe–S cluster scaffold proteins also provides a rationalization for the apparent disparity concerning the role of the second partly conserved cysteine residue in yeast Grx5 in *in vivo* and *in vitro* activity data. Mutagenesis results indicated that the second conserved cysteine is required for *in vitro* deglutathionylation activity, but is not required for *in vivo* Fe–S cluster biogenesis activity

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(Belli *et al*, 2002) or the assembly of a [2Fe–2S] cluster on GrxS14. Nevertheless, among all natural or mutated plant CGFS Grxs tested, those which do not possess this additional cysteine (GrxS15 and GrxC4 CGFS) were not able to complement the *S. cerevisiae* Δ*grx5* mutant. This indicates that, although not essential for cluster incorporation, this additional cysteine may be required for an efficient complementation with plant Grxs.

Two pieces of evidence argue against a scaffolding role for monothiol CGFS Grxs in *de novo* Fe–S cluster assembly. First, gene disruptions of known chloroplastic scaffold proteins such as the Nfu proteins and other intrinsic components of the chloroplastic Fe–S assembly machinery are generally associated with a dwarf phenotype or abnormal development (Touraine *et al*, 2004; Yabe *et al*, 2004; Balasubramanian *et al*, 2006; Xu and Moller, 2006; Van Hoewyk *et al*, 2007). In the case of GrxS14, the phenotype consists of a defect in early seedling growth under oxidative stress (Cheng *et al*, 2006) and is not as strong as that associated with *nfu* gene disruptions. Taking into account the large number of Fe–S proteins in the chloroplast, one possibility is that GrxS14 has specific target proteins whose functions are not essential for plant development. Alternatively, the absence of GrxS14 may be compensated by other potential scaffold proteins such as GrxS16 or SufA in the loss-of-function mutant. To address this issue, it will be necessary to investigate the phenotypes of gene disruptions resulting in depletion of other chloroplast proteins, both individually and together, for example, GrxS16, GrxS14/GrxS16, SufA/GrxS14 and SufA/GrxS16. Second, the role of Grx5 in yeast mitochondrial Fe–S cluster biogenesis does not appear to be dependent on GSH, which is required for the assembly of a [2Fe–2S] cluster on GrxS14. Depletion of GSH was found to affect the maturation of cytosolic Fe–S proteins, but had no significant effect on mitochondrial Fe–S cluster biogenesis (Sipos *et al*, 2002). However, the significance of this observation remains to be established as there is currently no reliable information on the type of cluster or the requirements for cluster assembly on Grx5, or the level of GSH that is required to support mitochondrial Fe–S cluster assembly. A recent paper by Picciocchi *et al* (2007) reported *in vitro* assembly of a Fe–S cluster in yeast Grx5 and other CGFS Grxs in the presence of GSH. However, variability in the reported UV–visible absorption spectra, coupled with the absence of Mössbauer, CD, resonance Raman or quantitative EPR data, leaves the cluster content of these samples unresolved.

Alternative functions for monothiol CGFS Grxs

The ability of CGFS Grxs to accommodate [2Fe–2S] clusters and transfer them to acceptor proteins is also consistent with a role in storage of preformed Fe–S clusters or as Fe–S cluster delivery systems that mediate cluster transfer from other potential scaffold proteins (i.e. SufA, SufB and Nfu in chloroplasts and Isa/IscA, Nfu and Isu in mitochondria) to specific acceptor proteins. Such a role is consistent with yeast Δ*grx5* immunoprecipitation studies, which suggested that Grx5 facilitates transfer of clusters assembled on the Isu scaffold protein to acceptor proteins (Mühlenhoff *et al*, 2003). This would also explain why a strong phenotype may not be associated with gene disruption, because the transfer would still occur although at a lower rate. In addition, yeast two-hybrid studies indicate an *in vivo* interaction between Grx5 and Isa1 (Vilella *et al*, 2004) and the crystal structure of an IscA protein with an asymmetrical subunit-bridging [2Fe–2S] cluster has recently been published (Morimoto *et al*, 2006). Hence, it is possible that IscA/SufA-type proteins hand off clusters to monothiol Grxs for delivery to acceptor proteins and that this cluster assembly pathway would only be completely shut down by deletion of both genes. *In vitro* cluster transfer experiments involving chloroplast [2Fe–2S] SufA and [2Fe–2S] Nfu proteins and apo GrxS14 and kinetic studies of the effect on apo GrxS14 on the rates of cluster transfer from chloroplast [2Fe–2S] SufA and [2Fe–2S] Nfu to apo Fd are in progress to test this hypothesis.

In light of the proposed role for Grx3 and Grx4 in the regulation of Fe homeostasis in yeast (Ojeda *et al*, 2006), an alternative rationalization for the cluster-binding ability of chloroplast Grxs is that they play a role in the regulation of the SUF Fe–S cluster biogenesis machinery by facilitating assessment of the chloroplast Fe–S cluster status. Using bioinformatics, Huynen *et al* (2005) proposed that BolA proteins act as reductases that interact with monothiol Grxs in the oxidative stress response. Nevertheless, such a function seems unlikely in plants as BolA proteins do not possess conserved cysteines. Nevertheless, among the three genes encoding SufE proteins in *A. thaliana*, SufE1 (At4g26500) contains a C-terminal BolA domain (Xu and Moller, 2006; Ye *et al*, 2006a). SufE proteins are sulphur transferases that serve as activators for the NifS and SufS cysteine desulphurases in the initial steps of chloroplast Fe–S cluster biosynthesis (Xu and Moller, 2006). Hence, it is possible that GrxS14 acts as a Fe–S cluster-dependent regulator of the SUF machinery by interacting with SufE1 via the BolA domain. Under this scenario, Fe–S cluster incorporation into GrxS14 would occur under Fe–S cluster-replete conditions, resulting in enhanced interaction with SufE1 to limit the activity of the SUF Fe–S cluster biogenesis machinery.

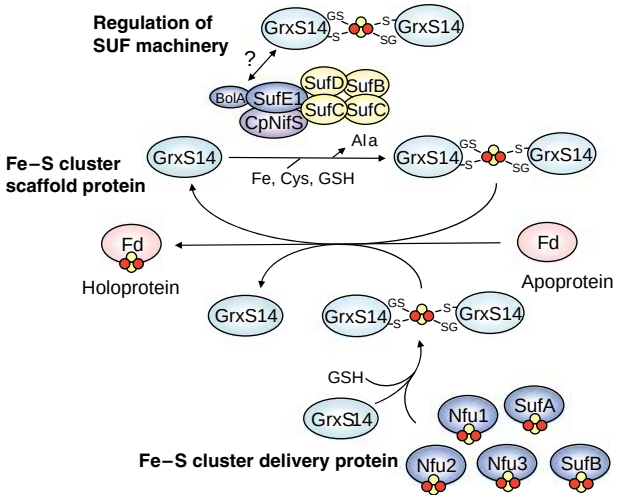

Figure 9 Working model for the potential roles of GrxS14 and S16 in chloroplastic Fe–S cluster assembly. GrxS14 and S16 could function as scaffold proteins for *de novo* synthesis and transfer of Fe–S clusters, as Fe–S cluster delivery proteins for mediating the transfer of Fe–S clusters from other potential scaffold proteins (Nfu1, 2 and 3, SufA and SufB) to acceptor proteins, or as regulators of the SUF machinery by interacting with the BolA domain of SufE1 in the cluster-bound form.

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The three possible roles for CGFS Grxs in chloroplast Fe–S cluster biogenesis are summarized in Figure 9. It is important to emphasize that these three roles are not mutually exclusive and that all three could be operative in chloroplasts with GrxS14 and S16 having different specificity for acceptor proteins, cluster-donor proteins or cysteine desulfurases. Such functional variability and specificity differences may also be responsible for anomalous behaviour of GrxS15 and the truncated form of GrxS17 in yeast Grx5 complementation studies.

Materials and methods

Heterologous expression and purification of CGFS Grxs in E. coli

The cloning and aerobic purification procedures of CGFS Grxs are described as Supplementary data. Anaerobic purification of poplar GrxS14 and AtGrxS16 was carried out under Ar in a Vacuum Atmospheres glove box at oxygen levels <2 p.p.m. The cell pellet was resuspended in 100 mM Tris–HCl pH 8 with 1 mM GSH (buffer B), sonicated and centrifuged at 39 700 *g* for 1 h at 4°C to remove the cell debris. The reddish-brown cell-free extract containing holo GrxS14 was subjected to 40% ammonium sulphate cut followed by centrifugation. The brown pellet was resolubilized in buffer B and loaded onto a 10 ml Phenyl Sepharose column (GE Healthcare). The protein was eluted with a 1–0 M $(NH_4)_2SO_4$ gradient using buffer B. The purest fractions, as judged by SDS–PAGE analysis, were pooled and $(NH_4)_2SO_4$ was removed by ultrafiltration dialysis using a YM10 membrane and buffer B.

Reconstitution of a Fe–S cluster in apo poplar GrxS14

Reconstitution of poplar apo GrxS14, 0.4 mM in buffer B, was accomplished in a glove box under anaerobic conditions by incubating at room temperature for 150 min with 5 mM GSH, 2 mM DTT, 12-fold excess of Fe(II) (ferrous ammonium sulphate), L-cysteine and catalytic amounts of *E. coli* IscS (20 μM). Reagents in excess were removed by loading onto a High-Trap Q-Sepharose column (GE Healthcare) and eluting with a 0–1 M NaCl gradient in buffer B. The holo protein was eluted between 0.45 and 0.55 M NaCl and was desalted using ultrafiltration dialysis.

Yeast plasmids and strains

Grx sequences were cloned in-frame in the yeast plasmid pMM221, which contains the *S. cerevisiae* mitochondrial targeting sequence of Grx5 plus a C-terminal 3HA/His_6 tag, under the control of the doxycycline-regulatory $tetO_2$ promoter (Molina *et al*, 2004) (Table I). pMM54 (Rodríguez-Manzaneque *et al*, 2002) contains a yeast *GRX5-3HA* construction under its own promoter. *S. cerevisiae* strains are described in Table II. Plasmids were linearized by *Cla*I digestion previous to chromosomal integration.

Growth conditions for S. cerevisiae cells

S. cerevisiae cultures were grown as described in Molina *et al* (2004). Samples were taken from cultures grown exponentially for at least 10 generations at 30°C. Sensitivity to oxidants was determined onto YPD plates containing the indicated concentration of the agent, by spotting 1:5 serial dilutions of exponential cultures and recording growth after 2 days of incubation at 30°C.

Table I New plasmids employed in this study

Plasmids	Characteristics
pMM628	Sequence coding from amino acid 2–117 of GrxC1 cloned between *Not*I–*Pst*I sites of pMM221
pMM630	Sequence coding from amino acid 2–117 of GrxC1(G32P) cloned between *Not*I–*Pst*I sites of pMM221
pMM632	Sequence coding from amino acid 2–113 of GrxC4 cloned between *Not*I–*Pst*I sites of pMM221
pMM634	Sequence coding from amino acid 37–174 of GrxS15 cloned between *Not*I–*Pst*I sites of pMM221
pMM657	Sequence coding from amino acid 66–173 of GrxS14 cloned between *Not*I–*Pst*I sites of pMM221
pMM676	Derivative of pMM628 with the sequence coding for CGFS instead of CGYC in GrxC1
pMM712	Sequence coding from amino acid 85–296 of GrxS16 cloned between *Not*I–*Pst*I sites of pMM221
pMM713	Sequence coding from amino acid 1–492 of GrxS17 cloned between *Not*I–*Pme*I sites of pMM221
pMM714	Sequence coding from amino acid 398–492 of GrxS17 cloned between *Not*I–*Pme*I sites of pMM221

Table II Yeast strains employed in this study

Strains	Relevant phenotype	Comments
W303-1A	*MAT***a** *ura3-1 ade2-1 leu2-3,112 trp1-1 his3-11,15*	Wild type
W303-1B	As W303-1A but *MAT*α	Wild type
MML100	*MAT***a** *grx5*::*kanMX4*	Rodríguez-Manzaneque *et al* (2002)
MML240	*MAT***a** *grx5*::*kanMX4* [pMM54(*GRX5-3HA*)]::*LEU2*	Rodríguez-Manzaneque *et al* (2002)
MML755	*MAT*α [pMM628(*GrxC1-3HA*)]::*LEU2*	Integration of linear pMM628 in W303-1B
MML756	*MAT*α [pMM630($GrxC1_{G32P}$-*3HA*)]::*LEU2*	Integration of linear pMM630 in W303-1B
MML757	*MAT*α [pMM632(*GrxC4-3HA*)]::*LEU2*	Integration of linear pMM632 in W303-1B
MML758	*MAT*α [pMM634(*GrxS15-3HA*)]::*LEU2*	Integration of linear pMM634 in W303-1B
MML759	*MAT*α [pMM657(*GrxS14-3HA*)]::*LEU2*	Integration of linear pMM657 in W303-1B
MML761	*MAT***a** *grx5*::*kanMX4* [pMM628(*GrxC1-3HA*)]::*LEU2*	Spore from a cross MML100 × MML755
MML763	*MAT***a** *grx5*::*kanMX4* [pMM630($GrxC1_{G32P}$-*3HA*)]::*LEU2*	Spore from a cross MML100 × MML756
MML765	*MAT***a** *grx5*::*kanMX4* [pMM632(*GrxC4-3HA*)]::*LEU2*	Spore from a cross MML100 × MML757
MML767	*MAT***a** *grx5*::*kanMX4* [pMM634(*GrxS15-3HA*)]::*LEU2*	Spore from a cross MML100 × MML758
MML769	*MAT***a** *grx5*::*kanMX4* [pMM657(*GrxS14-3HA*)]::*LEU2*	Spore from a cross MML100 × MML759
MML779	*MAT*α [pMM676($GrxC1_{CGFS}$-*3HA*)]::*LEU2*	Integration of linear pMM676 in W303-1B
MML780	*MAT***a** *grx5*::*kanMX4* [pMM676($GrxC1_{CGFS}$-*3HA*)]::*LEU2*	Spore from a cross MML100 × MML779
MML786	*MAT*α [pMM712(*GrxS16-3HA*)]::*LEU2*	Integration of linear pMM712 in W303-1B
MML787	*MAT*α [pMM713(*GrxS17-3HA*)]::*LEU2*	Integration of linear pMM713 in W303-1B
MML788	*MAT*α [pMM714($GrxS17_{398-492}$-*3HA*)]::*LEU2*	Integration of linear pMM714 in W303-1B
MML806	*MAT***a** *grx5*::*kanMX4* [pMM712(*GrxS16-3HA*)]::*LEU2*	Spore from a cross MML100 × MML786
MML808	*MAT***a** *grx5*::*kanMX4* [pMM713(*GrxS17-3HA*)]::*LEU2*	Spore from a cross MML100 × MML787
MML810	*MAT***a** *grx5*::*kanMX4* [pMM714($GrxS17_{398-492}$-*3HA*)]::*LEU2*	Spore from a cross MML100 × MML788

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Other methods

Mitochondria were purified and subfractionated (Diekert *et al*, 2001) from exponential yeast cultures in YPLactate medium at 30°C. Aconitase and malate dehydrogenase were assayed as described in Robinson *et al* (1987), in extracts prepared (Molina-Navarro *et al*, 2006) from cells growing exponentially in YPGalactose medium.

In vivo subcellular localization by GFP fusions

Full-length open reading frames were cloned in 5′ of the GFP sequence under the control of a double 35S promoter into the plasmid pCK S65C between *Nco*I and *Bam*HI sites (underlined) using primers described in Supplementary Table I. *Nicotiana benthamiana* cells were transfected by bombardment of leaves with tungsten particles coated with plasmid DNA and images were obtained with a Zeiss LSM510 confocal microscope.

Analytical and spectroscopic methods

Protein concentrations were determined by the *DC* protein assay (Bio-Rad), using BSA as a standard. Iron concentrations were determined using bathophenanthroline under reducing conditions, after digestion of the protein in 0.8% $KMnO_4$/0.2 M HCl (Fish, 1988). Sample concentrations and extinction coefficients are based on protein monomer and samples were in 100 mM Tris–HCl pH 8 with 1 mM GSH, unless indicated otherwise. Samples for spectroscopic studies were prepared under Ar in a Vacuum Atmospheres glove box ($O_2 < 2$ p.p.m.). UV–visible absorption and CD spectra were recorded at room temperature using a Shimadzu UV-3101PC spectrophotometer and Jasco J-715 spectropolarimeter, respectively. Resonance Raman spectra were recorded as previously described (Cosper *et al*, 2004), using an Instruments SA Ramanor U1000 spectrometer coupled with a Coherent Sabre argon ion laser, with 20 μl frozen droplets of 2–3 mM sample mounted on the cold finger of an Air Products Displex Model CSA-202E closed cycle refrigerator. Mössbauer spectra were recorded by using the previously described instrumentations (Ravi *et al*, 1994). The zero velocity of the spectra refers to the centroid of a room temperature spectrum of a metallic Fe foil. Analysis of the Mössbauer data was performed with the WMOSS program (Web Research).

Fe–S cluster transfer experiments

Synechocystis Fd for cluster transfer experiments was heterologously expressed in *E. coli* and purified according to published procedures (Glauser *et al*, 2004). Apo Fd was prepared by treating the holo protein with EDTA and potassium ferricyanide at room temperature under anaerobic conditions and removing excess reagents by ultrafiltration dialysis using a YM10 membrane and buffer B. The time course of cluster transfer from [2Fe–2S] GrxS14 to apo Fd was monitored under anaerobic conditions in 1 cm cuvettes at 23°C using UV–visible CD spectroscopy. The reactions were carried out in buffer B with 5 mM DTT and apo Fd was added 60 min prior to initiation of the cluster transfer reactions by addition of [2Fe–2S] GrxS14. Changes in the CD spectra at 423 nm were used to assess the concentration of holo Fd formed as a function of time. The time course of holo Fd formation was analysed by fitting to second-order kinetics, based on the initial concentrations of GrxS14 [2Fe−2S] clusters and apo-Fd in the reaction mixture, using the Chemical Kinetics Simulator software package (IBM).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

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DISCUSSION GENERALE ET CONCLUSIONS

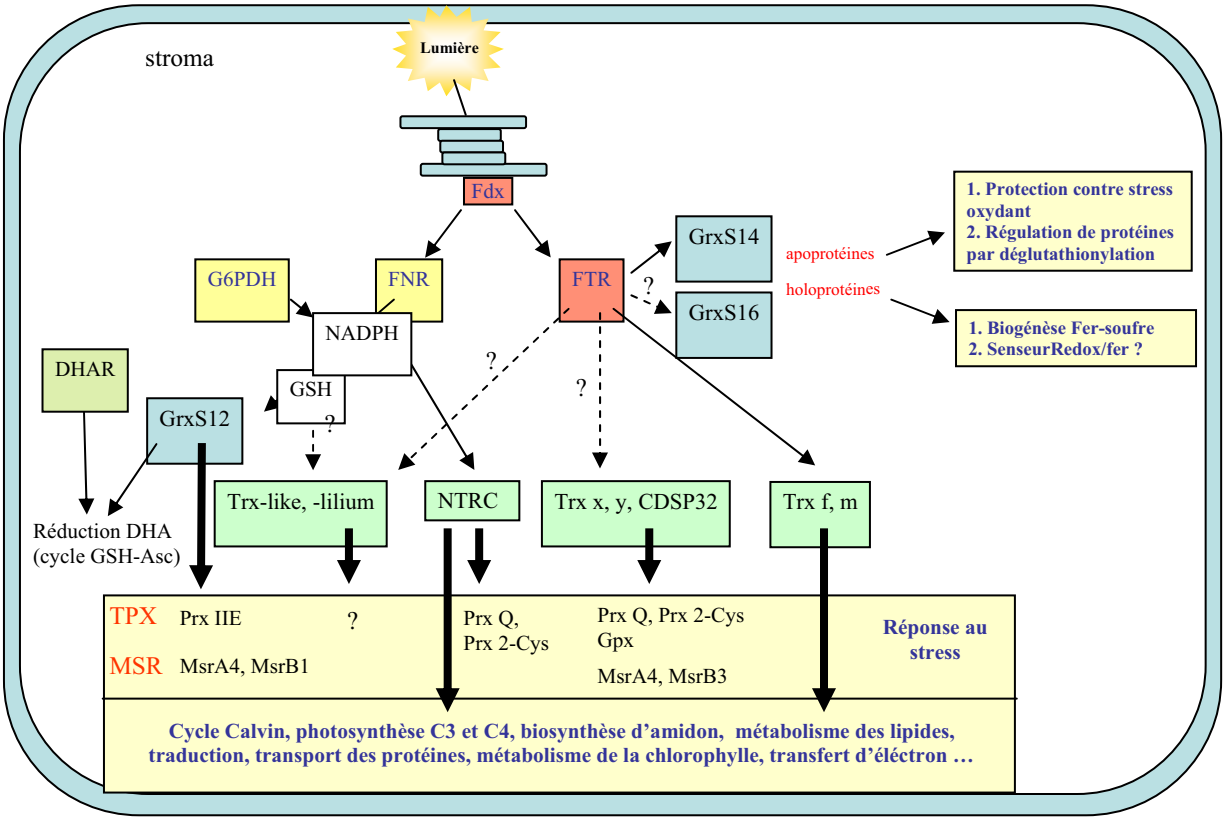

Figure 23: Spécificité entre oxydoréductases et protéines cibles au niveau du chloroplaste.
Parmi les systèmes antioxydants, il existe une certaine spécificité des Prxs vis-à-vis de leurs réducteurs. Les PrxIIE acceptent préférentiellement des électrons des Grxs et Trx-like et lilium, alors que les Prx Q, Prx 2-Cys et Gpx utilisent les Trx, x, y, CDSP32 et NtrC. Par ailleurs, les Grxs S14 et S16 participent à la biosynthèse des centres fer-soufre, aux mécanismes de déglutathionylation et à la protection contre le stress oxydant.

Discussion générale et conclusions

Les travaux menés au sein de l'équipe du professeur Jean-Pierre Jacquot concernent la caractérisation des systèmes antioxydants thiol dépendants du peuplier. Chez les plantes, ces systèmes sont assez complexes, comprenant de nombreuses protéines appartenant à des familles multigéniques. Je me suis plus particulièrement intéressé au cours de ma thèse à deux types d'enzymes: les peroxyrédoxines et les glutarédoxines. Il est très clair à présent que ces enzymes jouent un rôle dans de nombreux processus physiologiques, en réponse à des conditions de stress oxydant. La figure 23 décrit la complexité et les specificités qui existent, au niveau du chloroplaste, entre les systèmes de réduction (Trx et Grx) et les protéines cibles, en particulier les Prxs et les Msrs. En effet, seules quelques protéines de ces familles avaient été caractérisées au commencement de ma thèse, et il est évident qu'une connaissance plus approfondie des rôles spécifiques de ces enzymes peut nous éclairer sur le fonctionnement et les mécanismes précis de ces systèmes antioxydants.

La première partie du travail de recherche durant ma thèse a donc consisté en l'étude des peroxyrédoxines de type II. Les résultats obtenus nous ont permis d'avoir des informations sur le mécanisme de régénération des Prx II et sur les contacts moléculaires entre les Prxs et Grxs d'une part et entre les Prxs et les Trxs d'autre part. Le point de départ de ces recherches a été l'étude d'une protéine de fusion naturelle (hybride Prx-Grx chez *Neisseria meningitidis*).

Bien que la production de quantités utilisables de protéines multimodulaires et solubles sous forme recombinante soit parfois difficile à obtenir, j'ai pu produire et purifier 3 formes de protéines hybrides de peuplier composées d'un module Prx en position N-terminale couplé soit à un module Trx (PrxTrx) soit à un module Grx (PrxGrx1 et PrxGrx2). Seuls les hybrides PT et PG1 ont permis d'obtenir des protéines avec une activité proche de celle obtenue en mélangeant les protéines indépendantes. La protéine PG2 étant dépourvue d'une partie du N-

terminale du module Grx, on peut donc émettre l'hypothèse que c'est cette absence qui provoquerait cette baisse d'activité. La Grx C4 étant régénérée par le GSH, il est possible aussi que la délétion introduite provoque une baisse de l'affinité pour le GSH due à des changements conformationnels au niveau la protéine hybride. Les Grxs de plantes possèdent une extension N-terminale qui est absente chez certaines glutarédoxines bactériennes et qui pourrait être responsable de la bonne efficacité catalytique de la protéine. Les protéines chimériques PT et PG1 ont été correctement produites, avec une bonne structuration de la protéine, sous forme dimérique et non en tétramère comme cela est le cas pour la protéine fusion décrite par (Kim *et al*., 2003). En ce qui concerne l'enzyme de Kim *et al*, les interfaces Prx–Prx, Prx–Grx, et Grx–Grx ont été décrites et le module Prx d'un monomère semble réduit par le module Grx d'un autre monomère. Nos expériences ne permettent pas d'interpréter la façon dont est réalisé le transfert d'électrons entre les modules Grx ou Trx et le module Prx. Cette organisation en dimère peut impliquer une structure de type « tête-à-queue » où l'extrémité N-terminale du module Trx ou Grx se retrouve en face de l'extrémité C-terminale du module Prx. Lorsque ces résultats ont été publiés aucune expérience pour pouvoir confirmer ou infirmer cette hypothèse n'avait été réalisée mais des études de structure 3D étaient prévues afin de pouvoir répondre à cette interrogation. Malheureusement, les protéines n'ont jamais cristallisé.

Comme pour bon nombre de Prx, nous avons observé que le module PrxII peut être suroxydé et également glutathionylé. De tels résultats ont déjà été obtenus pour d'autres Prxs (Biteau *et al*., 2003; Georgiou and Masip, 2003; Woo *et al*., 2005). Ces modifications post-traductionnelles pourraient être à la base de mécanismes de signalisation cellulaire à l'instar de ce qui a déjà été démontré pour d'autres Prxs d'organismes différents (Rhee *et al*., 2005).

Par la suite, j'ai caractérisé deux protéines de peuplier, les Prxs IIF et IIE. Seule la Prx IIB cytosolique avait été étudiée auparavant dans ce sous-groupe de Prx. Les deux enzymes en question sont respectivement mitochondriale et chloroplastique, et le fait que ces deux compartiments soient des sites privilégiés de synthèse d'EOR, notamment par le biais des chaînes de transfert d'électron, renforce l'intérêt de l'étude de ces protéines.

Tout d'abord nous avons étudié les réducteurs et les substrats potentiels. La Prx IIB était déjà connue pour être réduite par le système Trx ou le système Grx, mais pas par le GSH (Rouhier *et al*., 2001). La Prx IIF d'*A. thaliana* est réduite par la Trx o mitochondriale, et non par l'isoforme cytosolique, la Trx h (Gama *et al*., 2007). De plus, le système Grx ou le GSH seul peuvent également régénérer cette protéine (Finkemeier *et al*., 2005, Rouhier *et al*., 2005b). En revanche, aucun réducteur de la Prx IIE d'*A. thaliana* n'était connu. Contrairement à la Prx IIB de peuplier, utilisée en contrôle, la Prx IIF de peuplier n'est réduite par aucune Trx testée (Trx h1, h3 et h5 cytosoliques ou Trx h2 mitochondriale). La Trx o est également mitochondriale, mais n'a pas pu être produite sous forme recombinante. La Gpx3 mitochondriale n'est elle non plus ni réduite par la Trx h2, ni par la Trx o d'*A. thaliana* (Gelhaye *et al*., 2004). Nous avons également testé le système Grx comme réducteur potentiel, le GSH seul montre une efficacité qui est accrue lorsqu'on ajoute la GrxC4 cytosolique (résultats identiques à la Prx IIF d'*Arabidopsis*). Cette propriété diffère de celle de la Prx IIB qui n'est pas réduite par le GSH seul. Ceci nous amène à penser les Prxs de type II seraient en premier lieu glutathionylées et ensuite une Grx ou une autre molécule de GSH viendrait réduire le pont disulfure et restaurer une Prx fonctionnelle. À la différence de la Prx IIF mitochondriale, la Prx IIE de peuplier n'est pas réduite par le GSH seul, mais est très efficacement régénérée par le système GSH/Grx. Elle se rapproche en cela de la Prx IIB, qui

est réduite par les Trxs ou par le système GSH/Grx, ce dernier étant le plus efficace. En gardant l'hypothèse que c'est bel et bien le GSH qui réduit l'acide sulfénique, cela voudrait dire que cette Prx glutathionylée serait réduite par une Grx et non pas du GSH. Ces données sont en accord avec la description de formes glutathionylées de la Prx IIB de peuplier (Noguera-Mazon *et al*., 2006). Le fait que certaines Prxs de type II ne possèdent pas de deuxième cystéine (par exemple, la deuxième cystéine est apparemment absente dans la Prx IIC d'*O. sativa* et remplacée par une leucine) abonde dans le sens que cette régénération passe par adduit de glutathion et non par un pont disulfure intramoléculaire. Cependant, il est tout à fait possible que les mécanismes varient en fonction des isoformes. Ce mécanisme de régénération serait compatible autant pour des Grxs monothiol que dithiol. Nous avons testé différentes Grxs chloroplastiques, la Grx S12 avec un site actif WCSYS, les Grx S14 et S16 avec des sites actifs CGFS et la Grx C4 cytosolique avec un site actif CPYC. Aucune Grx CGFS n'a montré d'activité réductrice alors que les Grx S12 et C4 étaient capables de régénérer la Prx IIE *in vitro*. En cherchant dans les banques de données, on s'aperçoit que cette Grx S12 est exprimée au niveau des mêmes organes que la Prx IIE, et elle pourrait donc être son réducteur naturel. Cependant, on ne peut pas exclure que cette Prx possède d'autres réducteurs physiologiques, telles que la Grx C5 présente chez *Arabidopsis* et plus généralement chez les Brassicacées ou que les Grxs de classe III de type CC, ni que les Grxs téstées puissent être réduite par la FTR. De plus, la mutation des cystéines du site actif de la Grx C4 nous prouve qu'elle utilise un mécanisme monothiol pour régénérer les Prxs ; à ce jour il n'a toujours pas été démontré que les Grxs réduisent les ponts disulfure de substrats protéiques. Par ailleurs, deux Trxs cytosoliques de peuplier (Trx h1 et h3) réduisent peu efficacement la Prx IIE. Comme la Trx h1 n'est pas très efficace pour la régénération de la Prx IIE, mais néanmoins plus active que des Trxs chloroplastiques pour réduire les Prx Q et Gpx 1 et 3.2 de peuplier (chloroplastiques),

nous pouvons supposer que les Trxs plastidiales ne sont pas de bons partenaires physiologiques, bien que nous ne puissions pas exclure complètement la possibilité que certaines Trxs plastidiales non identifiées pourraient être de meilleurs catalyseurs. Comme les Trxs chloroplastiques ne sont pas réduites par la NTR, le test basé sur l'oxydation du NADPH ne peut pas être utilisé. Une alternative aurait été d'utiliser la méthode FOX mais la Prx IIE est directement réduite par le DTT présent dans le milieu masquant l'effet des Trxs chloroplastiques.

Ensuite, la spécificité pour différents substrats a été mesurée en présence d'un système Grx saturant et avec différents peroxydes (H_2O_2, t-BOOH ou CuOOH). Le meilleur substrat pour la Prx IIF est t-BOOH suivi par H_2O_2 et CuOOH. La vitesse et l'efficacité catalytique (kcat/Km autour de 10^4 $M^{-1}.s^{-1}$) sont dans la même gamme que celles déterminées pour d'autres Prxs déjà testées à l'état stationnaire, c'est-à-dire une activité inférieure aux autres systèmes de détoxication. Si l'on se réfère à un système analogue, la réduction des MSRs par les Trxs, il est vraisemblable que la réaction limitante est la réduction de l'enzyme cible oxydée par la réductase à thiol (Boschi-Muller *et al*. 2008). En réalité, l'étape dite réductase, qui correspond à la réduction du substrat est beaucoup plus rapide (kcat/Km autour de 10^7 $M^{-1}.s^{-1}$) comme cela a pu être démontré pour plusieurs Prxs (Parsonage *et al*., 2008). Toutefois, cette grande variété de substrats susceptibles d'être réduits par les Prxs est essentielle puisque les catalases et ascorbate peroxidases sont des enzymes qui réduisent seulement H_20_2, ce qui semble confirmer que le nombre d'isoformes de Prxs est une réponse à la diversité des peroxydes à réduire. En effet, le nombre relativement important de peroxydases présentes chez la plante semble permettre la réduction de composés autres que le peroxyde d'hydrogène de manière tout aussi efficace, bien que leur efficacité reste a priori relativement faible. La réduction de lipides oxydés dans la mitochondrie et le chloroplaste est essentielle pour leur intégrité, en particulier

dans des situations de stress. Il a été déjà démontré que la Prx IIF d'*A. thaliana* est capable de réduire des hydroperoxydes phospholipidiques bien que l'efficacité soit inférieure à l'H_2O_2 par exemple (Finkemeier *et al*., 2005). Du point de vue de la spécificité de substrats, la Prx IIE peut réduire l'H_20_2 ainsi que des peroxydes plus complexes tels que le tBOOH, mais est incapable de réduire le CuOOH, qui inactive la protéine sans doute par suroxydation. D'un point de vue physiologique, la réduction préférentielle de la Prx IIE dans le chloroplaste par un système NADPH/GR/GSH/Grx-dépendant semble importante puisque ce mécanisme pourrait fonctionner à l'obscurité contrairement à la réduction dépendante de la lumière d'autres thiol-peroxydases utilisant la FTR et les Trxs chloroplastiques pour leur régénération. On peut aussi émettre l'hypothèse que cette voie NADPH-dépendante serait utilisée dans les tissus non-chlorophylliens, alors que le système dépendant de la FTR, et donc de la lumière est utilisé préférentiellement dans les tissus photosynthétiques. Cependant, les Grxs sont également exprimées au niveau des organes verts et le maintien de plusieurs systèmes de régénération pourrait être une assurance contre la défaillance d'une des voies de réduction.

Grâce à l'utilisation des banques de données de Genevestigator nous avons pu étudier les profils d'expression de la Prx IIF *d'A. thaliana* suivant les organes et les conditions de stress. Cette Prx IIF (At3g06050) est exprimée dans tous les organes de la plante. Parmi les conditions de stress présentées, l'expression de l'enzyme ne change pas considérablement comparée aux conditions témoins. Ces données indiquent que la Prx IIF semble être exprimée de façon constitutive comme cela a déjà été suggéré dans des précédentes expériences de RT-PCR (Brehelin *et al*., 2003) et donc que son rôle ne serait pas spécifique à un organe ou quelques organes.

Nous avons ensuite étudié les profils d'expression de la Prx IIF aussi bien chez *A. thaliana* que chez *P. trichocarpa*, en utilisant un anticorps dirigé contre la protéine d'*A.*

thaliana mais qui réagit également spécifiquement avec les Prx IIF d'autres plantes. Chez *A. thaliana*, la protéine est exprimée de manière similaire dans tous les tissus testés avec une quantité légèrement plus élevée dans les jeunes feuilles et les racines. En revanche, chez le peuplier, la quantité de protéine est sensiblement plus élevée dans les feuilles comparé aux tiges et encore plus même comparé aux racines et aux pétioles. De plus, en utilisant des extraits bruts mitochondriaux de feuilles de peuplier, nous avons confirmé que la Prx IIF est présente dans les mitochondries de peuplier. Ce résultat soutient des observations précédentes qui indiquaient que la Prx IIF d'*A. thaliana* est mitochondriale et serait donc chargée de protéger cet organite lors des stress oxydants. Cette localisation est retrouvée pour des extraits bruts mitochondriaux d'*A. thaliana*, de *Pisum sativum* et de *Solanum tuberosum*. Le fait que l'anticorps reconnaisse des orthologues d'autres espèces n'est pas surprenant puisqu'on remarque une identité de séquences d'environ 80%.

Les niveaux d'accumulation de cette protéine ont ensuite été étudiés suite à différentes conditions de stress. Les Prx IIB et Prx Q de peuplier ayant fait l'objet d'expériences montrant leur régulation lors d'infection du peuplier par le champignon pathogène *Melampsora larici populina* (Rouhier *et al*., 2004b), nous avons examiné d'abord les changements de quantités des protéines Prx IIF et Prx 2-Cys au cours de réactions compatibles ou incompatibles de plants de peuplier contaminés par ce champignon. Dans la réaction incompatible, la quantité de protéine Prx IIF diminue clairement entre 12 et 24 heures et augmente ensuite pour atteindre un niveau proche du niveau initial à 96 heures. En revanche, les niveaux de Prx 2-Cys totaux ne varient pas beaucoup avec le temps d'infection, bien que l'intensité de sa forme dimérique augmente entre 12 et 24 heures. Dans la réaction compatible, la quantité de Prx IIF augmente durant les 6 premières heures et atteint des très hauts niveaux après 96 heures d'infection. Pour la Prx 2-Cys, l'expression du monomère ne change pas alors que la quantité de dimère diminue entre 6

et 96 heures. Ces résultats indiquent que la quantité de Prx 2-Cys ne change pas suite à l'infection, conformément aux expériences précédentes qui ont montré que les niveaux de cette protéine restent remarquablement constants. La quantité de Prx IIF est en revanche perturbée pendant l'infection et varie de manière opposée dans les réactions compatible et incompatible. Ces résultats se distinguent de ceux décrits pour les Prx IIB et Prx Q, dont l'expression augmente dans une réaction incompatible et diminue dans une réaction compatible (Rouhier *et al*., 2004). Le sens physiologique de ces variations reste incertain puisque nous avions anticipé une augmentation de l'abondance des Prxs provoquée par la formation en masse de molécules oxydantes suite à la réaction incompatible et un comportement opposé dans les réactions compatibles. Cependant, cela indique que la régulation des Prxs est différente selon les conditions de stress testés.

Nous avons examiné ensuite les variations d'abondance de la Prx IIF d'*A. thaliana* en réponse à divers stress abiotiques. Suite à une exposition à des métaux lourds, aucun changement n'a été remarqué. De la même façon, aucun changement n'a été observé suite au traitement entraînant un stress photo-oxydant. En situation de déficit hydrique, la quantité de Prx IIF augmente légèrement dans les feuilles adultes, mais pas dans les jeunes feuilles. En réponse au froid, le niveau de Prx IIF augmente également légèrement après 1 et 5 jours de traitement mais de façon plus considérable après 10 jours de traitement. Enfin, la sénescence des feuilles ne produit aucune modification du taux de Prx IIF, contrairement à la Prx 2-Cys, dont les diminutions d'expression après 144 h d'induction de sénescence sont notables. Le résultat concernant la Prx 2-Cys était attendu puisque le nombre de chloroplastes et donc des protéines chloroplastiques à ce point de la sénescence.

Des expériences similaires ont été réalisés pour la Prx IIE, ainsi que les Prxs IIB, IIC et IID et les Prxs chloroplastiques Q et 2-Cys. La Prx IIE est présente de façon constitutive dans tous les tissus analysés chez *A. thaliana*, avec un niveau tout de même inférieur au niveau des racines. En condition de stress (déficit hydrique et traitement photo-oxydant) et sans tenir compte des espèces utilisées (*A. thaliana*, la tomate et l'orge), il n'y a presque aucune variation de quantité. Au niveau transcriptionnel, l'expression des Prx IIB et IIC est augmentée suite à des conditions de stress oxydant, mais également en condition de stress causé par un apport de sel, alors que l'expression de la Prx IIE d'*A. thaliana* n'est pas affectée par ces conditions de stress oxydant, mais diminue en réponse à l'application d'ascorbate et en réponse à un stress salin (Horling *et al*., 2003).

En comparant le profil d'expression des différentes Prxs plastidiales suivant les stress testés, deux types de comportement peuvent être soulignés bien que les analyses d'expression aient été faites essentiellement chez *A. thaliana* et qu'il soit difficile d'étendre les conclusions à d'autres organismes. Les quantités de Prx 2-Cys et de Prx IIE ne varient généralement pas dans les feuilles suite à un stress biotique ou abiotique (Collin *et al*., 2005; Gama *et al*., 2007; Havaux *et al*., 2007), alors que l'expression de la Prx Q est modifiée dans les deux types de stress. Ainsi, ces résultats suggèrent que parmi les thiol peroxydases du chloroplaste, la Prx Q et les Gpx, mais ni Prx 2-Cys ni Prx IIE, semblent surtout impliquées en réponse aux contraintes de l'environnement (Navrot *et al*., 2006). L'expression de Prx 2-Cys et de Prx IIE pourrait néanmoins être modifiée dans certaines conditions de stress oxydant, c'est ainsi le cas des stress salins où la quantité de transcrits pour les deux gènes diminue fortement (Horling *et al*., 2002). Cependant, nous ne pouvons pas exclure que des modifications post-traductionnelles pourraient contrôler l'activité des Prxs en condition de stress, bien que les quantités de protéines seraient inchangées. Effectivement, il a été montré que la suroxydation

de la cystéine catalytique en acide sulfinique ou acide sulfonique en ce qui concerne la Prxs 2-Cys aboutirait à l'inactivation de l'enzyme et la proportion de Prx suroxydée dépendrait des conditions de l'environnement indépendamment de l'abondance de la protéine (Rey *et al*., 2007). Les suroxydations éventuelles des Prxs pourraient permettre à ces enzymes de jouer un rôle dans la signalisation cellulaire en réponse à des conditions de stress, aboutissant à un stress oxydant comme cela a été montré récemment avec la Prx 2-Cys d'*A. thaliana* (Iglesias-Baena *et al*., 2010). Cette protéine sous forme suroxydée, et régulée par une sulfirédoxine (Srx), semble avoir un rôle dans la signalisation cellulaire sous certaines conditions de croissance.

La deuxième partie de mon travail de thèse a concerné l'étude d'un sous-groupe de Grxs encore très peu étudié, les Grxs possédant un site actif CGFS et regroupée dans la classe II (Couturier *et al*., 2009). La figure 24 résume les différents rôles physiologiques des glutarédoxines au niveau des cellules végétales en se basant sur les connaissances actuelles (Cheng, 2008; Couturier *et al*., 2009; Pedrajas *et al*., 2010; Rouhier *et al*., 2004) . Elles participent à la réponse au stress oxydant en alimentant les systèmes antioxydants (GrxS12, GrxC1) ou en interagissant avec des facteurs de transcriptions (GrxC7, GrxC9). Enfin, les Grxs de classe II (GrxS14 à S17) sont impliquées dans la biogenèse des centres fer-soufre ou dans le sensing du fer.

Au commencement de ma thèse, seules les Grxs de classe I avec des sites actifs du type CXXC/S avaient été caractérisées. Dans un premier temps, l'étude des Grxs CGFS était justifiée par le fait que certaines Prxs puissent être alimentées en pouvoir réducteur par ces protéines. Cependant, très rapidement l'information que ces glutarédoxines pouvaient jouer un rôle dans la biogenèse des centres fer-soufre s'est imposée et nous avons alors décidé de nous focaliser sur ce nouveau et excitant développement. Des études menées sur la Grx5 de *S.*

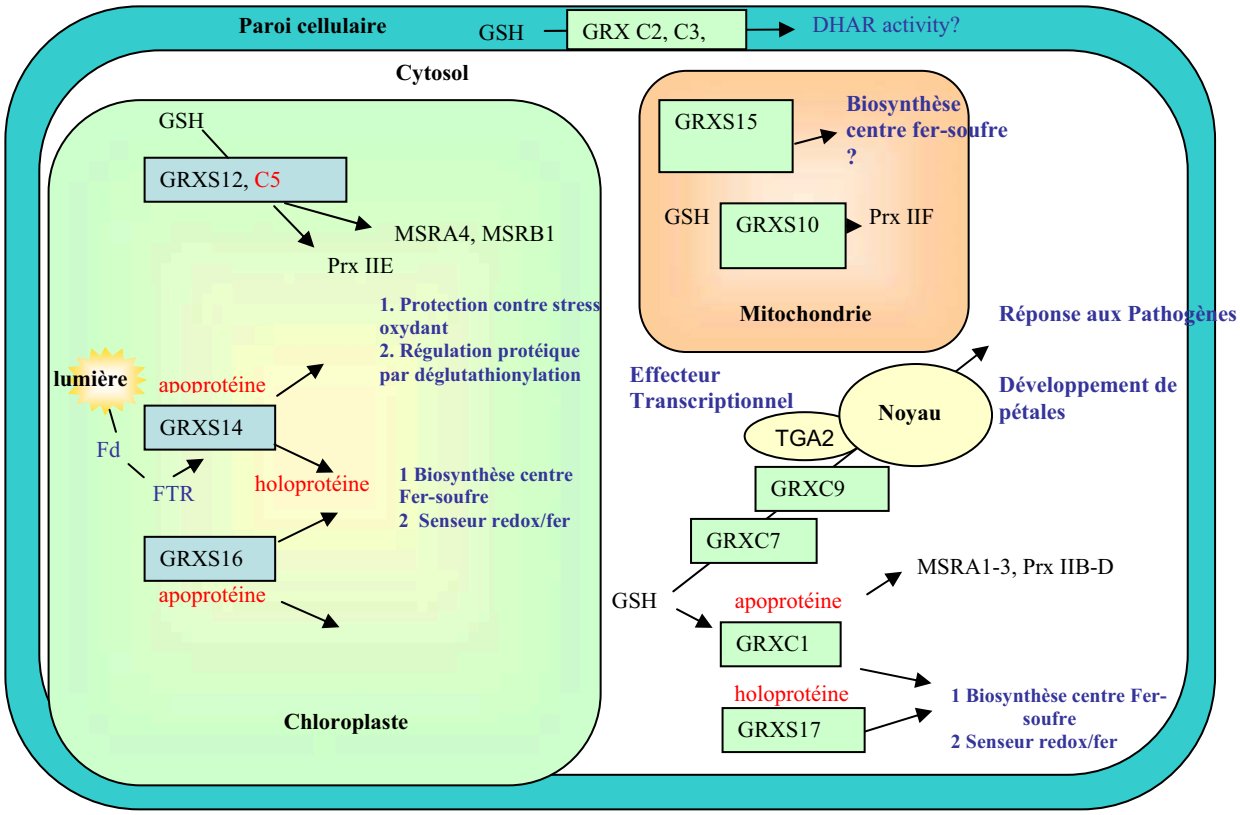

Figure 24 : Les rôles avérés ou supposés des glutarédoxines dans les cellules végétales.
Les glutarédoxines font partie d'une famille multigénique composée d'environ 30 membres chez les plantes supérieures. Au niveau des chloroplastes, les Grxs S12 et C5 participent à la régénération de Prxs ou de MSR et vont donc protéger cet organite du stress oxydant. Par ailleurs, elles pourraient réguler un certain nombre d'autres enzymes en fonction des conditions redox grâce à leur activité de déglutathionylation.
Les Grxs S14 et S16 semblent participer à la biosynthèse des centres fer-soufre de protéines chloroplastiques. Ce rôle serait dévolu à la Grx S15 au niveau de la mitochondrie. Au niveau du cytosol, la GrxC1 réduit les MSRA1 et 3 et les Prx IIB à D dans le cadre de la réponse au stress oxydant. Pouvant intégrer un centre fer-soufre, elle jouerait en compagnie de la Grx S17 (site actif CGFS) un rôle similaire aux Grx S14, S15 et S16 au niveau de la machinerie d'assemblage des centres fer-soufre cytosolique ou alors un rôle de senseur du fer ou de l'état redox. Enfin, les Grx C7 et C9 et certainement d'autres Grxs de type CC participent à la réponse aux pathogènes et au développement des pétales à travers la régulation de facteurs de transcription de type TGA (Transcriptor Gene Activator).

cerevisiae, notamment par délétion du gène codant cette protéine, ont permis de montrer qu'elle jouait un rôle dans la biosynthèse mitochondriale des centres fer-soufre (Rodriguez-Manzaneque *et al*., 2002) et des études d'immunoprécipitation ont suggéré qu'elle facilitait le transfert de ces centres de la protéine d'échafaudage Isu vers des protéines acceptrices (Mülhenhoff *et al*., 2003). Plus tard, il a été montré que certaines glutarédoxines pouvaient former des holodimères liant un centre [2Fe-2S] grâce à l'atome de soufre de la cystéine catalytique de chaque monomère et ceux de deux molécules de GSH (Feng *et al*., 2006; Johansson *et al*., 2007; Rouhier *et al*., 2007). A la lumière de ce résultat, il est surprenant que le rôle de la Grx5 mitochondriale de levure dans la biosynthèse des centres ne semble pas dépendre du GSH qui est exigé pour l'assemblage de ces centres. En effet, une diminution des niveaux de GSH affecte la maturation des protéines cytosoliques à centre fer-soufre, mais n'a aucun effet significatif sur la biosynthèse mitochondriale de ce même type de protéine (Sipos *et al*., 2002). Toutefois, les quantités résiduelles de GSH pourraient être malgré tout suffisantes pour que la machinerie fonctionne. Une autre explication pourrait être que la ligation de centre fer-soufre soit différente *in vivo* et n'implique pas le glutathion.

Dans les travaux présentés, les localisations subcellulaires de chaque Grx CGFS de peuplier ont été étudiées ainsi que leur capacité à complémenter la souche de levure délétée pour Grx5. Toutes les Grxs CGFS, excepté Grx S15, sont capables de complémenter ce mutant alors que les Grxs dithiols sont inefficaces. La Grx C1 contient pourtant un centre [2Fe-2S] mais cela ne semble pas suffisant pour complémenter la levure délétée. Ces résultats sont un peu surprenants car les 2 Grxs les plus efficaces (Grx S14 et S16 de peuplier) sont des protéines chloroplastiques alors que Grx S15 est mitochondriale et que la Grx5 de levure l'est également. Il semble donc qu'il n'existe pas une spécificité importante entre les machineries SUF et ISC. A l'époque de la publication de nos résultats, l'absence de complémentation

observée avec la Grx S15 de peuplier était cohérente avec son incapacité à incorporer un centre fer-soufre lors de son expression hétéroloque dans *E. coli*. Toutefois, bien que très labile, on a maintenant des évidences suite à des expériences de reconstitution *in vitro* que cette Grx peut en fait incorporer un centre fer-soufre. Ce résultat reste encore incompris d'autant que l'orthologue d'*A. thaliana*, appelé Grx4, semble complémenter le phénotype du mutant délété de la Grx5 chez la levure (Cheng, 2008). Néanmoins, les travaux de Cheng indiquent que la protéine serait localisée dans le chloroplaste alors que la GrxS15 de peuplier est adressée vers la mitochondrie. Ces résultats opposés nous ont fait penser qu'ils pouvaient avoir inversé ou mélangé des constructions. Pour en avoir confirmation, nous avons effectué des tests de complémentations avec les Grxs CGFS d'*A. thaliana*. Toutes les protéines sauf GrxS15 complémentent effectivement la souche de levure délétée pour Grx5 (résultats non publiés).

La capacité des Grxs monothiols à intégrer un centre [2Fe-2S] et à le transférer à des protéines acceptrices suggèrent plusieurs fonctions possibles. Elles pourraient avoir un rôle de protéines d'échafaudages, un rôle dans le transfert des centres Fe-S à partir des autres protéines d'échafaudage potentielles (c'est-à-dire SufA, SufB et Nfu au niveau des chloroplastes et Isa/IscA, Nfu et Isu dans les mitochondries) vers des protéines acceptrices spécifiques. Cette dernière hypothèse concorde avec les travaux de génétique menés chez la levure (Mülhenhoff *et al*., 2003). Enfin, elles pourraient contribuer au stockage de groupements Fe-S préformés en condition de stress et/ou de manière basale. Puisque la suppression de ces gènes ne semble pas létale, on peut penser qu'il existe d'autres protéines avec des fonctions analogues qui assureraient toujours la formation et le transfert de cluster, bien qu'à un taux inférieur par rapport à la souche sauvage. Des études d'interactions réalisées *in vivo* chez la levure par double hybride indiquent qu'il existe une interaction entre la Grx5 et les protéines de type Isa (Vilella *et al*., 2004). On peut donc formuler l'hypothèse que des transferts de centres fer-

soufre pourraient avoir lieu entre les 2 partenaires et que le processus d'assemblage pourrait être complètement annihilé par la délétion simultanée de ces gènes.

Les études spectroscopiques réalisées sur AtGrxS14 et AtGrxS16, soit purifiées en conditions anaérobie soit reconstituées en présence de L-cystéine, Fe^{2+} et GSH et d'une cystéine désulfurase, indiquent que ces protéines intègrent également un centre [2Fe-2S] mais dans un environnement qui semble être légèrement différent, bien qu'impliquant le GSH. Des données structurales obtenues pour la Grx 4 d'*E. coli* ont confirmé que la protéine s'organise en homodimère avec un cluster stabilisé lui aussi par deux molécules de GSH (Iwema *et al*., 2009). Cependant, les deux protomères ont une conformation légèrement différente du fait de la présence d'une insertion de quelques acides aminés devant le site actif, ce qui expliquerait la nature plus labile du cluster et sa propension à être transféré. Ceci est en accord avec les expériences de transfert que nous avons réalisées puisque la Grx S14 pouvait transférer son centre à une apo-ferrédoxine avec une efficacité jamais observée jusqu'ici, alors que Grx C1 (site actif CGYC) en était incapable. Lorsque l'on compare les séquences de la Grx C1 de peuplier, de la Grx S14 d'*A. thaliana* et de la Grx 4 d'*E. coli*, on s'aperçoit que l'extension en question est conservée dans Grx14 ce qui laisse à penser que la protéine pourrait adopter la même structure que la Grx4 d'*E. coli* (Figure 25). Par ailleurs, des résultats de mutagenèse dirigée ont indiqué que la deuxième cystéine en position C-terminale et assez conservée dans un motif GGCD est nécessaire pour l'activité déglutathionylation *in vitro* (Belli *et al*., 2002), mais n'est cependant pas exigée pour la machinerie d'assemblage des centres fer-soufre. Ceci semble confirmé pour AtGrx S14 et S16, puisque la mutation de ces résidus n'empêche pas la fixation du cluster (Bandyopadhyay *et al*., 2008). La différence entre GrxC1 et GrxS14 et S16 pourrait être minime puisqu'une forme mutée de GrxC1 avec un site CGFS peut complémenter la souche $Grx5^-$, bien que ne présentant pas l'insertion. Il serait intéressant d'effectuer des

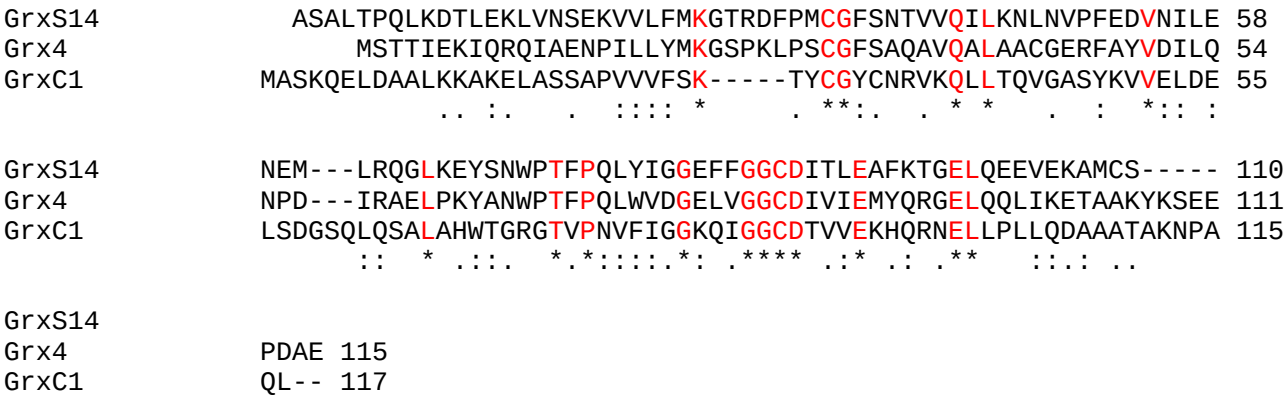

Figure 25 : Comparaison de séquences des GrxC1 de peuplier, GrxS14 d'*A. thaliana* et Grx4 d'*E. coli.*
En rouge sont figurés les acides aminés strictement identiques qui correspondent essentiellement aux résidus nécessaires à la fixation du GSH sur la protéine.

expériences de transfert avec cette forme mutée même si le fait que la protéine soit stable au cours d'une purification aérobie n'est pas en faveur d'un transfert efficace.

Parmi les glutarédoxines, il faut distinguer donc les Grxs de classe I qui incorporent des clusters plutôt stables et les Grxs de classe II qui incorporent des clusters labiles (Rouhier *et al*., 2007). Plusieurs travaux de mutagenèse dirigée ont démontré que la séquence du site était déterminante pour la capacité des Grxs à intégrer un tel centre (Rouhier *et al*., 2007; Couturier *et al*., 2009). Lillig et collaborateurs ont soumis l'hypothèse que la forme holodimérique de la Grx2 humaine était un senseur de stress régulant l'accumulation et l'activation de sa forme apo-monomérique (Lillig *et al*., 2005). Par ailleurs, on a constaté que les deux autres Grxs à site actif CGFS de la levure, Grx3 et Grx4, étaient des protéines nucléocytoplasmiques principalement impliquées dans la régulation de la localisation nucléaire du régulateur transcriptionnel du régulon fer, Aft1 (Ojeda *et al*., 2006; Pujol-Carrion *et al*., 2006). Cette régulation implique au minimum une autre protéine de la famille BolA (Kumanovics *et al*., 2008). Il a ainsi été montré que les Grx 3 et 4 de levure sont capables de former un complexe hétérodimérique avec la protéine Fra2, homologue de BolA, contenant un centre [2Fe-2S] (Li *et al*., 2009). Ce centre est lié à la cystéine de la Grx et à une molécule de GSH alors qu'elle se fixe à une histidine du monomère Fra2. La présence d'un centre fer-soufre dans ce complexe jouerait le rôle de senseur de la quantité cellulaire en fer pour ensuite réguler le facteur de transcription Aft1 (Kumanovics *et al*., 2008). Ces données nous ont poussé à regarder si l'orthologue des plantes, GrxS17, pourrait interagir de la même manière avec une protéine de type BolA. Au cours d'analyses bioinformatiques préliminaires, plusieurs isoformes de BolA ont été identifiées, BolA1, A2, A3 et SUFE1, une protéine de la machinerie d'assemblage chloroplastique qui possède un domaine BolA du coté N-terminal d'un domaine SufE (Xu and Moller, 2006). Puisque les protéines BolA sont prédites pour être localisée dans le cytosol

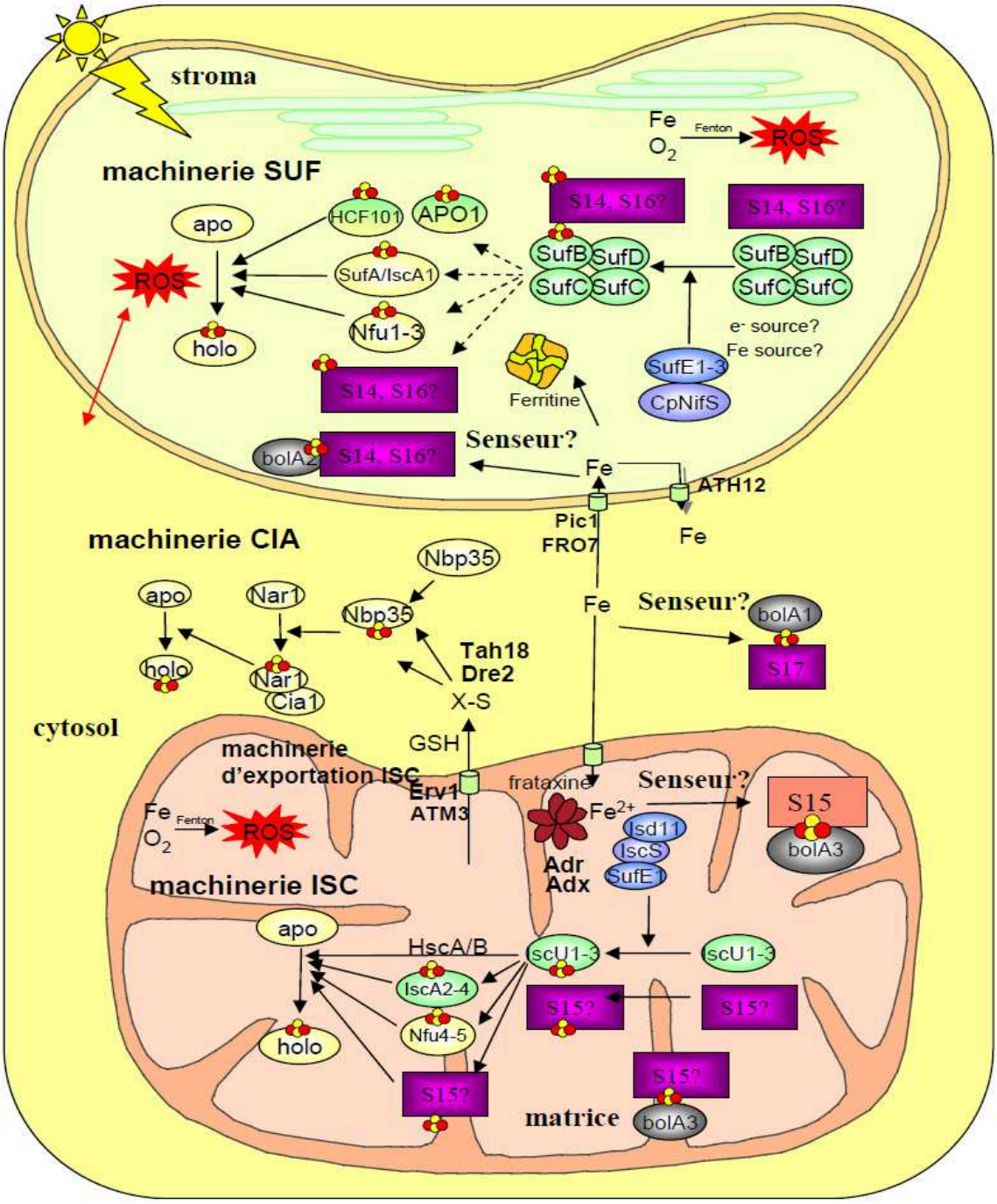

Figure 26 : Les mécanismes de biosynthèse et d'intégration des centres fer-soufre chez les plantes et les rôles supposés des glutarédoxines.
Cette biosynthèse s'effectue aussi bien dans la mitochondrie (machinerie ISC) que dans le chloroplaste (machinerie SUF) et le cytosol (machinerie CIA). Les Grxs S14 et S16 participent à la maturation des centres fer-soufre soit en tant que protéine d'échafaudage soit en tant que protéine de transfert. De plus, elles pourraient jouer un rôle de senseur du fer dans le chloroplaste grâce à son interaction avec la protéine BolA2 ou SUFE1. Au niveau de la mitochondrie, les processus semblent conservés, sauf que les acteurs sont GrxS15 et BolA3.
La fonction de Grx S17 pourrait être liée à la machinerie cytosolique ou au sensing du fer en interaction avec BolA1.

(BolA1), les plastes (Bol2 et SUFE1) et les mitochondries (BolA3) où se trouvent également des Grx de classe II, il sera crucial d'examiner si une interaction est possible. La figure 26 résume les connaissances actuelles concernant le rôle des Grxs lors de la formation des centres fer-soufre et replace également les protéines BolA. Dans cette figure, aucune des hypothèses citées précédemment n'est écartée, et le rôle et donc la position des glutarédoxines dans la machinerie d'assemblage restent incertains. La présence de protéines BolA dans la plupart des compartiments cellulaires et d'un domaine BolA dans SUFE1 pourrait également suggérer une fonction de sensing et de régulation des niveaux en fer ou en centre fer soufre. De manière très intéressante, une étude protéomique menée chez *E. coli* a identifié les Grx3 et 4 comme partenaires de deux paralogues, SufE et CsdE (Bolstad *et al*., 2010). La protéine SufE d'*E. coli* ne possède pas de domaine BolA, qui ne serait pas requis dans ce cas. Afin de comprendre le rôle exact de ces Grxs, une approche fonctionnelle a été entreprise comprenant entre autres l'étude de plantes mutées ou surexprimant ces gènes. Une autre direction prometteuse sera la recherche d'interactions entre les Grxs et les protéines de la machinerie de formation des centres et notamment avec les protéines de type Suf ou BolA par des approches candidates, de type double hybride ou par des approches sans a priori également de type double hybride par criblage de banque de cDNA ou de type co-immunoprecipitation.

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Résumé : Les variations de l'environnement peuvent influer sur le métabolisme de la plante, notamment à travers la formation des espèces oxygénées réactives (EOR) nocives à forte concentration. Des systèmes enzymatiques vont dégrader ces EOR mais aussi réparer les molécules oxydées par ces réactifs. Ils sont composés notamment de peroxydases (glutathion peroxydases utilisant les thiorédoxines comme réducteur, et les peroxyrédoxines utilisant les glutarédoxines et les thiorédoxines pour leur régénération) dont l'activité s'appuie sur des échanges dithiol-disulfure. Ces systèmes présentent une complexité liée à l'existence de familles multigéniques. Des hybrides Prx-Grx actifs ont été produits de façon recombinante, donnant des indications sur les interactions entre Prx et Grx. Les études enzymatiques réalisées sur les PrxIIE et IIF ont montré qu'elles sont capables de réduire de nombreux substrats autres que le peroxyde d'hydrogène avec des efficacités variables. De plus, leurs localisations tissulaires sont différentes ainsi que leurs réactions en réponse à divers stress oxydant. Les glutarédoxines pourraient participer aussi à la signalisation par le mécanisme de glutathionylation, impliqué dans la protection de cystéines critiques par fixation d'une molécule de glutathion. Outre leur fonction de réducteurs, les glutarédoxines aident à la formation et l'intégration des centres fer-soufre et participent donc à la maturation des protéines fer-soufre. Les Grxs chloroplastiques S14 et S16 intègrent de façon naturelle un centre fer-soufre et complémentent des mutants de levure déficients en Grx5, une glutarédoxine CGFS nécessaire pour l'assemblage des centres fer-soufre. Enfin, la GrxS14 est capable de transférer efficacement son centre à une protéine acceptrice suggérant qu'elle pourrait soit avoir un rôle de donneur de centre fer-soufre, soit aider au transfert de ces centres. Ces résultats nous permettent de mieux comprendre la redondance de ces familles : ces systèmes modulent différents processus physiologiques, dont certains encore inconnus, en réponse au stress oxydant.

Mots-clés : Peuplier, Stress oxydant, Peroxyrédoxines, Glutarédoxines, centre fer-soufre

Abstract: Environmental variations can modify plant metabolism in particular by production of reactive oxygen species (ROS) which are harmfull at high level. Enzymatic systems can degrade these ROS but also repair oxidized molecules. They are composed by peroxidases (thioredoxin-dependent glutathione peroxidases and thioredoxin- or glutaredoxin-dependent peroxiredoxins) which use dithiol-disulfide exchange reaction for their regeneration. These systems present a complexity according to their multigenic family origin. Recombinant Prx-Grx hybrids have been produced giving clue to interactions between Prx and Grx. Enzymatic studies showed that PrxIIE and IIF can reduce a wide range of substrates other than hydrogen peroxide, with variable efficiencies. Furthermore, they are not expressed in the same plant tissues and they react to oxidative stress in different ways. Grx could also participate to cell signalling by glutathionylation, a mechanism which protects cysteines by fixating a GSH molecule. Besides their reductase activity, Grxs help produce iron-sulfur clusters and play a role in the maturation of iron-sulfur proteins. Plastidial GrxS14 and S16 can naturally bind an iron-sulfur cluster and complement a yeast mutant deficient for Grx5, a mitochondrial ortholog known to participate to iron-sulfur protein maturation. Finally, GrxS14 is able to transfer efficiently his cluster to an acceptor protein, suggesting that it could act either as a scaffold or a carrier protein. These results allow us understanding better these multigenic families regulating several physiological processes, some still unknown, in oxidative stress.

Key Words: Poplar, Oxidative stress, Peroxiredoxins, Glutaredoxins, iron-sulfur cluster