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par **Nicolas NAVROT**

Etude fonctionnelle des glutathion peroxydases de peuplier, une famille de peroxydases thiorédoxine-dépendantes

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SOMMAIRE

ABBREVIATIONS

ADN : acide désoxyribonucléique (chlDNA, ADN chloroplastique ; mtDNA, ADN mitochondrial)

Apx : ascorbate peroxydase

Asc : ascorbate

CAT : catalase

CDSP32 : chloroplastic drought stress protein of 32kDa

DHA : déhydroascorbate

DHAR : déhydroascorbate réductase

EST : expressed sequence tag (séquence exprimée)

ETC : electron transfer chain (chaîne de transfert d'électrons)

FAD : flavine adénine dinucléotide

FBPase : fructose 1,6-bisphosphatase

Fd : ferrédoxine

FTR : ferrédoxine thiorédoxine réductase

GFP : green fluorescent protein (protéine fluorescente verte)

Gpx : glutathion peroxydase

GR : glutathion réductase

Grx : glutarédoxine

GSH/GSSG : glutathion réduit/oxydé

GST : glutathion S-transférase

LA : acide lipoïque

LHC : light harvesting complex (antenne collectrice de lumière)

MAP kinase : mitogen activated protein kinase

MDH : malate déshydrogénase

MDHA : monodéhydroascorbate

MDHAR : monodéhydroascorbate réductase

MSR(ase) : méthionine sulfoxyde réductase

NAD(P)H : nicotinamide adénine dinucléoside (phosphate) réduit

NDK : nucléotide diphosphate kinase

NO : oxyde nitrique

Nrx : nucleorédoxine

NTR : NADPH thiorédoxine réductase

PDI : protéine disulfide isomérase

PHGpx : phospholipide hydroperoxyde glutathion peroxydase

Prx : peroxyrédoxine

PSI : photosystème 1

PSII : photosystème 2

RNR : ribonucléotide réductase

ROS : reactive oxygen species (espèces actives de l'oxygène)

RT-PCR : retrotranscription-polymerase chain reaction

Sec : sélénocystéine

SECIS : selenocysteine insertion system (système d'insertion de sélénocystéine)

SOD : superoxyde dismutase

Srx : sulfirédoxine

Trx : thiorédoxine

INTRODUCTION

Une différence majeure entre les organismes aérobies et anaérobies stricts est la capacité d'utiliser et de tolérer l'oxygène gazeux et ses formes dérivées. Les organismes photosynthétiques aérobies ont développé un métabolisme basé sur l'utilisation de l'oxygène au travers de la respiration et de la photosynthèse. Dans ces deux voies métaboliques, des chaînes de transport d'électrons permettent la génération d'O_2 à partir d'H_2O (photosynthèse) ou la réduction d'O_2 en H_2O (respiration).

Lors des réactions liées à la photosynthèse ou à la respiration, des intermédiaires réactionnels de l'oxygène (ROS, Reactive Oxygen Species) sont souvent générés, ces ROS étant susceptibles d'endommager les macromolécules constituant les cellules vivantes. La production de ROS en grande quantité est aussi une conséquence au niveau cellulaire d'un stress environnemental. Les végétaux, de part leur immobilité, sont particulièrement exposés à ces changements plus ou moins rapides de leur environnement.

Au niveau cellulaire, les conséquences de cette accumulation de ROS sont nombreuses: ainsi, l'oxydation des bases de l'ADN, et notamment celle de la thymine qui est très sensible, pourrait conduire à des mutations qui sont susceptibles de perturber le fonctionnement cellulaire (Bao et al 1997). Les membranes cellulaires sont aussi sensibles à l'oxydation à travers des peroxydations en chaîne au sein des couches lipidiques qui peuvent aboutir à une perte de l'intégrité membranaire ou à la rigidification des membranes (Stark 2005). Enfin, les protéines cellulaires peuvent subir différents types d'altération suite à leur

réaction avec des ROS. Des dommages peuvent être causés par l'oxydation de différents acides aminés, et la perturbation des structures tertiaire et quaternaire par une oxydation excessive et non contrôlée peut aussi causer une perte de l'activité de la protéine et sa dégradation par la cellule (Davies 2005). Tous ces effets nocifs sont maintenant connus depuis plusieurs décennies, et sont dus à la présence de ROS à des concentrations relativement élevées (Halliwell et Gutteridge 1990).

Cependant, l'émergence de nouvelles techniques, ainsi que l'étude plus fine des relations entre ROS et métabolisme cellulaire, ont conduit à relativiser ce rôle uniquement délétère des ROS dans les cellules. En effet, les cellules peuvent utiliser ces ROS pour empêcher le développement d'organismes pathogènes, par exemple lors de réactions incompatibles entre les végétaux et diverses races de pathogènes, et il est maintenant admis que ces molécules ont un rôle essentiel de transduction des signaux à l'intérieur de la cellule, mais aussi entre les cellules d'un organe, comme par exemple pour le peroxyde d'hydrogène (H_2O_2) dans le mécanisme de fermeture des stomates (Zhang et al 2001). Ainsi, à l'instar de l'oxygène moléculaire, qui possède un potentiel énergétique aussi grand que son pouvoir destructeur, les espèces oxygénées réactives ont deux facettes, l'une découle de leur réactivité importante et les rend nocives pour les cellules, l'autre a été mise en place par les cellules, qui vont les utiliser comme molécules de signalisation, tant que leur concentration peut être contrôlée par les systèmes antioxydants. La molécule d'H_2O_2 en particulier est impliquée dans l'induction de la transcription de nombreux gènes, tels que ceux de la glutathion S-transférase (GST), ou encore dans l'activation de kinases (Desikan et al 1999), elles-mêmes impliquées dans la réponse des cellules aux stress extérieurs. Les études de transcriptomique montrent aussi qu'un très grand nombre de gènes voient leur

expression stimulée ou réprimée par l'H_2O_2 (Desikan et al 2001). D'autres études utilisant des mutants d'*Arabidopsis thaliana* ont mis en évidence une réponse rapide et spécifique du transcriptome à l'oxygène singulet (op den Camp et al 2003). Il devient donc possible de déterminer les groupes de gènes qui sont stimulés ou réprimés spécifiquement selon l'espèce oxygénée utilisée comme stimulus (Gadjev et al 2006).

Les compartiments cellulaires qui contiennent les chaînes de transfert d'électrons sont les principales sources mais aussi les principales cibles de ces ROS. Chez les animaux, les mitochondries sont considérées comme la principale source de ROS dans la cellule. Chez les végétaux en revanche, le chloroplaste est le principal lieu de production de ROS, au moins pendant la journée. Pendant les phases d'obscurité, le rôle de la mitochondrie devient prépondérant. Les lieux de production des ROS au sein des chaînes de transfert d'électrons sont étudiés depuis plusieurs décennies et sont encore un sujet d'intenses investigations. Ils sont détaillés dans le paragraphe ci-dessous.

I Production des ROS

I.A Au niveau du chloroplaste :

Dans cet organite se trouvent les composants d'une chaîne d'électrons qui va fonctionner dans un environnement riche en oxygène. Ceci induit un risque important de transfert d'électrons vers l'oxygène moléculaire, aboutissant à la

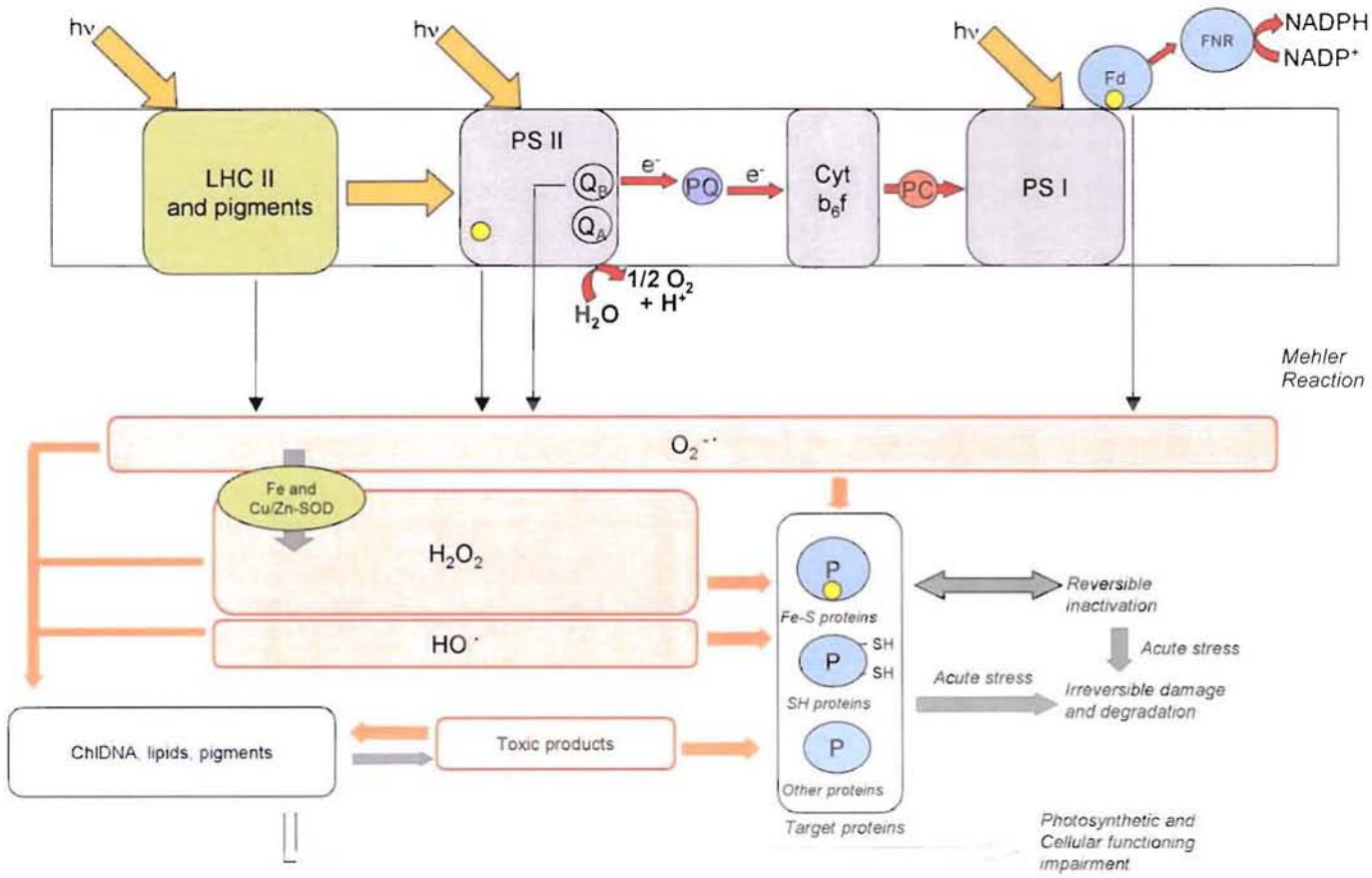

Figure 1. Origine et effets des ROS produits dans le chloroplaste. Les flèches rouges indiquent un transfert d'électrons. Les cercles jaunes figurent les centres Fer-Soufre. Les quinols sont en violet. Les complexes protéiques en vert peuvent intervenir dans la protection contre le stress oxydant. Les molécules en bleu représentent des cibles de l'oxydation. Les flèches fines indiquent une production de ROS, les flèches oranges un dommage oxydatif, les grises une conversion moléculaire.

formation de ROS. Plus particulièrement, cette fuite d'électrons peut tout d'abord se produire au niveau du photosystème I (PSI). En cas de surcharge de la chaîne de transfert d'électrons (ETC), la ferrédoxine (Fd) réduit directement l'oxygène moléculaire (O_2) en anion superoxyde (O_2^-) au cours de la réaction de Mehler (Figure 1). La réduction d'O_2 peut aussi se produire au niveau des centres Fer-Soufre (2Fe-2S et 4Fe-4S) du photosystème I, ainsi qu'au niveau du site accepteur de quinones Q_B du PSII (Zhang et al 2003). En résumé, la présence de métaux de transition comme le fer, ou de groupements quinols, va favoriser les fuites d'électrons de la chaîne de transfert d'électrons photosynthétique vers l'O_2. Les pigments comme la chlorophylle vont aussi pouvoir réduire l'oxygène moléculaire quand ils se trouvent dans un état excité. L'anion superoxyde produit peut réagir directement avec les molécules biologiques, ou se dismuter en H_2O_2 spontanément ou via une superoxyde dismutase à fer ou à cuivre et zinc (FeSOD ou Cu/ZnSOD). Des réactions de type Fenton entre H_2O_2 et des ions métalliques, libres ou incorporés dans diverses enzymes ou transporteurs, vont également générer des radicaux hydroxyles (OH), eux aussi très nocifs (Edreva 2005). La cellule a mis en place des voies spécifiques pour réguler la concentration en ROS du chloroplaste et pour maintenir un fonctionnement optimal de la photosynthèse (Mittler 2002). L'une d'elles est la photoinhibition, spécifique au chloroplaste, qui va empêcher un flux d'électrons trop important dans la chaîne de transfert en cas de forte illumination. Ce phénomène se déroule au niveau du photosystème II, dont l'un des composant principaux, le protéine D1, va subir des dommages irréversibles en cas d'exposition prolongée de la plante à une lumière intense. L'inactivation des photosystèmes II endommagés, qui vont ensuite être progressivement réassemblés avec des nouvelles protéines D1 synthétisées *de novo*, va diminuer l'activité

photosynthétique. Dans les cas les plus sévères, ou si elle est associée à d'autres facteurs de stress, cette inhibition de la photosynthèse peut avoir un effet négatif sur la croissance des végétaux. La chélation du fer libre, notamment par des protéines comme la ferritine, permet aussi d'éviter la production de radical OH (Briat et al 1999). En outre, des molécules de faibles poids moléculaires, dont la principale est l'ascorbate, ainsi que des systèmes enzymatiques, vont assurer la protection du chloroplaste contre les ROS. Ces systèmes sont détaillés ultérieurement dans ce manuscrit.

I.B Au niveau de la mitochondrie :

La production des ROS au sein de la mitochondrie est détaillée dans l'article I (Navrot et al 2007a). Elle a lieu principalement au niveau du complexe I et III de la chaîne respiratoire. La réduction excessive du pool membranaire d'ubiquinone par le complexe I peut aussi favoriser la réduction d'O_2 par ces molécules, ainsi que le fonctionnement rétrograde de la chaîne de transport, c'est-à-dire la réduction du complexe I par les quinones qui entraîne la formation d'anion superoxyde. Les systèmes antioxydants mitochondriaux, en particulier les sytèmes thiol-dépendants, sont aussi décrits dans l'article I.

Article I : ROS generation and antioxidant systems in plant mitochondria

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Cet article de synthèse décrit les systèmes qui génèrent les espèces oxygénées de l'oxygène (ROS) dans les mitochondries végétales. Plus particulièrement, l'implication de la chaîne respiratoire de transfert d'électrons est décrite, ainsi que les systèmes spécifiques des végétaux qui permettent la régulation du transfert d'électrons. Dans une seconde partie, les systèmes qui vont réguler la concentration et éliminer les ROS dans la matrice mitochondriale sont détaillés. Il s'agit des systèmes dépendants du glutathion, de l'ascorbate, de l'acide lipoïque, ainsi que des systèmes enzymatiques. Une analyse plus fine des systèmes thiorédoxines- et glutarédoxines-dépendants est présentée. Ces systèmes vont jouer un rôle prépondérant dans la détoxication des ROS dans la mitochondrie, notamment en réduisant les peroxyrédoxines et glutathion peroxydases présentes dans ce compartiment. De plus, ces systèmes sont sans doute impliqués dans la régulation d'enzymes telles que l'alternative oxydase, ou dans l'incorporation de centres fer-soufre dans les protéines mitochondriales.

REVIEW

Reactive oxygen species generation and antioxidant systems in plant mitochondria

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In living cells, reactive oxygen species (ROS) play a key role in signaling but these compounds can also damage macromolecules. As in other compartments, the mitochondrial ROS concentrations need to be tightly controlled. Plant mitochondria contain several antioxidant systems that are not only able to scavenge ROS and limit their production but also to repair damages to macromolecules and possibly to serve as redox sensors. They comprise ascorbate- and glutathione-dependent pathways as well as systems based on thioredoxin (TRX)- and glutaredoxin (GRX)-like molecules. This review describes the various mitochondrial redox pathways for ROS control in plants with special emphasis on the poorly studied GRX and TRX systems and provides perspectives for future research in this area.

Introduction

Land plants like other aerobic photosynthetic organisms, appeared and evolved in the presence of atmospheric oxygen and, as a consequence, developed metabolic pathways that were able to use the great energetic potential of O_2 and also to limit the deleterious side effects of this oxidant and its active derived molecules, known as reactive oxygen species (ROS). These ROS generated by aerobic metabolism include singlet oxygen ($^1O_2$), superoxide ions ($O_2^{\cdot -}$) and peroxides, the most widely distributed being hydrogen peroxide (H_2O_2). On the one hand, plants need to control the levels of these oxidants because of their harmful nature, but on the other hand, they also use ROS as signaling molecules especially in response to various stresses or threats to the plant integrity, as pathogen attacks, or non-optimal growth conditions. These molecules can thus act as messengers to trigger protein de/activation, or induce gene transcription (Desikan et al. 2001). It has been shown recently that singlet oxygen, superoxide ions and H_2O_2 all induce the transcription of specific sets of genes in plant cells (Gadjev et al. 2006). This signaling system also interacts with other pathways, like phosphorylation cascades by kinases or Ca^{2+} signaling, and is a part of the regulation network that connects the cell to its environment. Thus, plants are not only able to take advantage of the powerful O_2 molecule for their energy needs, but they also make use of some of its derivatives for intra- or intercellular signal transduction pathways. This review will essentially describe the mechanisms for generation and control of ROS in plants but will not deal further with the signaling aspects, which have been extensively treated in recent reviews (Foyer and Noctor 2003, Laloi et al. 2004).

Abbreviations – Aco, aconitase; AOX, alternative oxidase; APX, ascorbate peroxidase; ASC, ascorbate; CAT, catalase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; ETC, electron transfer chain; GPX, glutathione peroxidase; GR, glutathione reductase; GRX, glutaredoxin; GSH, glutathione; GSSH, oxidized glutathione; H_2O_2, hydrogen peroxide; MDHAR, monodehydroascorbate; MnSOD, manganese-containing superoxide dismutase; MSR, methionine sulfoxide reductase; NO, nitric oxide; NADH, nicotinamide adenine dinucleotide; NTR, NADPH-dependent thioredoxin reductase; PCD, programmed cell death; PRX, peroxiredoxin; ROS, reactive oxygen species; SOX, sulfhydryl oxidase; TRX, thioredoxin; UCP, uncoupling protein.

In plant cells, most of the ROS produced originate from chloroplasts or peroxisomes, but in non-green tissues, or in the dark, mitochondrial ROS production becomes predominant although a blue light-induced generation of ROS has been recorded in animal mitochondria (King et al. 2004). Moreover, although mitochondria are clearly not the major site of ROS production in plants, they are nevertheless a primary target for the ROS-induced damages because some ROS species diffuse freely through the cellular compartments (Bartoli et al. 2004, Dutilleul et al. 2003, Sweetlove et al. 2002). Recent studies indicate that plant mitochondria are at a cross-point in the signaling pathways involving ROS, especially those concerning apoptosis (Jones 2000). As the field of mitochondrial ROS is becoming very large, we will discuss here briefly the generation of oxidized species in mitochondria, their negative effect on biological processes and detail more extensively the various redox-active ROS regulation systems present in plant mitochondria. Reviews on related topics have also been published recently (Foyer and Noctor 2003, Laloi et al. 2004, Millar et al. 2001a, Møller 2001).

Generation and nature of ROS in plant mitochondria

In the respiratory process, oxygen becomes reduced to H_2O and different pathways are involved in this reduction process. Some enzyme systems reduce oxygen via tetravalent mechanisms [e.g. cytochrome *c* oxidase, alternative oxidase (AOX)] but some others reduce it in a stepwise manner, i.e. oxygen is accepting electrons one by one only. This leads to the formation of a reactive intermediate molecule, namely the superoxide anion $O_2^{\cdot -}$. The major site of superoxide formation in mitochondria lies in the electron transfer chain (ETC), especially at the level of Complex I and Complex III. It was shown in animal mitochondria that the flavine mononucleotide (FMN)-containing subunit and an iron–sulfur cluster of the nicotinamide adenine dinucleotide (NADH) dehydrogenase of Complex I are the sites of $O_2^{\cdot -}$ generation (Chen et al. 2005), especially when this complex is glutathionylated after oxidative stress (Taylor et al. 2003). This complex could amplify ROS production and participate in the regulation of ROS concentrations in the whole cell. The overreduction of the ubiquinone pool by Complex I can also lead to a reverse functioning of the chain, and to the formation of large amounts of ROS. In Complex III, the overreduction state of the ubiquinone pool can lead to a direct electron transfer to molecular oxygen, and to the formation of superoxide anions (see Fig. 1).

Most of the superoxide ions produced are efficiently converted to H_2O_2 by a manganese-containing superoxide dismutase (MnSOD). H_2O_2 can then react with iron or copper ions in the Fenton reaction to generate hydroxyl radicals $OH^{\cdot}$. Iron–sulfur containing proteins, like aconitase, may also be involved in these reactions, as it releases Fe^{2+} upon inactivation of the enzyme by $O_2^{\cdot -}$, also leading to Fenton-type reactions (Vasquez-Vivar et al. 2000). Other ROS targets are high-molecular mass molecules, such as membrane lipids or mitochondrial DNA, with the formation of lipid or nucleotide peroxides, especially at the level of thymine (Cullis et al. 1987). Furthermore, mitochondrial DNA is more sensitive to oxidative damage than nuclear DNA, in particular because of the absence of chromatin organization and lower mitochondrial DNA repair activities (Yakes and Van Houten 1997).

Adverse environmental conditions generate this kind of oxidative stress. For example, pathogen attacks of the plant (Kuzniak and Sklodowska 2004), senescence or saline stress (Maxwell et al. 2002, Mittova et al. 2004), as well as accumulation of the metal ion aluminum (Yamamoto et al. 2002), initially result in an accumulation of ROS. In turn, these species impair the mitochondrial metabolism but they also constitute an intracellular signal, especially involved in the transcription regulation in the nucleus.

Besides ROS, the cells also contain reactive nitrogen species. They are products of the reaction of nitric oxide (NO) with different biological molecules, and as ROS they can be harmful to the cell. NO itself is also involved in metabolic regulation. It was shown to interfere with the function of Complex III, leading to the production of $O_2^{\cdot -}$ (Yamasaki et al. 2001). Interestingly, the mitochondrial respiratory chain from root cells can also be a source of NO, via nitrite reduction under anoxic conditions (Gupta et al. 2005). This is probably part of the yet poorly understood role of this molecule and its derivatives in signal transduction, and will not be developed further here.

Finally, mitochondria play an essential role in the programmed cell death (PCD) pathway, via cytochrome *c* release (Jones 2000, Vianello et al. this issue). This mechanism is similar to the one present in mammal cells which involves mitochondrial thioredoxin (TRX) (Tanaka et al. 2002) and possibly AOX (Robson and Vanlerberghe 2002). In the case of heat-shock-induced PCD, it is also likely that mitochondrial respiration impairment is one of the earliest events (Vacca et al. 2004). High ROS production as well as cytochrome *c* release from mitochondria and increased NO production are observed when a hypersensitive response is triggered by eliciting compounds (Krause and Durner 2004).

Thus, this organelle is a major sensing component in the cell, also coordinating the cell response to an oxidative stress from the enhancement of gene transcription

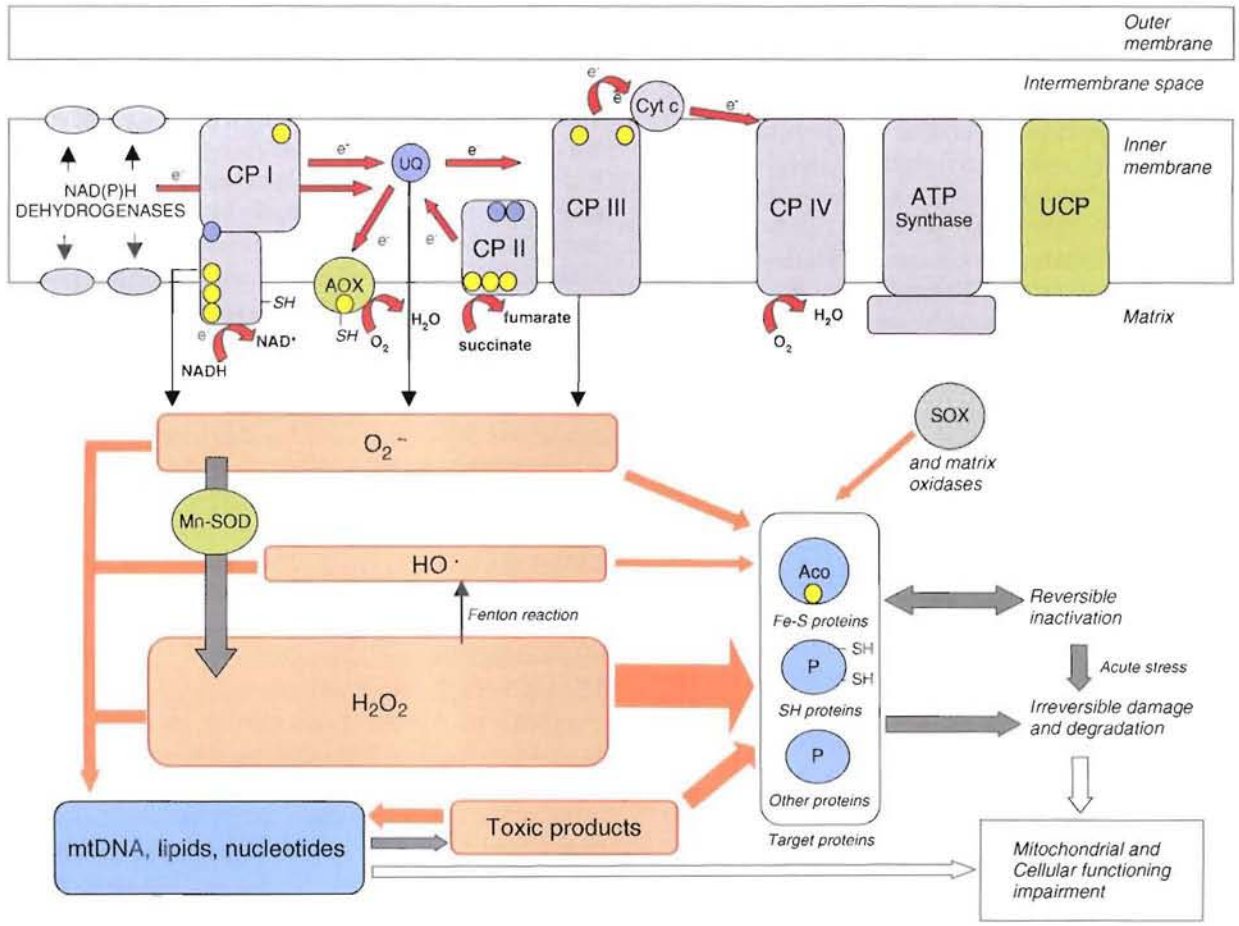

Fig. 1. Origins and effects of reactive oxygen species (ROS) produced in mitochondria I, II, III, IV respiratory complexes. Electron transfer along the respiratory chain are indicated with red arrows. Fe–S centers are yellow circles. Ubiquinol cofactors are figured in purple circles. Thin black arrows indicate the production of ROS, orange arrows oxidative damage, gray arrows molecular conversions. Proteins in green color are involved in protection of mitochondria against oxidative stress. Molecules in blue colors are sensitive targets for oxidative damage. S symbols indicates thiol-dependent regulation. Aco, aconitase; SOX, sulfhydryl oxidase; mtDNA, mitochondrial DNA; ATP, adenosine triphosphate; UQ, ubiquinone; SH, thiol group.

up to PCD (Dutilleul et al. 2003, Maxwell et al. 2002).

Analysis of the mitochondrial proteome

The biological systems present in mitochondria share homologies with the ones found in the cytosol or the chloroplast, but there are also specificities linked to the unique properties of plant mitochondria. Large-scale proteomic studies have been performed to follow the evolution of these complex systems, especially under stress conditions (Clifton et al. 2005, Lister et al. 2004). Many mitochondrial proteins are putative targets of different peroxides and some of them are found in an oxidized form (Kristensen et al. 2004, Møller and Kristensen 2006). Oxidation can simply affect the enzyme activity in a reversible way and participate in signaling pathways, when occurring on cysteines or methionines. In the case of other amino acids, the chain breakdown probability and the protease attack susceptibility are increased via the presence of carbonyl groups (Kristensen et al. 2004). This can affect the mitochondrial metabolism in a significant manner. After treatments leading to oxidative stress, protein breakdown products can also be found in mitochondrial extracts. The abundance of numerous mitochondrial proteins is also altered after such treatments. Among the oxidation targets, tricarboxylic acid (TCA)-cycle enzymes and Fe–S cluster containing enzymes are likely to be affected first in oxidative stress situations (Heazlewood et al. 2003, Millar et al. 2001b, 2004, 2005, Møller and Christensen 2004, Sweetlove et al. 2002).

Mitochondrial energy-dissipating systems

AOXs, as well as plant uncoupling mitochondrial proteins (UCP) are two mitochondrial energy-dissipating systems, encoded by multigenic families in plants, which are differentially expressed and regulated (Nogueira et al. 2005). These systems allow a fine tuning of the mitochondrial membrane potential, and can thus decrease ROS production caused by ETC overreduction (Borecky et al. 2006, Camacho et al. 2004). As mammalian UCPs, plant UCPs are involved in thermogenesis but they are rather required to generate a decrease of the membrane potential because they are upregulated in the presence of ROS (Considine et al. 2003, Hourton-Cabassa et al. 2004, Kowaltowski et al. 1998). Their regulation could also influence the TCA cycle (Smith et al. 2004). Considering the growing number of studies and the diversity of AOXs and UCPs, differential roles in plant mitochondria could emerge in the future. Specificities of plant AOX will be discussed in a subsequent paragraph.

Nevertheless, considering the protection of the organelle from ROS, these two proactive systems can play a significant protective role in mitochondrion-generated oxidative stress, but they cannot prevent damage because of ROS diffusion from the cytosol.

Ascorbate and lipoic acid-dependent systems

Ascorbate (or vitamin C) is a small antioxidant molecule that has been extensively studied for decades. Its antioxidant capacities are reinforced by its very high concentration in plant cells, usually between 10 and 100 m*M* (Noctor and Foyer 1998) depending on the subcellular compartments considered, and even higher, around 300 m*M*, under some conditions (Streb et al. 2003). As a consequence, it plays a preponderant role in the cell redox poise and metabolic regulations. Although it is more abundant in the chloroplast, it is present in the rest of the cell, and particularly in mitochondria. The ascorbate concentrations in mitochondria are similar to those found in other cell compartments and it is present essentially in the reduced form (Jimenez et al. 1997). The mechanism of peroxide detoxication by ascorbate has been deciphered, and the enzymes needed for its regeneration have been identified. A couple of years ago, it was shown that mitochondria possess a complete ascorbate–glutathione (GSH) pathway, making this organelle able to regulate its internal H_2O_2 concentration (Fig. 2). Mitochondria contain GSH, ascorbate, as well as GSH reductase (GR) and dehydroascorbate reductase (DHAR) in the matrix, and monodehydroascorbate reductase and ascorbate peroxidase (APX) bound to the membranes (Jimenez et al. 1997, Teixeira et al. 2006). This pathway is found in both chloroplasts and mitochondria, as Chew et al. (2003) showed that in *Arabidopsis thaliana*, the ascorbate–GSH cycle enzymes are dually targeted. As the presence of a specific transport of the reduced form (ascorbate) into mitochondria has not been proven, ascorbate is likely to enter mitochondria in its fully oxidized form (DHA), its transport being dependent on a glucose transporter (Szarka et al. 2004). Interestingly, the enzyme catalyzing the final step of ascorbate synthesis (galacto-lactone dehydrogenase) is located in the inner membrane of mitochondria (Bartoli et al. 2000). It uses cytochrome *c* as an obligate substrate

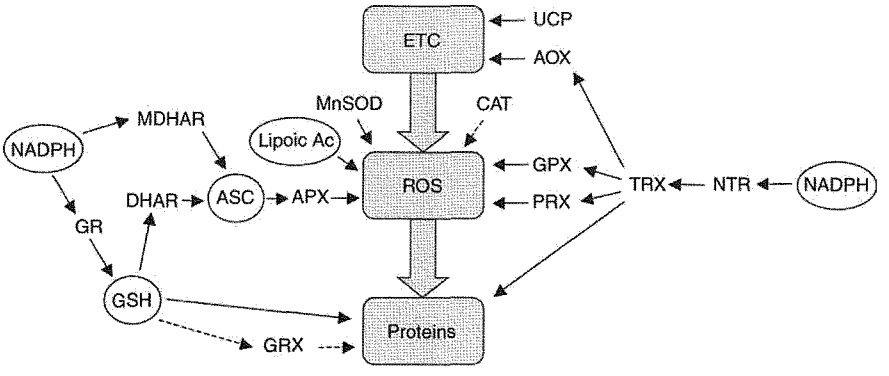

Fig. 2. Representation of the different mitochondrial antioxidant pathways and their level of action. Different systems can be distinguished: electron transfer chain proactive enzymes (alternative oxidase and uncoupling proteins), nicotinamide adenine dinucleotide phosphate-independent reactive oxygen species (ROS) molecules [manganese-containing superoxide dismutase, molecules like lipoic acid, and possibly catalase (CAT)], and NADPH-dependent pathways. Among these, on the left part of the figure are the glutathione- and ascorbate- (ASC) dependent systems, and on the right side the thioredoxin-dependent systems. The NADPH-dependent pathways act either by reducing ROS directly or by protecting or regenerating oxidized proteins. Metabolites are circled. In dotted lines are figured the pathways whose relevance is still under debate or unproven. MDHAR, monodehydroascorbate reductase.

and is associated with the ETC, which could regulate its activity via interaction with Complex I (Millar et al. 2003). Thus, ascorbate synthesis is associated with mitochondria. Ascorbate could be synthesized in the intermembrane space, and oxidized before being transported into the mitochondrial matrix, where it enters the ascorbate–GSH cycle to be reduced. Then, ascorbate is used by APX to reduce H_2O_2 to H_2O, releasing a molecule of DHA. The latter is reduced to ascorbate via the ascorbate–GSH cycle, also called the Halliwell–Asada cycle. The regeneration of ascorbate takes place at the expense of two molecules of GSH, oxidized into oxidized GSH (GSSG) (see Fig. 2).

Lipoic acid is a sulfur-containing molecule, which acts as a coenzyme for some mitochondrial enzymes involved in pyruvate and glycine metabolism (e.g. pyruvate dehydrogenase complex, glycine decarboxylase complex). It has been studied extensively and is known to chelate transition metals, and to reduce $HO^{\cdot}$ and $O_2^{\cdot -}$ (Packer et al. 1995). In its reduced form, lipoic acid was shown to play a role in the cell protection against lipid peroxidation in animal mitochondria (Lapenna et al. 2003). On the other hand, because of this reactivity, some lipoic acid-dependent enzymes, like glycine decarboxylase, are strongly inhibited by lipid peroxidation products because these peroxides can easily form an adduct with lipoic acid (Taylor et al. 2002).

Enzymatic ROS-scavenging pathways

SOD and catalase

The mitochondrial matrix contains an MnSOD, which catalyzes the reduction of $O_2^{\cdot -}$ to H_2O_2. This enzyme can provide protection to the soluble matrix enzymes, especially the ones with metallic cofactors, against oxidation by the superoxide anions, but the mechanism of subsequent H_2O_2 scavenging is still unclear (Rubio et al. 2001, 2002, 2004). Catalase activity was proposed to be present in plant mitochondria at a much lower level compared with animal mitochondria (Peixoto et al. 2004). Still, the presence and role of this enzyme in mitochondria is a matter of controversy, and some reports contradict this proposal. Other redox-dependent systems described below have been found to regulate H_2O_2 concentration in this compartment.

GSH-, glutaredoxin- and TRX-dependent mitochondrial systems

GSH is present in the mitochondrial matrix at concentrations up to the millimolar range. This organelle is thought to be devoid of GSH synthesis pathway, though Moran et al. (2000) found a GSH synthetase in nodule mitochondria. Different transporters assume the function of maintaining a constant pool of GSH in the compartment by uptake from the cytosol (Chen and Lash 1998). A specific GSH-dependent mechanism for the protection of proteins from oxidative damage is known as glutathionylation. The reversible adduction of a molecule of GSH protects the cysteines from being overoxidized to sulfenic, sulfinic or sulfonic acid forms, the latter being an irreversible modification. In general, glutathiolated proteins are inactive, and this mechanism could also be a regulation pathway in case of oxidative stress. This glutathionylation/deglutathionylation is mainly catalyzed by enzymes such as glutaredoxin (GRX) in mammalian mitochondria (Beer et al. 2004). At this point, GRXs have not been formally identified in plant mitochondria, but several sequences bear a putative mitochondrial transit peptide and it is thus likely that these proteins are present in this organelle (Rouhier et al. 2004).

It is now established that plant mitochondria possess their own complete TRX system, comprising at least one o-type TRX, and two forms of NADPH-dependent TRX reductase, NTRA and NTRB (Laloi et al. 2001, Reichheld et al. 2005). In poplar, one o-type and one h-type (TRX h2) are probably present in mitochondria (Gelhaye et al. 2004). The two types of mitochondrial NTR were shown to be able to reduce in vitro both *A. thaliana* TRX o1 and poplar TRX h2. These two TRX can interact with a mitochondrial target, namely AOX (Gelhaye et al. 2004).

GRX- and TRX-dependent targets in mitochondria

The involvement of TRX in apoptosis in animal cells has generated considerable interest for these systems in plant mitochondria. Following the discovery of the GRX and TRX systems in plants, one question arising was the search and analysis of their respective targets, and their role at the organellar or cellular level. It was shown not only that the mitochondrial TRX system is similar to what is found in the other compartments of the cell, but also that it regulates/reduces a specific set of mitochondrial enzymes. This was achieved thanks to emerging techniques as the cysteine-trap method, using single cysteine TRX or GRX mutants or by using two-dimensional electrophoresis associated to monobromobimane, a thiol-specific fluorescent probe. The isolated peptides are then analyzed by mass spectrometry, allowing the identification of mitochondrial TRX and GRX targets (Balmer et al. 2004, Rouhier et al. 2005). TRXs interact with, and most likely regulate numerous mitochondrial enzymes from different pathways. Among the different targets, it is

possible to discriminate between enzymes, which require TRX for their catalysis and regeneration, mostly oxidoreductases, and enzymes regulated via TRX (Fig. 2).

In the first category, TRX-dependent enzymes are peroxiredoxins (PRXs), ribonucleotide reductase, methionine sulfoxide reductase (MSR) and plant glutathione peroxidases (GPXs). MSR are present in human mitochondria, but until now no such protein homolog was found in plants, even at the genomic level. PRX are TRX-dependent peroxidases, which are present in nearly all plant cell compartments. In plants, they are commonly divided into four types, namely type II PRX, 2-Cys PRX, 1-Cys PRX and PRX Q. Type II PRXs constitute the largest subfamily. Depending on the organism and cellular compartment considered, PRX II can be reduced either by GSH alone or by the GRX or the TRX system (Brehelin et al. 2003, Finkemeier et al. 2005, Rouhier et al. 2001). In *A. thaliana*, for example, PRX II B, C, D are cytosolic, PRX II E is chloroplastic and PRX II F is the only mitochondrial isoform (Gama et al. this issue, Horling et al. 2002). In mammalian tissues, a 2-Cys PRX is present in mitochondria (PRX III in human), but this type has not been reported in plant mitochondria (Miranda-Vizuete et al. 2000). Apart from the four types of PRX, a fifth type of TRX-dependent peroxidase has been characterized more recently. It belongs to the so-called GPX proteins, which in plants are likely to function with TRX rather than GSH for their activity (Herbette et al. 2002, Jung et al. 2002, Tanaka et al. 2005). From sequence analysis, it was predicted that plant mitochondria contain at least one isoform of this enzyme (Rodriguez Milla et al. 2003), and recently, with a green fluorescence protein fusion approach, it was observed that a poplar GPX is present in mitochondria (Navrot et al., unpublished data). For this protein, a mitochondrial physiological electron donor is still missing, as it has been shown to be reduced in vitro by cytosolic or chloroplastic TRX, but not by either mitochondrial h2 or o-type TRXs (Gelhaye et al. 2004). Thus, the PRX family and their reducing partners seem to form a tight network, quite complex in mitochondria as in other cell compartments parts, and able to control ROS concentration in different conditions. They are also likely to play a role in redox sensing and signal transduction (for review, see Rouhier and Jacquot 2005).

Thanks to the above-mentioned proteomic techniques, we now have a catalog of proteins that are potentially regulated by TRX in mitochondria. These targets belong to a number of metabolic pathways, including the citric acid cycle, photorespiration, fatty acid metabolism, hormone involving processes, sulfur or nitrogen metabolism, transport regulation and protein assembly (Balmer et al. 2004). One of the interesting targets is an anion channel transporter, linked to permeability transition pore formation in PCD. The most studied of the targets is undoubtedly AOX. Belonging to the ETC, AOX is an iron-containing protein, which catalyzes the reduction of O_2 to H_2O, via oxidation of ubiquinol, and without formation of adenosine triphosphate (also known as cyanide-resistant respiration). It helps to prevent over-reduction of the ubiquinol pool, thus decreasing the production of $O_2^{\cdot -}$ and oxidative stress in mitochondria in non-optimal conditions (Camacho et al. 2004). This plant-specific crucial protein is also involved in mitochondrial-dependent PCD (Robson and Vanlerberghe 2002). It exists as a dimeric complex, anchored to the inner membrane of mitochondria. Each subunit possesses a matrix loop, which bears conserved cysteines. Two cysteines of the complex can be oxidized in a disulfide bridge, leading to dimerization and consequent inactivation of the enzyme (Umbach and Siedow 1993). NADPH-dependent isocitrate dehydrogenase was found to be able to reduce and activate the enzyme, linking activation to both the TCA cycle and the matrix redox state (Gray et al. 2004). Mitochondrial TRX was also found to be able to reduce the disulfide and activate the enzyme (Gelhaye et al. 2004). Once reduced, alpha-keto acids regulate the enzyme activity, acting on the dimerization-involved cysteine or on a second conserved one (Rhoads et al. 1998, Umbach et al. 2006). This strongly indicates that there is a cross talk between redox regulation in the matrix via TRX, the redox state of the ETC and the membrane potential.

In different organisms, but not in plants, mitochondrial GRX has been shown to be involved in apoptosis, or in iron–sulfur assembly in proteins. In plant cells, no GRX has yet been found in this compartment, but it would not be a surprise to find members of this family with identical roles. Some plant GRX isoforms are predicted to carry a mitochondrial signal peptide (Rouhier et al. 2004), and by a proteomic approach, a protein able to bind cations and belonging to the large GRX family, was found in *A. thaliana* mitochondria (Herald et al. 2003). In addition, using the cysteine-trap method and a cytosolic poplar GRX, Rouhier et al. (2005) identified numerous GRX mitochondrial targets, and some of them, as a GR and a glycyl transfer RNA synthetase, could be specific targets of GRX as they do not belong to the mitochondrial TRX targets previously identified (Balmer et al. 2004). Probably sequence variations have kept these proteins out of our sight until now. More generally, GRXs participate in the glutathionylation and deglutathionylation of proteins, protecting the redox-sensitive cysteines by GSH adduction (Starke et al. 2003). They can also help maintain the redox poise of this compartment by reduction of low-molecular weight redox molecules, like GSSG, and dehydroascorbate (Washburn and Wells 1999).

Heme and iron–sulfur assembly into mitochondrial proteins

The assembly of iron–sulfur clusters is essential for the generation of functional ETCs. It is also required for enzymes like aconitase to be active. Human mitochondrial GRX2 has been shown to contain an iron–sulfur cluster, which disappears upon oxidation, thus releasing the active enzyme (Lillig et al. 2005). This could be a possibility of regulation for those enzymes, for example, in situation of oxidative stress where the cluster is more likely to be cleaved off. Recently, Fe–S-containing GRX have also been discovered in plants (Feng et al. 2005, 2006). As it is now established that (Balk and Lobreaux 2005), as human or yeast, plant mitochondria contain their own Fe–S cluster assembly machinery, an emerging topic in plant research is the possible involvement of thiol-dependent systems as GRX and TRX in protein assembly and maturation. To date, a GRX from yeast has been shown to participate in this Fe–S assembly machinery but its role is still not completely deciphered (Rodriguez-Manzaneque et al. 2002). Studies are in progress to identify the target proteins of GRX in this iron–sulfur center assembly. One of these targets could be a cysteine desulfurase (Nfs1), which is proposed to be regulated by Grx5 in yeast (Alves et al. 2004). GRX may also constitute a sink for matrix iron, participating in the homeostasis of this element, as yeast grx mutants accumulate iron in the matrix. TRX could be linked to these processes as well, as it can interact with AtErv1p, a sulfhydryl oxidase supposedly part of the Fe–S assembly machinery in *A. thaliana* mitochondria (Levitan et al. 2004). Considering protein maturation, it has also been shown that TRX is able to reduce a protein involved in heme assembly and maturation of cytochrome *c*, namely CCMH (Meyer et al. 2005).

Concluding remarks

Step by step, our understanding of the physiology of mitochondria is progressing, thanks to technical improvements and emergence of new experimental designs. This starts with improved methods for mitochondrial purification, from different plants, as for the model plant *A. thaliana* (Escobar et al. 2006, Keech et al. 2005). New methods have also emerged in proteomics for medium or large-scale studies (Kristensen et al. 2004, Kruft et al. 2001, Millar et al. 2005). It appears now that mitochondrial homologs of most of the cytosolic antioxidant pathways exist (Fig. 2). Thus, ROS concentrations can be regulated as tightly as in other compartments.

Among these, TRX-dependent systems have been an intense research field in recent years, and now the complete system, NTR, TRX, PRX/GPX, is known to be present in mitochondria. The characterization of this system is still in progress, and features like its relation to ETC or its involvement in PCD look particularly promising. Mitochondrial GRX or GRX-like proteins are also under scrutiny, and it is likely that they will soon be included in our current representations of mitochondrial antioxidant systems.

Undoubtedly, the putative involvement of GRX in the assembly of iron–sulfur centers into plant mitochondrial proteins promises to be an exciting field of research in the near future. Another area, which looks extremely promising, will be to test the effect of TRXs or GRXs in the redox regulation of ion channels. The recent review of Scholz-Starke et al. (2005) presents several lines of evidence suggesting that ROS and the reducing systems have indeed the capacity to control the activity of transporters in plants. Whether the mitochondrial ion channels are also subject to this regulation is yet unknown. The analysis of conserved cysteines in the amino acid sequences and the biochemical properties of these transporters will help answer these questions. Another interesting approach will be to generate knockdown mutants of the documented mitochondrial TRXs and GRXs and evaluate the impact of these mutations on the redox state of mitochondria.

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I.C Au niveau du peroxysome :

Un autre compartiment cellulaire, le peroxysome, est aussi source de ROS. Ils vont être produits par le métabolisme oxydatif au sein de cet organite, au niveau de la xanthine oxydase, ou encore lors de la beta-oxydation des acides gras ou de la photorespiration. Enfin, au niveau de la membrane du peroxysome, des enzymes du type monodéhydroascorbate réductase (MDHAR) ou encore un cytochrome de type b, sont susceptibles de générer des anions superoxydes (del Rio et al 2006).

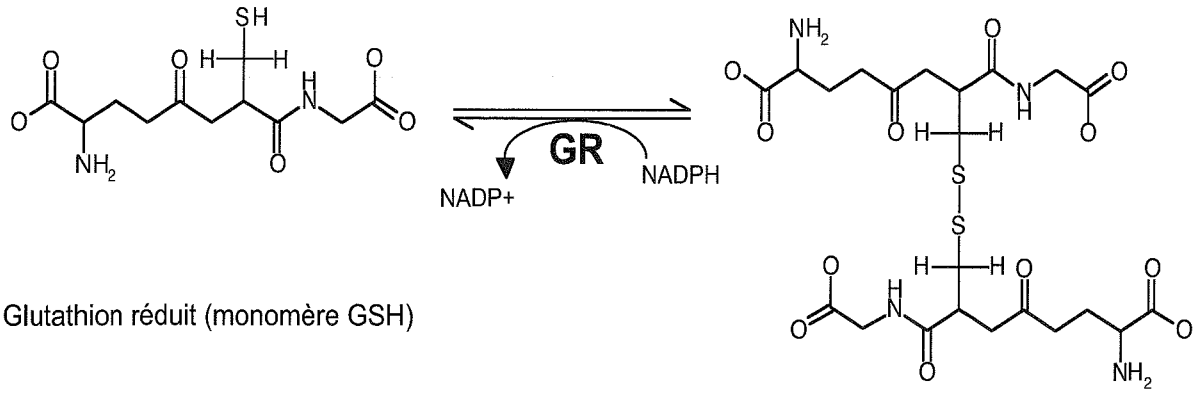
II Production des RNS - L'oxyde nitrique et ses dérivés

L'oxyde nitrique (NO) et ses rôles biologiques ont été découverts il y a une vingtaine d'années. Depuis, l'intérêt porté à cette molécule a été croissant, et a révélé de multiples rôles dans le fonctionnement des cellules et des organismes, par exemple, à travers la nitrosylation des protéines. Comme l'oxygène, ce gaz peut aussi réagir avec de nombreuses molécules pour former une variété de composés chimiques plus ou moins complexes appelées couramment RNS (reactive nitrogen species). En particulier, la réaction entre l'anion superoxyde O_2^- et le NO va aboutir à la formation de peroxynitrite. Le NO est présent chez tous les organismes vivants, et semble induire une voie de signalisation universelle. Chez les plantes, deux enzymes sont capables de synthétiser l'oxyde nitrique : une nitric oxide synthase mitochondriale, impliquée dans la réponse au stress oxydant a été identifiée chez *Arabidopsis thaliana* (Guo et al 2003, Guo et Crawford 2005). La nitrate réductase

semble aussi posséder une activité annexe lui permettant de produire du NO (Morot-Gaudry-Talarmain et al 2002), en particulier en réponse à l'acide abcissique dans les cellules de garde des stomates (Desikan et al 2002).

III Systèmes de réduction des ROS

Les organismes supérieurs ont développé des systèmes pour réguler la concentration intracellulaire en ROS, afin de protéger le matériel cellulaire mais aussi pour assurer un ajustement fin de la signalisation cellulaire par les ROS. Cela a pu se faire en limitant la production et la concentration des formes réactives de l'oxygène (ROS) hors des chaînes de transport d'électrons, et aussi en restaurant les molécules ayant réagi avec des ROS. Certaines molécules simples vont avoir une action directe de détoxication, tandis qu'on trouvera aussi des systèmes enzymatiques complexes que l'on peut usuellement classer selon leur utilisation de voies thiol-dépendantes ou non pour leur fonctionnement. Ces systèmes enzymatiques sont décrits en détail pour notre modèle d'étude le peuplier quand les données correspondantes sont disponibles. D'autres systèmes prennent en charge la réparation des molécules endommagées lors d'un stress oxydant. Il s'agit d'enzymes telles que les méthionine sulfoxyde réductases, qui vont réduire les méthionines oxydées au sein des protéines afin d'en restaurer l'activité (Boschi-Muller et al 2005). La restauration de bases de l'ADN hydroperoxydées est réalisée par des enzymes telles que les Phospholipid Hydroperoxide Glutathion peroxydases (PHGpx) ou les Glutathion S-transférases (GST) chez les mammifères



Glutathion réduit (monomère GSH)

Glutathion oxydé (dimère GSSG)

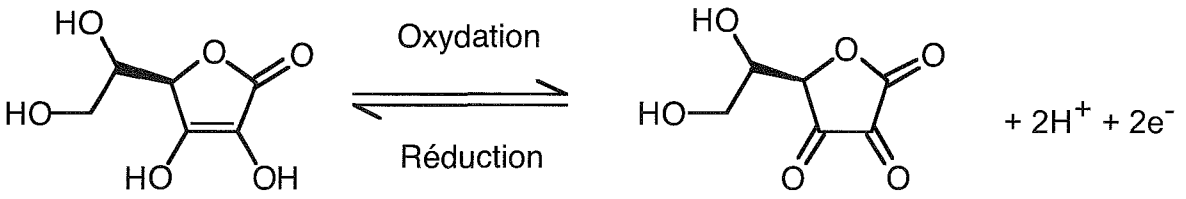
Figure 2. Formes réduites et oxydées du glutathion. Le glutathion oxydé GSSG va être réduit par la glutathion réductase (GR), aux dépens d'une molécule de NADPH.

(Bao et al 1997) ou encore des phospholipides membranaires. Ces systèmes de réparation ne sont pas détaillés ici.

III.A Les systèmes non-enzymatiques

1- Le glutathion :

Le glutathion est un tripeptide (gamma-Glutamyl-Cystéine-Glycine) omniprésent dans les cellules (Figure 2). Il est la plus importante forme de thiols cellulaires, hormis les protéines. Sa concentration à l'intérieur des cellules peut varier de 0,2 jusqu'à 10 mM. Il est considéré comme un véritable « tampon redox » qui va influencer l'état d'oxydation de nombreuses autres molécules. Il peut réduire directement certains composés, et occupe une place prépondérante dans le cycle de l'ascorbate-glutathion (décrit ultérieurement). Il sert aussi d'élément de base à la synthèse des phytochélatines, qui sont des polymères de GSH impliqués notamment dans la séquestration des métaux lourds dans les celules végétales (Zenk 1996). Enfin, au-delà de ces rôles dans les défenses antioxydantes, le GSH est aussi impliqué dans la régulation du métabolisme et notamment du développement cellulaire (Ogawa 2005, Cairns et al 2006). Le glutathion est présent sous deux formes, l'une réduite et monomérique (GSH), qui est la forme prépondérante dans les cellules en conditions optimales; l'autre, forme oxydée, est le dimère GSSG dans lequel deux molécules sont liées par un pont disulfure. La réduction du GSSG est effectuée par une flavoprotéine spécifique, la glutathion réductase NADPH-dépendante (GR) avec utilisation d'une molécule de NADPH pour obtenir deux molécules de GSH. Chez le peuplier, 3 gènes codant pour des



ASCORBATE DEHYDROASCORBATE

Figure 3. Molécules d'ascorbate et sa forme oxydée, déhydroascorbate, après réaction avec une molécule de peroxyde.

GR ont été identifiés, et ces séquences pourraient coder pour des isoformes destinées à différents compartiments cellulaires (Rouhier et al 2006a).

Sous sa forme réduite, le GSH est utilisé comme réducteur par diverses protéines, que sont les glutarédoxines (Grx), les glutathion-transférases (GST), dans certains cas les glutathion peroxydases (Gpx), ainsi que certaines peroxyrédoxines (Prx).

Enfin, le GSH peut être fixé à certaines protéines par glutathionylation. Ce phénomène consiste en l'adduction réversible d'une molécule de GSH sur une ou plusieurs cystéines d'une protéine cible, dans un but de protection contre l'oxydation ou encore de régulation (Cabiscol et Levine 1996). Cette glutathionylation réversible s'effectue par la réaction d'une molécule de GSSG, ou peut être catalysée par des enzymes, telles que les Grx (Lind et al 1998). Jusque récemment, peu de protéines végétales glutathionylées étaient connues. C'est le cas d'une Trx f chloroplastique chez *Arabidopsis thaliana* (Michelet et al 2005), ou d'une Trx h2 mitochondriale de peuplier (ce travail, article IV, Gelhaye et al). Les méthodes dérivées de la protéomique, utilisant du GSH/GSSG marqué, permettent désormais d'identifier d'autres protéines potentiellement régulées par cette voie, notamment des enzymes du métabolisme carboné (Ito et al 2003, Dixon et al 2005).

2- L'ascorbate :

L'ascorbate (ou acide ascorbique) est une molécule de faible masse moléculaire de la famille des lactones (Figure 3), qui est présente dans tous les compartiments de la cellule végétale, en concentration particulièrement importante dans le chloroplaste. On le trouve aussi cependant dans le cytoplasme ou la

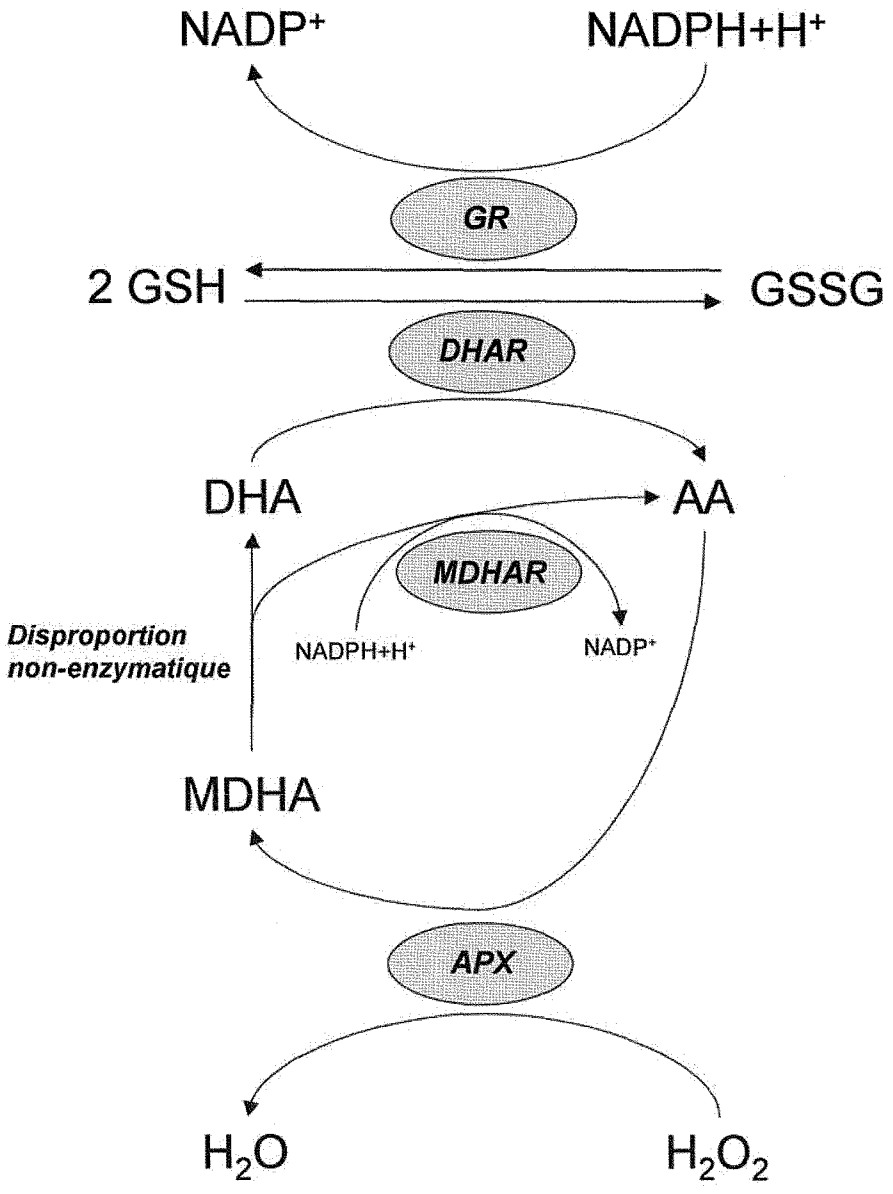


Figure 4. Cycle de l'ascorbate et du glutathion, d'après Noctor (1998). AA : acide ascorbique ; APX : ascorbate peroxydase ; DHA : déhydroascorbate ; DHAR : déhydroascorbate réductase ; GR : glutathion réductase ; GSH/GSSG : glutathion réduit/oxydé ; MDHA : monodéhydroascorbate ; MDHAR : monodéhydroascorbate réductase.

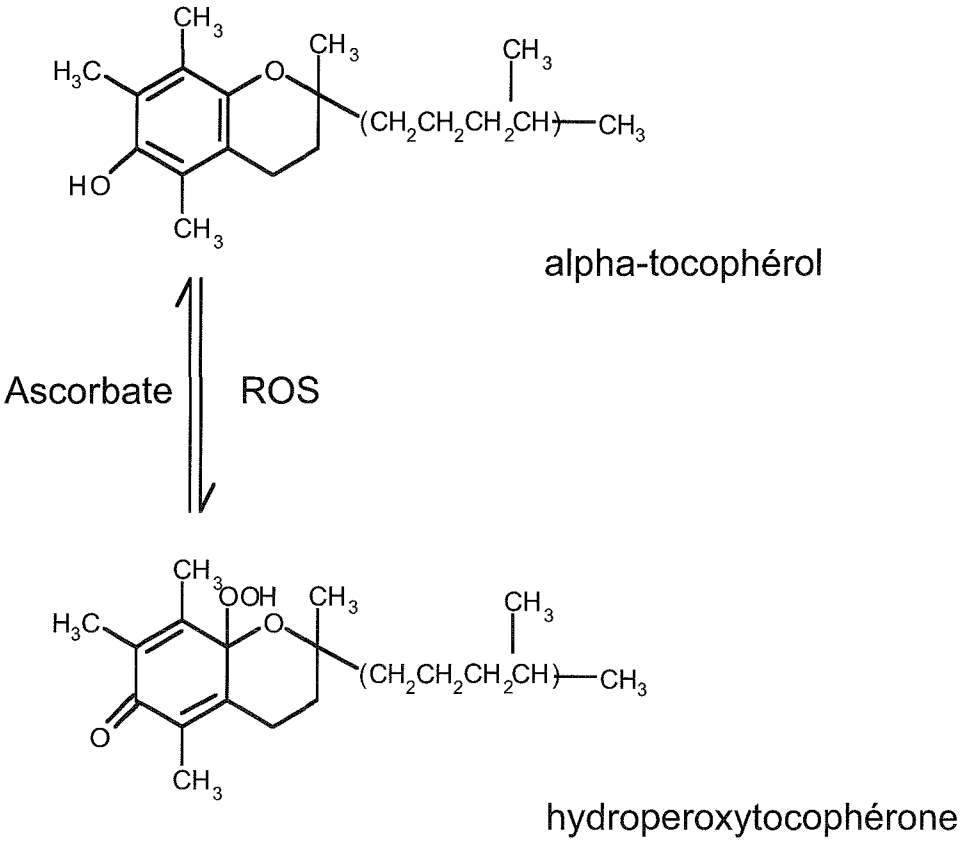


Figure 5 : Formules semi-développées de l'alpha-tocophérol sous sa forme réduite et hydroperoxyde.

mitochondrie. Il joue un rôle de substrat dans l'activité de l'ascorbate peroxydase (Apx), qui est l'enzyme chloroplastique prépondérante de détoxication du peroxyde d'hydrogène (H_2O_2). L'ascorbate se trouve alors oxydé sous forme de monodéhydroascorbate (MDHA) ou déhydroascorbate (DHA), qui est réduit en une molécule d'ascorbate grâce à une enzyme, la déhydroascorbate réductase (DHAR), qui tire son pouvoir réducteur du glutathion (GSH). Le MDHA, quand à lui, subit une disproportion non-enzymatique qui aboutit à la formation de DHA. Il peut aussi être pris en charge par une MDHA réductase, qui va le réduire directement en ascorbate, au prix de l'oxydation d'une molécule de NADPH. Ce cycle est appelé cycle de l'ascorbate et du glutathion, ou encore cycle d'Halliwell-Asada-Foyer (Figure 4). Il est notable que ce cycle constitue un point d'échange de pouvoir réducteur entre différents types de réducteurs, l'ascorbate qui est thiol-indépendant et le GSH qui appartient aux systèmes thiols-dépendants.

3- L'alpha-tocophérol :

L'alpha-tocophérol est la forme principale de la vitamine E. C'est un composé liposoluble formé d'un noyau polaire et d'une chaîne hydrophobe (Figure 5). Il se trouve exclusivement dans les membranes photosynthétiques chez les cyanobactéries et dans les chloroplastes des végétaux les plus évolués. Il a un rôle d'élimination des ROS, et en particulier de l'oxygène singulet $^1O_2$ et du radical peroxyl OH . Il peut réagir par un phénomène de résonance, qui va permettre l'élimination de nombreuses molécules, mais avec une transformation simultanée et irréversible du tocophérol en forme quinone ou époxyde. Une autre voie de détoxication implique la réaction chimique du tocophérol avec des radicaux peroxyl

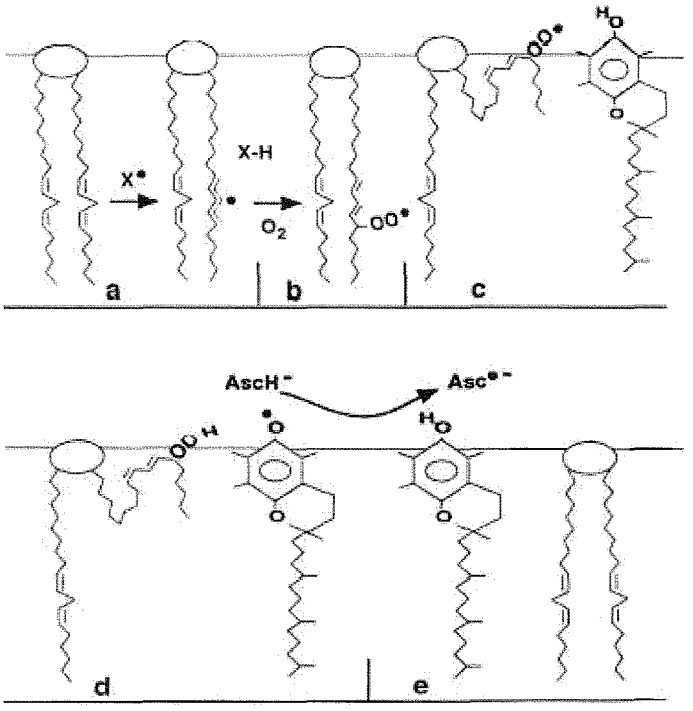
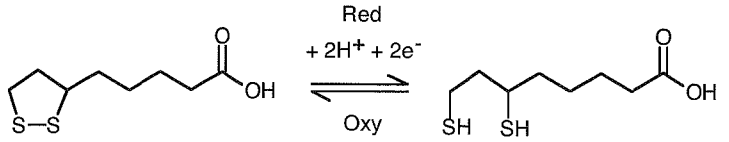


Figure 6. Schéma simplifié de l'action des tocophérols au niveau des membranes. a : un radical X réagit avec un phospholipide membranaire, formant un radical alkyl au niveau de la chaîne hydrophobe du phospholipide. b : la réaction avec l'oxygène va donner un radical peroxyl. c : déplacement de la chaîne hydrophobe vers la phase aqueuse. d : l'alkyl réagit avec le tocophérol pour former une molécule de péroxyde et un radical tocophéroxyl. e : réduction du peroxyde par les systèmes antioxydants cellulaires, réduction du tocophéroxyl par une molécule d'ascorbate (adapté du site http://ethesis.helsinki.fi/julkaisut/mat/bioti/vk/blokhina/ch1.html).



Acide lipoïque (LA) Acide déhydrolipoïque (DHLA)

Figure 7. Formules semi-développées de l'acide lipoïque sous forme réduite et oxydée.

de lipides, qui va former un peroxyde et une molécule de tocophéroxyl, qui peut ensuite être réduite en tocophérol par une molécule d'ascorbate (Figure 6). Cette voie permet la régénération de l'alpha-tocophérol et la formation de peroxydes, qui vont être pris en charge et réduits par d'autres systèmes de détoxication (Munné-Bosch 2005). L'alpha-tocophérol va participer à la protection du photosystème II contre la photoinhibition et des membranes contre la photooxydation (Havaux et al 2005).

4- L'acide lipoïque :

L'acide lipoïque (LA) est une molécule qui est utilisée comme coenzyme par de nombreuses enzymes, comme le complexe pyruvate déshydrogénase ou le complexe glycine décarboxylase. Il est présent sous forme oxydée (acide lipoïque), ou sous forme réduite (acide dihydrolipoïque (DHLA)) dans les cellules (Figure 7). Il possède des propriétés antioxydantes, ce qui a stimulé la recherche sur cette molécule, en particulier en médecine. L'acide lipoïque est notamment capable d'éliminer les molécules $^1O_2$ et OH . Il est aussi capable de chélater certains ions métalliques et donc de prévenir leur action pro-oxydante. Cependant, certaines études ont aussi montré un rôle pro-oxydant de l'acide lipoïque, certaines enzymes étant rapidement inhibées en présence de peroxydes, à cause de la réactivité de leur cofacteur acide lipoïque qui va fixer des peroxydes de lipides en particulier (Packer et al 1995).

5- Les caroténoïdes :

Les caroténoïdes, molécules en C40 (tétraterpènes), regroupent de nombreux pigments dont les carotènes et les xanthophylles, dérivant d'une molécule commune, le lycopène. Ces pigments vont jouer un rôle mineur dans l'absorption de la lumière au niveau des chloroplastes. En revanche, ils vont dissiper l'énergie excessive absorbée par les molécules de chlorophylle par des réactions de conversion moléculaire (époxydation/ dé-époxydation), dans les situations d'éclairement excessif. Ils vont ainsi permettre de réduire le nombre de molécules d'oxygène singulet produites au niveau des membranes des thylacoïdes, protégeant de ce fait la chaîne de transport d'électrons photosynthétique.

III.B Les systèmes enzymatiques

1 Thiols-indépendants :

a- Superoxyde dismutases (SOD)

Les superoxyde dismutases sont des enzymes qui vont catalyser la réaction de dismutation de l'anion superoxyde en peroxyde d'hydrogène. Elles sont présentes dans les différents compartiments cellulaires, et permettent d'éviter l'accumulation d'anion superoxyde, qui diffuse difficilement à travers les membranes lipidiques. Les cellules végétales contiennent trois types de superoxyde dismutases, contenant différents métaux dans leur centre actif. Les Fe-SOD se trouvent dans les chloroplastes, les Cu/Zn-SOD sont cytosoliques et chloroplastiques, tandis que les Mn-SOD sont limitées à la mitochondrie (Fink et Scandalios 2001).

b- Catalases (CAT)

Les catalases sont des enzymes à hème, actives sous forme tétramérique, localisées principalement dans les peroxysomes. Elles catalysent la réduction d'H_2O_2 en H_2O, avec une très grande efficacité, et elles sont d'autant plus efficaces qu'elles ne vont pas utiliser de pouvoir réducteur cellulaire pour leur fonctionnement, et ne vont donc pas perturber l'équilibre redox intracellulaire. Ainsi, elles constituent une protection efficace pour la cellule, en éliminant l'H_2O_2 produit en grande quantité dans le peroxysome. Ces enzymes forment une famille multigénique chez les plantes, répondant différentiellement aux conditions de stress, contrairement à ce qui est observé chez les mammifères, qui ne possèdent qu'une isoforme (Scandalios 2005).

c- Ascorbate peroxydases (Apx)

Les ascorbate peroxydases sont des peroxydases à hème, de masse moléculaire d'environ 30 kDa, actives sous forme monomérique ou dimérique suivant l'isoforme considérée. Elles catalysent la réduction d'H_2O_2 en O_2 en utilisant le pouvoir réducteur d'une molécule d'ascorbate. L'ascorbate est oxydé en monodéhydroascorbate, et est ensuite régénéré par le cycle Halliwell-Asada-Foyer (cf paragraphe III.A.2). Elles se trouvent sous forme soluble dans le cytosol et le stroma des chloroplastes, et sous forme liée aux membranes dans le peroxysome ou au niveau des thylacoïdes. Chez les végétaux supérieurs, les Apx sont codées par des familles multigéniques, avec 9 isoformes chez l'arabette et 8 chez le riz

(Texeira et al 2006). L'expression des différents gènes est de plus régulée spatialement dans la plante, et différentiellement suivant les conditions environnementales (Mittler et al 2004, Texeira et al 2006). Récemment, il a été montré que des interactions entre ces enzymes et les systèmes Trx sont possibles, au moins dans le cytosol (Article V: Gelhaye et al 2007). Ces travaux démontrent qu'il existe des liens entre les différents systèmes de réduction des ROS, Trx et Apx. La régulation des ces dernières pourrait donc aussi être liée à l'état redox de la cellule, via les systèmes thiol-dépendants.

2 Thiol-dépendants :

a- Les systèmes réducteurs

Les systèmes thiol-dépendants vont utiliser le pouvoir réducteur généré au niveau des chaînes de transports d'électrons ainsi que lors du métabolisme carboné sous la forme de NAD(P)H (nicotinamide dinucléotide (phosphate)). La concentration en NAD(P)H et le rapport NADPH/NADP ou NADH/NAD sont aussi utilisés comme des indicateurs de l'état d'oxydo-réduction de chacun des compartiments cellulaires. Les effecteurs principaux de ces systèmes sont les thiorédoxines (Trx) et les glutarédoxines (Grx). Les Grx vont utiliser le glutathion (GSH) comme source de pouvoir réducteur. La régénération du GSH a été évoquée dans un paragraphe précédent (IIIA.1). Les Trx vont quant à elles fonctionner grâce au pouvoir réducteur fourni par différentes enzymes appelées thiorédoxine réductases.

b- Les thiorédoxine réductases :

Le transfert de pouvoir réducteur vers les systèmes thiorédoxine-dépendants se fait par l'intermédiaire de protéines différentes suivant les compartiments cellulaires considérés.

Dans le chloroplaste, ce transfert débute au niveau de la chaîne de transport d'électrons, qui réduit la ferrédoxine (Fd) au cours de son fonctionnement normal. Celle-ci va alors transférer séquentiellement deux électrons à la ferrédoxine thiorédoxine réductase (FTR) sur laquelle un pont disulfure intramoléculaire va être réduit en dithiol. Ce dithiol va pouvoir à son tour réduire les Trx chloroplastiques. Il est aussi notable que la Fd va être une source de NADPH dans le chloroplaste, en transférant son pouvoir réducteur à la Fd NADP oxydoréductase (Schürmann et Jacquot 2000). Une voie alternative, présente dans le chloroplaste et très similaire aux systèmes cytosolique et mitochondrial a été identifiée récemment. Cette NTRC est constituée d'une thiorédoxine réductase NADPH dépendante fusionnée à une thiorédoxine (Perez-Ruiz et al 2006) (voir III.B.2.c).

Dans le cytoplasme et la matrice mitochondriale, une flavoprotéine, la NADPH-thiorédoxine réductase (NTR), va réduire les Trx en utilisant le pouvoir réducteur du NADPH. La présence d'un cofacteur FAD dans la NTR va permettre le transfert de deux électrons à la fois, ce qui constitue une différence importante par rapport au fonctionnement de la FTR (Dai et al 1996).

Enfin, pour certaines isoformes de Trx, les glutarédoxines (Grx), qui font l'objet d'un paragraphe ultérieur (IIIB.2d), peuvent être un donneur d'électrons spécifique (Gelhaye et al 2003).

c- Les thiorédoxines (Trx) :

Les thiorédoxines sont des protéines de faible masse moléculaire, 12 kDa environ, qui ont été découvertes chez les plantes il y a une trentaine d'années. Ces enzymes sont des oxydoréductases qui possèdent un site actif de séquence WC(G/P)PC, et une structure tridimensionnelle très conservée et commune à d'autres protéines. Cette structure, formée de quatre hélices alpha entourant 5 feuillets beta antiparallèles, est appelée « Trx fold » (Eklund et al 1984, Coudevylle et al 2004). On la retrouve dans des enzymes comme les Grx, les protéines disulfide isomérases (PDI) ou encore les glutathion peroxydases (Gpx) animales (Ladenstein et al 1979). Les Trx sont capables de réduire des ponts disulfures sur de très nombreuses protéines et elles sont de ce fait très impliquées dans la régulation du métabolisme cellulaire. Elles ont depuis été un sujet de recherche d'intérêt croissant, et aujourd'hui encore elles font l'objet d'études chez tous les organismes vivants, en particulier dans la recherche médicale. Chez les mammifères, elles participent à l'activation et l'inactivation de facteurs de transcription tels que NFκB ou AP-1, et sont impliquées dans de nombreux mécanismes dont ceux qui contrôlent l'apoptose (Arner et Holmgren 2000). Chez les végétaux, les Trx régulent de nombreuses enzymes impliquées dans la photosynthèse, comme par exemple la fructose 1,6 bis-phosphatase (FBPase) ou encore la malate déshydrogénase (MDH) (Issakidis et al 1996, Jacquot et al 1997). Elles sont aussi impliqués dans la régulation d'enzymes impliqués dans le développement de la plante, en particulier au cours de la germination, pour la mobilisation des réserves notamment (Marx et al 2003, Wong et al 2003).

Les Trx végétales ont été étudiées extensivement, et l'émergence de nouvelles techniques et le développement de nombreux projets de séquençage du génome ont encore amélioré la qualité du travail de caractérisation. Ces progrès ont permis un recensement exhaustif des gènes codant pour les Trx, notamment chez *Chlamydomonas reinhardtii* (Lemaire et Miginiac-Maslow 2004), *Arabidopsis thaliana* (Meyer et al 2005), puis chez le peuplier et d'autres végétaux supérieurs. Les nombreuses isoformes ont été classées en familles et sous-familles selon leur séquence et celle de leur site actif. Toutes ne sont pas encore caractérisées en détail, et la question de la redondance de ces multiples isoformes dans le fonctionnement cellulaire reste encore peu éclaircie. Ces protéines sont ubiquitaires et jouent un rôle prépondérant dans la régulation de nombreuses voies métaboliques, dans la photosynthèse ou la germination entre autres. Elles sont aussi nécessaires au fonctionnement de nombreuses enzymes. Le système complexe des Trx chez les plantes supérieures est détaillé dans l'article II (Gelhaye et al 2005).

Article II : The plant thioredoxin system

Eric Gelhaye, Nicolas Rouhier, Nicolas Navrot and Jean-Pierre Jacquot

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Cet article décrit et analyse la famille des thiorédoxines chez les végétaux supérieurs. Grâce à la disponibilité des séquences du génome *d'Arabidopsis thaliana*, une étude globale de cette famille a été possible. Les groupes et sous-groupes qui la composent sont présentés suivant les différents compartiments cellulaires: chloroplastes, mitochondrie et cytosol. Les membres des différents sous-groupes sont aussi analysés chez les autres végétaux supérieures en fonction des séquences disponibles. Cette étude met en évidence la complexité du système thiorédoxine chez les plantes supérieures, la présence d'isoformes atypiques, et les liens entre les thiorédoxines et leurs cibles, ainsi qu'avec les autres systèmes redox, en particulier le glutathion et les glutarédoxines.

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Review

The plant thioredoxin system

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Abstract. Thioredoxins are small proteins catalyzing thiol-disulfide interchange and are involved in the regulation of the redox environment of the cell. In plants, the thioredoxin system is particularly complex since at least 20 thioredoxin isoforms are found in the plant model *Arabidopsis thaliana*. Based upon primary sequence analysis and subcellular localization, thioredoxins can be classified into different groups and subgroups. Different pathways allowing thioredoxin reduction also coexist in the plant involving ferredoxin-thioredoxin reductase, thioredoxin reductases and the glutathione/glutaredoxin system. This review discusses the literature of plant thioredoxins with emphasis on recent findings in the field.

Key words. Disulfide bridge; redox regulation; thioredoxins; thiol reduction.

Introduction

Several fundamental processes mediating and regulating enzyme activities are performed through sensor proteins and transmitters of redox signals. Oxidation-reduction of cysteinyl residues is a key factor in these processes, allowing the activity modulation of several enzymes. Cysteinyl residues can undergo oxidation to form disulfides (S-SR), sulfenic acid (S-OH), sulfinic acid ($-SO_2H$) or sulfonic acid ($-SO_3H$). The development of proteomic as well as other approaches led to the identification, in all kingdoms, of numerous post-translationally redox-regulated proteins. Apart from low molecular weight compounds such as glutathione and ascorbic acid, cellular redox control is mediated mainly by two sets of related proteins: the thioredoxins and glutaredoxins. The plant glutaredoxin system has been recently reviewed [1, 2], and we will focus in this review on current knowledge about the thioredoxin system in plants. Since several aspects of this topic have been reviewed recently [3–9], this review will only deal with most recent advances in the field.

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Sequence comparisons and thioredoxin diversity in plants

Thioredoxins are small proteins (around 12 kDa) that are present in all organisms. In addition to these 'classical' thioredoxins, numerous proteins also exhibit thioredoxin-like domains or multiple thioredoxin domains. These proteins differ from the classical thioredoxins and are not reviewed in this article. In plants, the thioredoxin system is particularly developed in comparison with other organisms. Indeed, in *Arabidopsis thaliana* at least 20 thioredoxin genes have been reported in the whole sequenced genome [10]. An alignment of the amino acid sequences of different thioredoxins found in *A. thaliana* is shown in figure 1. Primary structure analysis allows a classification of the reported isoforms: Trx*f*, Trx*h*, Trx*m*, Trx*o*, Trx*x* and Trx*y* thioredoxins. The increasing number of expressed sequence tags (ESTs) found in databases reveals that the different thioredoxin groups are present in all higher plants studied (poplar, pinus, tomato, soybean and so on) suggesting that the thioredoxin diversity found in *A. thaliana* is representative of all higher plants. Based on the primary structure analysis, a phylogenetic tree was constructed using Clustalw (http://clustalw.genome.

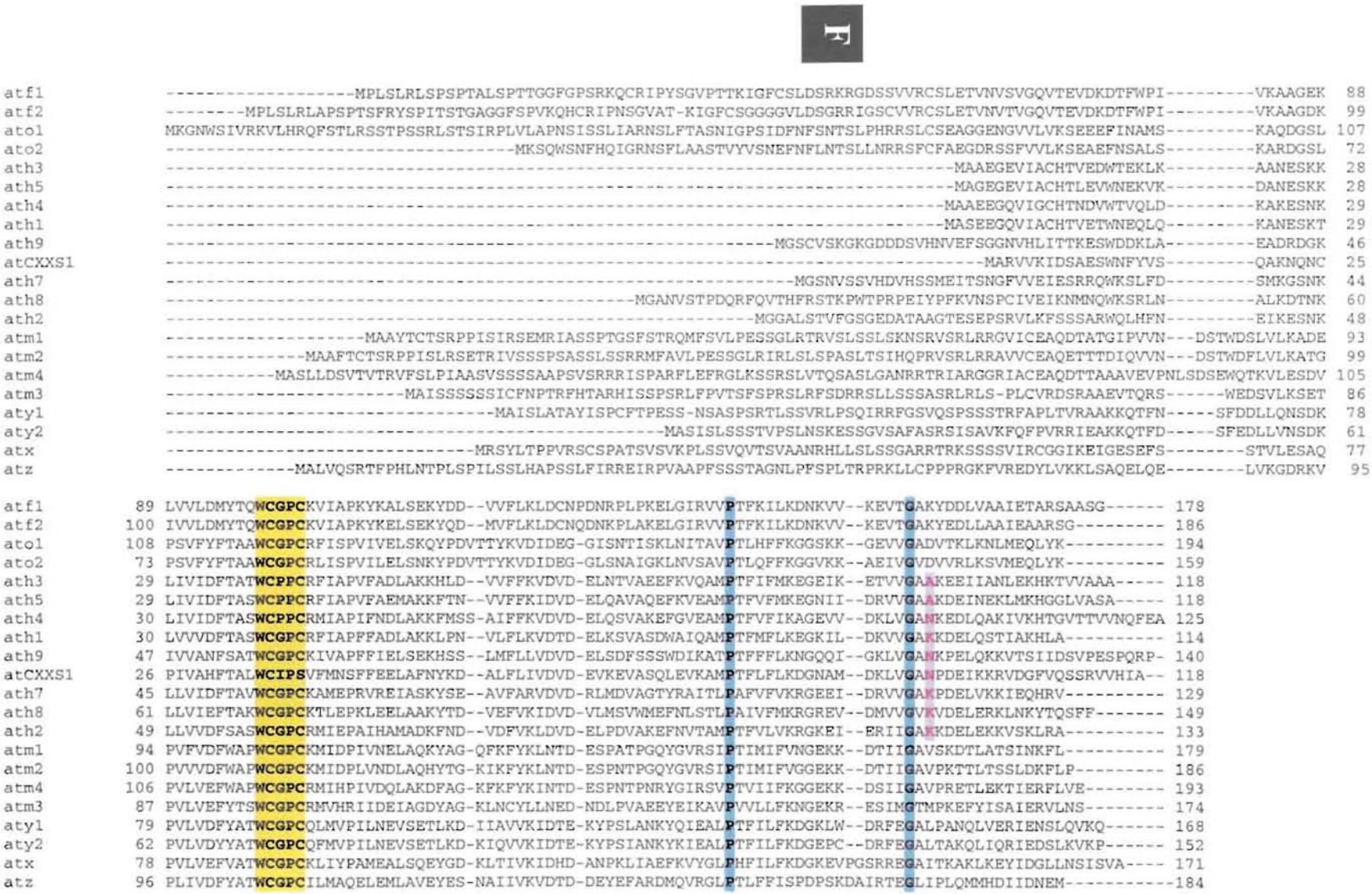

Figure 1. Amino acid sequence comparison of the thioredoxins of *A. thaliana*. The alignment was performed with ClustalW. The protein entry codes are the following: ath1: At3g51030 ; ath2: At5g39950; ath3: At5g42980; ath4: At1g19730 ; ath5: At1g45145; ath7: At1g59730; ath8: At1g69880; ath9: At3g08710; ato1: At2g35010; ato2: At1g31020; Atm1: At1g03680; Atm2: At4g03520; Atm3: At2g15570; Atm4: At3g15360; Atf1: At3g02730; Atf2: At5g16400; Atx: At1g50320; Aty1: At1g76760; Aty2: At1g43560; AtCXXS1: At1g11530. The atypical AtCXXS2 (At2g40790) has been omitted from this alignment. Catalytic sites are in yellow, strictly conserved amino acids in black on blue. The residue 101 important in the thioredoxin *h* classification (Ath3 numeration) is depicted in pink on gray.

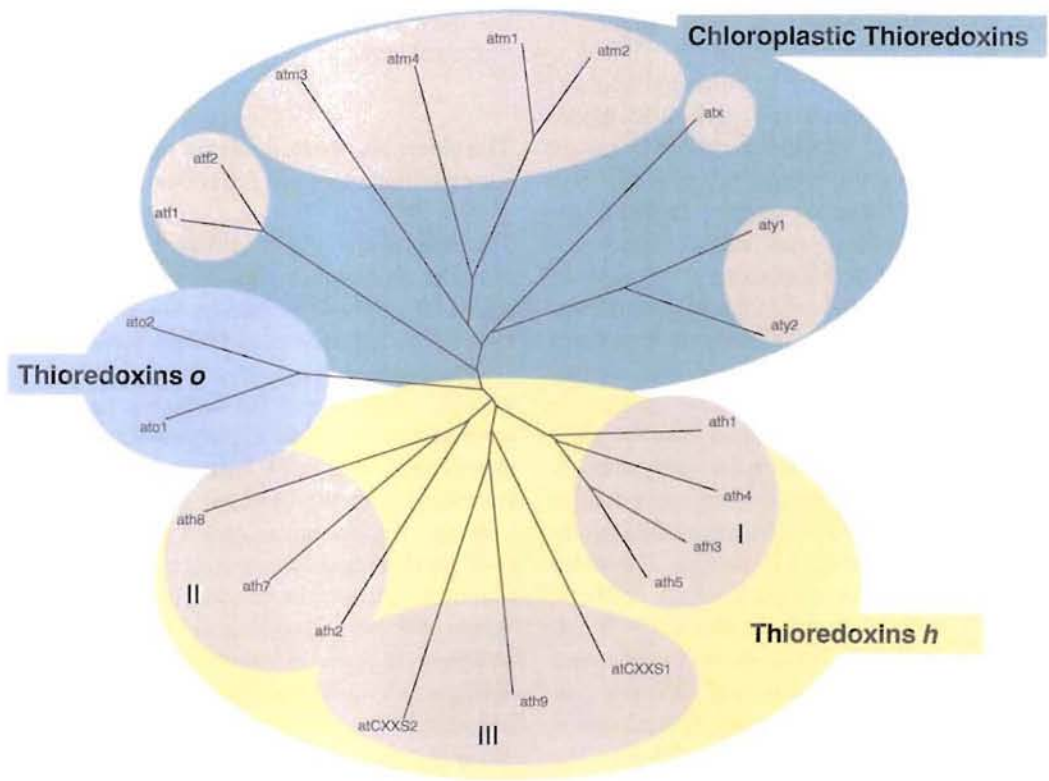

Figure 2. Phylogenetic tree of the *A. thaliana* Trx isoforms. This tree was drawn using ClustalW. The protein entry codes are identical to those of figure 1.

ad.jp/), encompassing the various thioredoxin isoforms found in *A. thaliana* (fig. 2). Comparisons with prokaryotic and eukaryotic sequences show that thioredoxins *m*, *x*, and *y* are of prokaryotic origin [10, 11]. In contrast, phylogenetic analyses suggest a eukaryotic origin for thioredoxins *f*, *h* and *o* [10, 12, 13].

In addition to primary structure analysis, subcellular localization the different thioredoxins is an important factor in understanding the plant thioredoxin system. The chloroplast contains the thioredoxins *f*, *m*, *x* and *y* and the atypical CDSP32. The corresponding amino acid sequences possess an N-terminal extension. These extensions are likely to correspond to a putative transit peptide for targeting to the chloroplast. All sequences are predicted to be addressed to the chloroplast with a very high score by Predotar, iPSORT and TargetP (links available at http://www.expasy.ch). The chloroplastic localization of *f*, *m* and *x* thioredoxins has been confirmed by fusion green fluorescent protein (GFP) experiments using the *A. thaliana* isoforms [14].

The thioredoxins *o*, at least AtTrx*o*1, have been recently demonstrated to be present in mitochondria. These isoforms, which exhibit a N-terminal extension, are predicted to be addressed to the mitochondria. This localization has been confirmed by mitochondrial import experiments [13] and by GFP fusion experiments for AtTrx*o*1 [14]. The mitochondrial system could be completed by at least one isoform of thioredoxin *h*. Based on primary structure analysis, thioredoxin *h* could be divided in to three subgroups [15, 16]. Our group has recently demonstrated that one poplar isoform (PtTrx*h*2) belonging to the subgroup 2 [17] is associated with mitochondria [18].

Besides this isoform, the thioredoxin *h* group found in *A. thaliana* constitutes a large disparate group that includes some thioredoxin isoforms exhibiting N-terminal extensions. Nevertheless, cell sorting prediction programs suggest that these isoforms are cytosolic. This point is in disagreement with previous results showing the purification of thioredoxin *h* from mitochondria [19, 20] and endoplasmic reticulum [19]. Furthermore, Trx*h*1 and Trx*h*2 from soybean belonging to the same subgroup as PtTrx*h*2 exhibit a hydrophobic N-terminal extension, suggesting that they could be bound to membranes [21]. Nuclear localization of thioredoxin *h* has been also demonstrated [22]. Finally, recent data demonstrate that subcellular localization may not depend only on the signal peptide presence [23, 24], suggesting that subcellular localization of thioredoxin *h* needs to be investigated.

Physical and mechanistic aspects of thioredoxin

A thioredoxin is defined both by its structural and catalytic characteristics. It is able to reduce disulfide bonds (the model used is insulin). In addition, thioredoxins are very stable proteins containing around 110 amino acids in their mature form (excluding the transit peptides of the nuclear encoded chloroplastic and mitochondrial isoforms). The major feature of thioredoxins is the presence of two cysteinyl residues involved in the very conserved catalytic site WC[G/P]PC. A few thioredoxin *h* isoforms belonging to subgroup 3 [10, 16] exhibit an unusual catalytic site where the C-terminal cysteine residue is changed to a serine (CXXS). In classical catalytic sites, both cysteinyl residues are involved in the catalytic activities of the enzyme, allowing disulfide bridge reduction of the target protein (fig. 3). The redox potential of thioredoxin is also critical in governing its activity. The redox potential of plant thioredoxins ranges between −285 and −350 mV [14, 25], with the notable exception of PtTrx*h*4. This poplar isoform belongs to thioredoxin *h* subgroup 3 [16] and exhibits a redox potential around −200 mV [unpublished results], explaining its unusual reduction pathway, which involves glutaredoxins (see below). The amino acid environment of the cysteinyl residues is critical for thioredoxin disulfide reductase activity. Protein disulfide isomerases (PDIs) catalyze the formation of disulfide bridges and also possess a CXXC active site (CGHC). Eukaryotic PDIs exhibit an E_m ranging from −147 to −175 mV [26]. In Dsba, which exhibits a CGHC catalytic site and oxidase activity, the pKa of the N-terminal active site cysteine is decreased in comparison to thioredoxin. Stabilization of the thiolate is due to an additional hydrogen bond with the active site histidine [27]. Different mutagenesis studies have shown that the sequences of the X–X dipeptides are a determinant of the redox potential of the thioredoxin superfamily of oxidoreductases and are also important in interactions with target proteins [28–30]. In particular, several thioredoxins *h* exhibiting the unusual catalytic site WCPPC have been reported [10]. In *A. thaliana*, among five isoforms belonging to subgroup I, three are WCPPC [10]. The redox potential of these isoforms is similar to the WCGPC isoforms (around −285 mV) [25]. Nevertheless, yeast complementation experiments have shown that modification of AtTrx*h*3 active site (WCPPC to WCGPC) restores a partial sulfate assimilation phenotype [31]. Furthermore, mutation of the PtTrx*h*3 active site (WCGPC to WCPPC) strongly modifies the protein conformation [32]. All these data suggest that the prolyl residue could play a role in the active site conformation, leading to specific interactions with target proteins.

Thioredoxins as well as the other members of the thioredoxin superfamily exhibit similar three-dimensional (3D) architecture. The description of these 3D structures has been reviewed recently [4, 6, 8] and will be not discussed here.

The chloroplastic thioredoxin system: Composition, mode of reduction and target enzymes

The multiplicity of the different thioredoxin isoforms found in chloroplasts 4*m*, 2*f*, 1*x* and 2*y* in *A. thaliana* raises the question of thioredoxin specificity and function. It is well documented that chloroplastic thioredoxins are associated with light regulation of carbon metabolism through regulation of the reducing pentose phosphate pathway and also of the C_4 pathway. Under oxidizing conditions prevailing in the dark, a number of enzymes are inactive due to the oxidized state of critical cysteinyl residues. Following a dark-to-light transition, these residues are reduced to the thiol state by thioredoxins present in the chloroplast stroma, leading in general to activation of the biocatalysts with the notable exception of glucose-6-phosphate dehydrogenase, which is inhibited during a dark-to-light transition [33]. The reducing power provided by light is mediated by the ferredoxin/thioredoxin system composed of ferredoxin, ferredoxin-thioredoxin reductase (FTR) and thioredoxins (fig. 3). This system has recently been reviewed and will not be detailed here [8, 34, 35]. Only two thioredoxin types have been implicated together with the ferredoxin/thioredoxin system, thioredoxins *f* and *m*. Historically, the Trx*m*s were

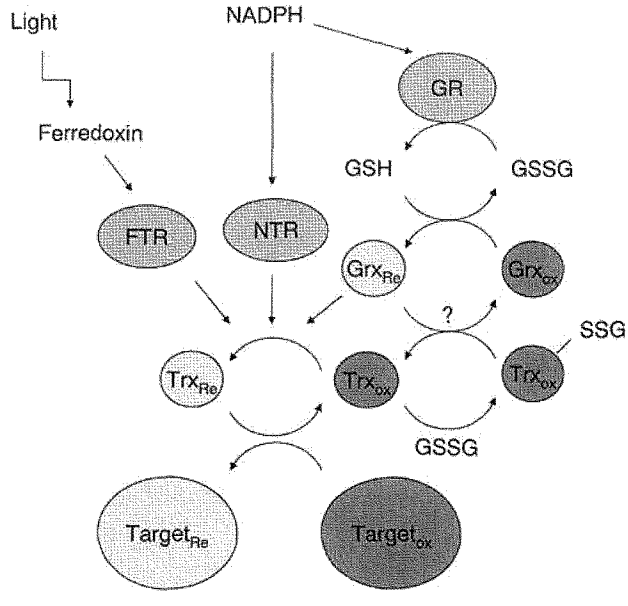

Figure 3. Different pathways of Trx reduction. The chloroplastic pathway involves ferredoxin-thioredoxin reductase (FTR). In mitochondria and cytoplasm, thioredoxins could be reduced by NADP-thioredoxin reductase (NTR). Particular thioredoxins could also be reduced via the glutathione (GSH/GSSG)/glutaredoxin (Grx) system. Glutathionylation of Trx has also been demonstrated.

so named because they were able to activate the well-characterized NADP malate dehydrogenase (NADP-MDH) [7], while Trx*f* has been demonstrated as a specific activator of fructose-1,6-biphosphatase (FBPase). Among the different protein targets biochemically characterized so far, many Calvin cycle enzymes are redox regulated via specific interactions with Trx*f* [7]. This suggests a specificity of thioredoxin *f* for photosynthetic carbon assimilation.

Among the four AtTrx*m*s, AtTrx*m*1, *m*2 and *m*4 have been shown to efficiently activate NADP-MDH [14]. Furthermore, using proteomic tools, these three isoforms have been found associated with the stromal side of the thylakoid membrane [36], even if this association seems to be very weak [37]. This localization has also been shown for 2-Cys peroxiredoxin [36–38]. Peroxiredoxins constitute a multigenic family involved notably in reactive oxygen species reduction (ROS) [39]. Among the nine expressed peroxiredoxins found in the *A. thaliana* genome, four isoforms are found in chloroplasts, two dimeric 2-Cys Prx, one type II Prx and one Prx Q [40]. Nevertheless, different chloroplastic thioredoxins (*f*, *m*, *x* and *y*) as well as one thioredoxin-like chloroplast drought-induced protein of 32 kDa [41] are able to reduce in vitro 2-Cys AtPrx as well as poplar PrxQ [14, 42]. AtTrx*x* is the most efficient thioredoxin tested in the reduction of 2-Cys Prx, suggesting that this isoform could be involved in vivo in peroxide detoxication [14].

AtTrx*m*3 exhibits specials properties. In in vitro tests, this isoform is not able either to activate NADH-MDH and FBPase or to reduce 2-Cys Prx [14], and its expression pattern differs from the other thioredoxins *m* [43], suggesting that this isoform could be involved in specific functions in non-green plastids.

One Trx*y* isoform conserved in photosynthetic organisms has been characterized in the green algae *Chlamydomonas reinhardtii* [11]. *Cr*Trxy is efficient in reduction of various Prxs [11, 42], but is probably not involved in, in vivo activation of FBPase or NADP-MDH [11].

The thioredoxin reduction pathway in chloroplasts is probably not only dependent on the FTR system. Indeed, *A. thaliana* genome analysis has suggested the presence of a putative NADPH-dependent thioredoxin reductase (NTR, see below and fig. 3) containing also a thioredoxin domain in chloroplasts [22, 44]. This protein has been recently characterized in rice as a bifunctional enzyme exhibiting both thioredoxin and NTR activity but not a NTR/thioredoxin system. Nevertheless, the NTR domain is unable to reduce the thioredoxins tested, particularly thioredoxins *f* and *m*. Knockout experiments have suggested that this protein is probably involved in protecting of chloroplasts against oxidative damage [45].

The diversity of chloroplastic thioredoxin isoforms is in accordance with the number of demonstrated or putative target proteins. Nevertheless, in most cases the specificity of these protein-thioredoxin interactions remains undefined. Recently, the development of proteomics coupled with the identification of redox-regulated proteins led to a dramatic increase of the number of potential thioredoxin targets in chloroplasts [46–48]. The potential thioredoxin-interacting proteins are listed in table 1. The number of potential functions cannot be detailed here and remain to be confirmed in several cases. Apart from its well-documented dark-to-light transition, the thioredoxin system participates in responses against environmental stresses. In most cases, abiotic or biotic stresses are linked to the production of ROS. The thioredoxin system is involved in ROS detoxication at least through several peroxiredoxin isoforms [38, 42], glutathione peroxidases [49] and peptide methionine sulfoxide reductases [50, 51].

The mitochondrial thioredoxin system: composition, mode of reduction and target enzymes

The plant mitochondrial thioredoxin system involving thioredoxin *o* and NTR was recently described in *A. thaliana* [13]. In this system, reducing equivalents for thioredoxin reduction are provided by NADPH through NTR (fig. 3). This system is ubiquitous, but despite their similarities the NTRs of plants and animals are fundamentally different. In animals, NTRs are very homologous to glutathione reductases [52]. These homodimers of 55-kDa subunits usually contain a selenocysteine residue present in a carboxy-terminal active site [53]. In plants, as well as in fungi, archea and bacteria, the NTR proteins are found as homodimers of 35-kDa subunits without selenocysteine residues [54, 55]. *Chlamydomonas reinhardtii* exhibits both NTR forms [56]. In *A. thaliana*, two genes encoding typical NTRs have been found in the whole sequenced genome and called *ntrA* and *ntrB* [10]. ntrA produced two different messengers: one shorter, encoding a cytosolic protein, and one longer, featuring a signal peptide that allows mitochondrial importation of the protein [13]. This mitochondrial NTRA has been shown to efficiently reduce AtTrxo1, the first characterized plant mitochondrial thioredoxin [13]. Two thioredoxins *o* have been described in *A. thaliana*, AtTrx*o*1 and AtTrx*o*2; nevertheless, only AtTrx*o*1 has been shown to be localized in mitochondria, while the subcellular localization of AtTrx*o*2 remains unclear [13]. Thioredoxin *o* genes have been found in other plant genomes [25]. Besides the *o* type of thioredoxins, plant mitochondria could also contain thioredoxin *h*. The poplar thioredoxin *h*2 (PtTrx*h*2) [17] was recently shown to be associated with mitochondria using both GFP fusion and immunolocalization experiments [18]. PtTrx*h*2 is reduced efficiently in vitro by AtNTRA, suggesting

Table 1. Potential target of thioredoxins in the chloroplasts.

	References		References
Carbon assimilation		**Photorespiration**	
Transketolase	[47]	Glycerate kinase	[93]
Triose phosphate isomerase	[47]		
Ribulose-P-3-epimerase	[47]	**Translation**	
Carbonic anhydrase	[47]	RB60	[94]
Sedoheptulose-1,7-biphosphatase	[78]	Elongation factor Tu	[47]
Phosphoribulokinase	[79]	Elongation factor g	[47]
Glyceraldehyde-3P- dehydrogenase	[80]	Ribonucleoprotein	[47]
Rubisco activase	[81]	30S ribosomal protein S1	[47]
Rubisco small subunit	[47]	Ribosomal protein S6 (PrpS6)	[47]
Fructose-1,6-biphosphatase	[82]		
		DNA replication/transcription	
Metabolism		ATP-dependent DNA helicase	[47]
Glutamate synthase			
3-deoxy-D-arabino-heptulosonate 7-phosphate synthase	[83, 84]	**Plastid division**	
		FtsZ protein	[47]
NADP-malate dehydrogenase	[85]		
Acetyl-CoA carboxylase	[86]	**Protein degradation**	
CF1 ATPase	[87]	ATP dependent clp protease	[47]
Glucose-6P-dehydrogenase	[88]	Magnesium chelatase	[47]
Cysteine synthase	[47]		
ADP-glucose pyrophosphorylase	[89]	**Vitamin biosynthesis**	
Cyclophilin	[90]	Thiamin biosynthesis protein	[47]
Phosphoglycerate dehydrogenase	[47]	Thiazole biosynthetic enzyme	[47]
Cysteine synthase	[47]		
6-phosphogluconate dehydrogenase	[47]	**Stress coupled reactions**	
Enolase	[91]	Peroxiredoxin Q	[46]
β-amylase	[47]	2-Cys Peroxiredoxin	[95]
		Glutathione peroxidase	[96]
Photosystem		Methionine sulfoxide reductase	[16]
LHCII protein kinase	[92]		

that this isoform could also be reduced in vivo. These data confirm previous studies demonstrating the presence of Trx*h* in mitochondria [19]. The presence of at least two distinct thioredoxins in mitochondria raises the question of the specificity of each type. Recent proteomics experiments have detected numerous potential thioredoxin targets in mitochondria involved in several fundamental processes [57]. Indeed, up to 50 potential Trx-linked proteins are potentially involved in 12 processes: photorespiration, citric acid cycle, lipid metabolism, electron transport, ATP synthesis, membrane transport, translation, protein folding, nitrogen metabolism, sulfur metabolism, hormone synthesis and stress-related reactions. Biochemical experiments now need to be performed to determine the possible specificity of each mitochondrial thioredoxin type towards these targets. In addition, PtTrx*h*2 as well as AtTrx*o*1 have been shown to activate alternative oxidase (AOX) [18]. AOX couples the oxidation of ubiquinol to the complete reduction of oxygen to water. This pathway does not contribute to ATP synthesis but can dampen the mitochondrial generation of ROS. AOX activity is submitted to post-translational regulation involving the reduction of an intersubunit disulfide bridge and allowing AOX activation by α-keto acids (for review, see [58]). This disulfide bridge reduction could be performed by thioredoxins. AOX could play a role in the regulation of the programmed cell death [59]. In addition, mitochondrial thioredoxins could play another role in apoptosis regulation, modulating porin conformation [57]. The mitochondrial redox state and also probably the mitochondrial thioredoxin system play a key role in plant redox homeostasis and particularly in stress resistance induction [59].

The thioredoxin *h* families and cytososolic thioredoxins

Based on primary structure analysis, thioredoxins *h* constitute a large disparate group which can be divided in the three different subgroups [6, 10, 16], called subgroups I, II and III [9].

The classification of thioredoxins h needs to be improved by considering both their subcellular localization and their participation in distinct reduction pathways. We also propose a more detailed classification, presented below.

The subgroup I comprises thioredoxins, which are believed to be cytosolic. An analysis of 41 subgroup I

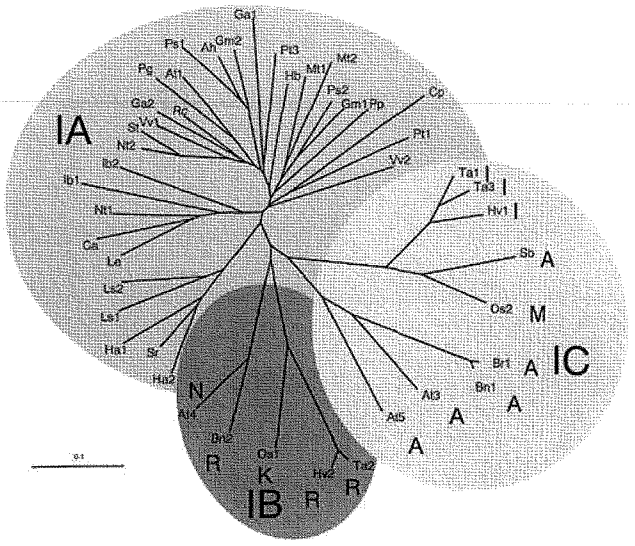

Figure 4. Phylogenetic tree of several thioredoxins *h* belonging to the subgroup I. This tree was drawn using ClustalW. Three clusters named IA, IB and IC can be distinguished according to their primary sequence. In clusters IB and IC, residue 101 is shown. The codes used are the following: Ah: *Arachnis hypogaea* (CD038084); At1: *A. thaliana* (At3g51030); At3: *A. thaliana* (At5g42980); At4: *A. thaliana* (At1g19730); At5: *A. thaliana* (At1g45145); Bn1: *Brassica napus* (U59379); Bn2: *Brassica napus* (U59380); Br1: *Brassica rapa* (AB10434); Ca: *Capsicum annuum* (AY496104); Cp: *Citrus paradisi* (AY271308); Ga1: *Gossypium arboreum* (BQ408049); Ga2: *Gossypium arboreum* (TC25963); Gm1: *Glycine max* (AI461219); Gm2: *Glycine max* (BI699372) Ha1: *Helianthus annus* (TC12357); Ha2: *Helianthus annus* (TC12867); Hb: *Hevea brasiliensis* (CB377001); Hv1: *Hordeum vulgare* (AY245454); Hv2: *Hordeum vulgare* (AY245455); Ib1: *Ipomoea batatas* (AY344230); Ib2: *Ipomoea batatas* (BJ561302); Le: *Lycopersicon esculentum* (TC116392); Mt1: *Medicago truncatula* (TC85696); Mt2: *Medicago truncatula* (TC87208); Ls1: *Lactuca sativa* (TC9289); Ls2: *Lactuca sativa* (BQ873424); Nt1: *Nicotiana tabacum* (X58527); Nt2: *Nicotiana tabacum* (Z11803); Os1: *Oryza sativa* (Q42443); Os2: *Oryza sativa* (Q9FRT3); Pg: *Peppermint glandular* (AW255457); Pp: *Prunus persica* (AF323593); Ps1: *Pisum sativum* (AY170650); Ps2: *Pisum sativum* (AJ310990); Pt1: *Populus tricocharpa cv. Trichobel* (AF483625); Pt3: *Populus tricocharpa cv. Trichobel* (BU822062); Rc: *Ricinus communis* (Z70677); Sb: *Sorghum bicolor* (TC87208); Sr: *Stevia rebaudiana* (BG525644); St: *Solanum tuberosum* (BM111010); Ta1: *Triticum aestivum* (CD886902); Ta2: *Triticum aestivum* (CD892602); Ta3: *Triticum aestivum* (BJ210524); Vv1: *Vitis vinifera* (CF216136); Vv2: *Vitis vinifera* (CB348011).

thioredoxins *h* shows that these proteins can be classified into three different clusters, which we have named IA, IB and IC (fig. 4). *A. thaliana* thioredoxins *h3*, *h4* and *h5* as well as brassica sequences are related to cereal thioredoxins in cluster IB and IC, whereas AtTrx*h*1 is present in cluster IA. A classification has been recently proposed based on the residue at position 101 [15, 61]. This residue could be present on the protein surface from ca. 12 Å of the active site [61]. AtTrx*h*3 and AtTrx*h*5 cluster in IC with thioredoxins exhibiting a hydrophobic residue in this position (A, I or M). In contrast, AtTrx*h*4 is related to thioredoxins harbouring a charged or hydrophilic residue (R, K or N) in cluster IB. This 101 residue is also involved in a structural motif [R_{101}KDD] critical for transfer from companion cells to sieve-tube elements [62, 63]. Indeed, thioredoxins *h* have been found abundantly in phloem sieve tubes from several plants [62, 64, 66]. Nevertheless, AtTrx*h*5 exhibits a [A_{101}KDE] motif and has also been reported to be expressed in vascular tissues [65]. Besides this C-terminal motif, the N-terminal sequence MAAEE also seems to be required for transfer through plasmodesmata [63].

Recent studies have shown that *A. thaliana* thioredoxins *h* differ by their cell type and specificity of expression [65, 67]. Among the thioredoxins *h* subgroup I, Attrx*h*1 and Attrx*h*4 expressions are correlated with the cell cycle, suggesting a role in redox control of cell proliferation [68]. In contrast, AtTrx*h*5 seems to be specifically involved in response of pathogens and to oxidative stresses [67]. In cereals, thioredoxins *h* are found throughout the plant but are more abundant in mature seeds [64]. During seed maturation, TaTrx*h*A has been detected in the nucleus of aleurone and scutellum cells, this localization corresponding to oxidative conditions in these tissues [22].

The second subgroup of thioredoxins *h* encloses proteins harbouring N-terminal extensions. Primary analysis of 28 sequences show that these thioredoxins can be divided in different clusters, one corresponding to cereal sequences called IIA, one containing the mitochondrial PtTrx*h*2, AtTrx*h*7 and AtTrx*h*8 (see above) named IIB and one containing thioredoxins related to AtTrx*h*2 named IIC (fig. 5). Concerning proteins of cluster IIB, the different reported sequences have been submitted to the prediction program (http://hypothesiscreater.net/iPSORT/), showing that these isoforms could also possess a signal peptide. Nevertheless, AtTrx*h*7, which belongs to this cluster, is not currently predicted to be associated with mitochondria, which does not exclude a possible mitochondrial localization. Despite this obvious exception, cluster IIB may include mitochondrial thioredoxins *h*.

Cluster IIC also constitutes a homogeneous group which exhibits a N-terminal extension. In soybean, a thioredoxin belonging to this subgroup has been shown to be anchored to the plasma membrane [21]. Very few studies have been performed on these thioredoxins, preventing any conclusion about the significance of this N-terminal extension. Complementation experiments have shown that AtTrx*h*2 allows yeast Trx mutants to grow on sulfate [70]. Furthermore, Attrx*h*2 gene expression is correlated to the cell cycle, exhibiting a peak of expression in G2 phase [68].

The third subgroup of thioredoxins *h* comprises the atypical thioredoxins CXXS and thioredoxins exhibiting the usual catalytic site WCGPC (fig. 6). The CXXS thioredoxins cannot be considered as true thioredoxins and are

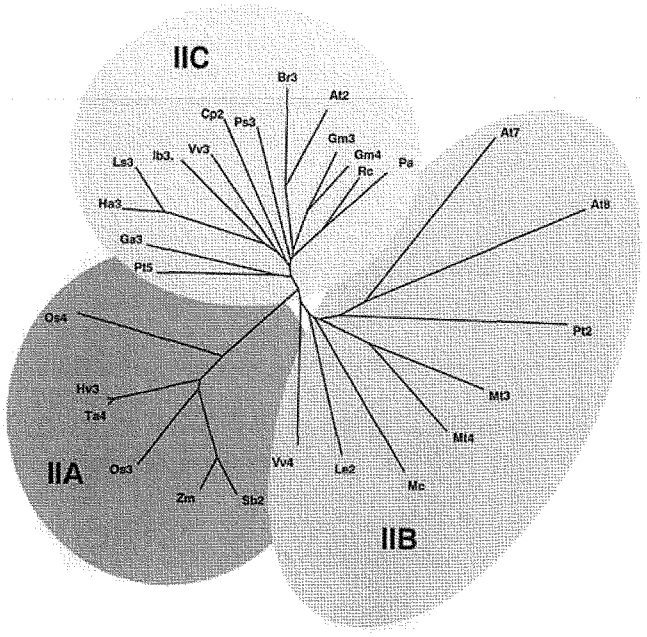

Figure 5. Phylogenetic tree of different thioredoxins *h* belonging to subgroup II. This tree was drawn using ClustalW. Three clusters named IIA, IIB and IIC can be distinguished according to their primary sequence. The codes used are the following: At2: *Arabidopsis thaliana* (At5g39950); At7: *Arabidopsis thaliana* (At1g59730); At8: *Arabidopsis thaliana* (At1g69880); Cp2: *Citrus paradisi* (CF837405); Br3: *Brassica rapa* (AF352030); Ga3: *Gossypium arboreum* (BG440056) Gm3: *Glycine max* (AW620807); Gm4: *Glycine max* (BM188930); Ha3: *Helianthus annus* (TC13569); Ib3: *Ipomoea batatas* (AY344228); Hv3: *Hordeum vulgare* (BI960260); Le2: *Lycopersicon esculentum* (TC135169); Ls3: *Lactuca sativa* (TC9551); Mc: *Mesembryanthemum crystallinum* (BE033809); Mt3: *Medicago truncatula* (AW560796); Mt4: *Medicago truncatula* (AW686237); Os3: *Oryza sativa* (AK062383); Os4: *Oryza sativa* (CB681257); Pa: *Prunus armeniaca* (CB818939); Ps3: *Pisum sativum* (AY170651); Pt2: *Populus tricocharpa cv. Trichobel* (AF483266); Pt5: *Populus tricocharpa cv. Trichobel* (BU869308); Rc: *Rosa chinensis* (BI978567); Sb2: *Sorghum bicolor* (TC76743); Ta4: *Triticum aestivum* (CD894811); Vv3: *Vitis vinifera* (CF513794); Vv4: *Vitis vinifera* (TC25464); Zm: *Zea mays* (AY104013).

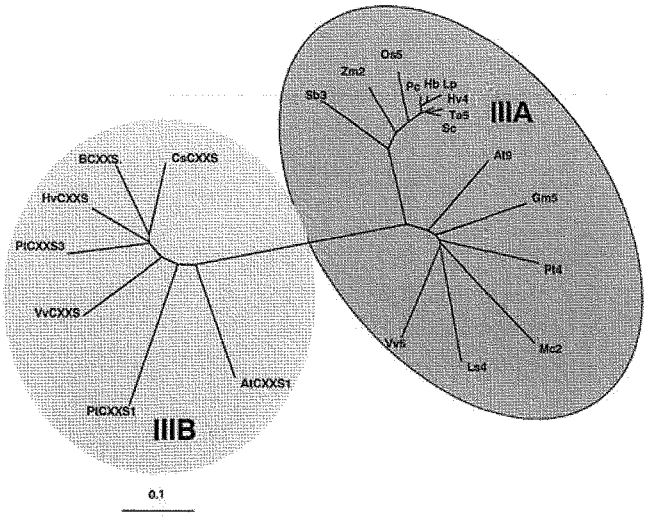

Figure 6. Phylogenetic tree of different thioredoxins *h* belonging to the subgroup III. Two clusters named IIIA and IIIB can be distinguished according to their primary sequence. Cluster IIIA enclosed the thioredoxins harbouring the usual active site WCGPC, whereas the members of cluster IIIB exhibit a CXXS catalytic site. The codes used are the following: At9: *Arabidopsis thaliana* (At3g08710); Gm5: *Glycine max* (CA799351); Hb: *Hordeum bulbosum* (AF159385); Hv4: *Hordeum vulgare* (AF435815); Lp: *Lolium perenne* (159387); Ls4: *Lactuca sativa* (TC9851); Mc2: *Mesembryanthemum crystallinum* (CA838461); Os5: *Oryza sativa* (AF435817); Pc: *Phalaris coerulescens* (AF159388); Pt4: *Populus tricocharpa cv. Trichobel* (BU835000); Sb3: *Sorghum bicolor* (TC72759); Sc: *Secale cereale* (AF159386); Ta5: *Triticum aestivum* (AF438359); Vv5: *Vitis vinifera* (CB004453); Zm2: *Zea mays* (AF435816); AtCXXS1: *Arabidopsis thaliana* (At1g11530); BpCXXS: Betula pendula (CD278293); CsCXXS: *Citrus sinensis* (BQ623126); HbCXXS: *Hevea brasiliensis* (AF133127); PtCXXS1: *Populus tricocharpa cv. Trichobel* (CA823821); PtCXXS3: *Populus tricocharpa cv. Trichobel* (BU874060); VvCXXS: *Vitis vinifera* (CF204852). The atypical AtCXXS2 clusters separately. Databases analysis did not reveal any plant AtCXXS2 orthologues. Consequently, AtCXXS2 has been omitted in the construction of this phylogenetic tree.

more likely related to the monothiol glutaredoxin superfamily [1]. Indeed, the absence of the second cysteinyl residue in the catalytic site prevents 'true' thioredoxin activity. In vitro experiments performed with PtCXXS3 have confirmed this hypothesis [16]. Nevertheless, these atypical thioredoxin genes have introns at the same positions at the other thioredoxins *h* [10]. In fact, the main feature of the third subgroup is possible interactions with the glutaredoxin/glutathione system. Indeed, in contrast with the other thioredoxins *h* (belonging to subgroups I and II), these proteins are not reduced by NTRs [15, 16]. PtCXXS3 is active in a glutathione:HED transhydrogenase assay, suggesting glutaredoxin-like activity [1]. Furthermore, PtTrx*h*4, which possesses a classical WCGPC active site, exhibits true thioredoxin activity but is reduced by glutaredoxins. An amino acid comparison of different PtTrx*h*4-related thioredoxins shows that a third cysteinyl is conserved in the N-terminal part of the protein (fig. 7). Mutagenesis experiments have shown that this third residue is probably involved in protein reduction by glutaredoxin [E. Gelhaye et al., unpublished]. PtTrx*h*4 exhibits an E_m value around −200 mV at pH 7 [E. Gelhaye et al., unpublished], a value quite elevated over the one reported for other thioredoxins [18, 25], but adequate to allow reduction by glutaredoxins [1]. This subgroup also constitutes a direct interconnection between the two major systems involved in cellular redox regulation.

The number of potential targets of thioredoxins *h* has considerably increased with the development of proteomics [71–75]. Nevertheless, the as yet undefined specificity of interactions between thioredoxins and target proteins prevents firm conclusions at this point.

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Sc      MGGCVG--KGR-SIVEEKLDFKGGNVHVITTKEDWDQKIEEANKDGKIVVANFSASWCGPCRVVAPVYAGMSKTYP
Ta5     MGGCVG--KDR-SIVEEKLDFKGGNVHVITTKEDWDQKIEEANKDGKIVVANLSASWCGPCRVIAPVYAEMSKTYP
Hv4     MGGCVG--KGR-GVVEEKLDFKGGNVHVITTKEDWDQKIEEANKDGKIVVANFSASWCGPCRVIAPVYAEMSKTYP
Hb      MGGCVG--KDR-SIVEDKLDFKGGNVHVITTKEDWDQKVAEANKDGKIVVANFSASWCGPCRVIAPVYAEMSKTYP
Lp      MGGCVG--KDR-SIVEDKLDFKGGNVHVITTKEDWDQKVAEANKDGKIVVANFSASWCGPCRVIAPVYAEMSKTYP
Os5     MGGCVG--KGRRHIEEDKLDFKGGNVHVITSKEDWDRKIEEANKDGKIVVANFSASWCGPCRVIAPIYAEMSKTYP
Zm2     MGGCAG--KVR-RDDEEKLDFKGGNVHIITSNEGWDQKIAEANRDGKTVVANFSASWCGPCRVIAPVYAEMSKTYP
Pt4     MGLCLDKYKRDADNDELHVEFAGGNVHLITTKESWDQKLSEASRDGKIVLANFSATWCGPCRQIAPFYNELSEKYP
Vv5     MGQCFMKHHNDDDDSDHNAAFASGNVHLITTKENWEEKLAEASKDGKIVIANFSATWCGPCKMIAPFYCELSEAHP
Ls4     MGICFSSTHNDGDESDHNAEFAGGNVTLVSSKDAWDQKLSEAKKDHKIVIANFSASWCGPCRMIAPYYIELSEKHP
Gm5     MGSCVSKNKARDNDSDHNVDFAAGNVKLITTKEAWDQYLEEARRDGKIVIANFSAAWCGPCKMIAPYYCELSEKYT
Mc2     MGNCLAKSRDRDNDSDQHVEFAAGNVALITTKEAWDQKLEEAKKDGKIVIANFSAVWCGPCKVIAPYYCEMSEKYT
At9     MGSCVSKGKGD-DDSVHNVEFSGGNVHLITTKESWDDKLAEADRDGKIVVANFSATWCGPCKIVAPFFIELSEKHS

Sc      QLMFLTIDVDDLMDFSSTWDIRATPTFFFLKNGQQIDKLVGANKPELEKKVQALGDGS----------------
Ta5     QLMFLTIDVDDLMDFSSTWDIRATPTFFFLKNGQQIEKLVGANKPELEKKVQALGDGS----------------
Hv4     QLMFLTIDVDDLMDFSSTWDIRATPTFFFLKNGQQIDKLVGANKPELEKKVQALGDGS----------------
Hb      QLMFLTIDVDDLMDFGSTWDIRATPTFFFLKNGQQIDKLVGANKPELEKKVQALGDGS----------------
Lp      QLMFLTIDVDDLMDFSSTWDIRATPTFFFLKNGQLIDKLVGANRPELEKKVQAIGDGS----------------
Os5     QLMFLTIDVDDLMDFSSSWDIRAKPTFFFIKNEKQVDKLVGANKPELEKKVQALADGS----------------
Zm2     QLMFLTIDVDDLMDFSSSWDIRATPTFFFLKNGQQIDKLVGANKPELEKKVLAAADASTS--------------
Pt4     SLLFLLVDVDELSDLSTSWEIKATPTFFFLRDGKQLEKLVGANKPELQKKITAIVDSLPPSDK-------------
Vv5     SLMFLTVDVDELSEFSSSWDIKATPTFFFLRDGQQVDKLVGANKPELQKKITAILDSMTQCNK-------------
Ls4     SLMFLSVDVDELTDFSTQWDIKATPTFFFLRNGEQFDKLVGANKPELLKKINAIVDSEPPRRAV-----------
Gm5     SMMFLVVDVDELTDFSTSWDIKATPTFFFLKDGQQLDKLVGANKPELQKKIVAINDSLPEYKQ-------------
Mc2     SMMFLLVDVDELTDFSTSWDIKATPTFFFLKDGQQLDKLVGANKPELEKKLVAIADSVPQISSDLFLFMFVDAT
At9     SLMFLLVDVDELSDFSSSWDIKATPTFFFLKNGQQIGKLVGANKPELQKKVTSIIDSVPESPQRP----------
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Figure 7. Amino acid sequence comparison of thioredoxins belonging to the cluster IIIA. The alignment was performed with ClustalW. The codes used are identical to those used in figure 6. Catalytic sites and the third conserved cysteine are in white on black.

F

Conclusion and perspectives

The thioredoxin system is particularly complex in higher plants, involving numerous isoforms present in all plant compartments. The chloroplast system is the best documented; nevertheless, the function of each isoform remains unclear. The development of proteomics tools led to the identification of numerous potential thioredoxin-regulated proteins; biochemical studies are needed to confirm these interactions. The plant mitochondrial system was discovered only very recently, and other thioredoxins may also be present in this organelle. Mitochondrial thioredoxins seem to be involved in fundamental processes in particular in apoptosis regulation; again these interactions have to be confirmed.

In the field of redox regulation, it is obvious that the different systems are directly connected. Indeed, human thioredoxin activity is modulated by glutathionylation [76], and yeast Grx5 may be reduced by Trx [77]. In plants, PtTrx*h*4 is directly reduced by Grx, and PtCXXS3 exhibits a glutaredoxin-like activity. Furthermore, it was recently established that PtTrx*h*2 redox potential is modulated by glutathionylation (fig. 3) [18]. These interconnections between major redox regulating systems are probably physiologically important. The study of these relationships will certainly lead to a better understanding of plant cell redox regulation.

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A côté de ces Trx *stricto sensu*, Il existe aussi des Trx atypiques, qui vont contenir un ou plusieurs motifs Trx fonctionnels à l'intérieur d'une séquence plus longue. C'est le cas de la protéine CDSP32 identifiée chez *Arabidopsis thaliana*, qui possède un motif Trx CxxC dans sa partie C-terminale et qui possède une activité Trx au sein du chloroplaste (Broin et al 2000, Broin et Rey 2003, Rey et al 2005). C'est le cas aussi de la NTRC d'*A. thaliana*, qui contient un module thiorédoxine réductase couplé à un module thiorédoxine, et qui est capable de réduire efficacement une Prx 2-Cys en présence de NADPH (Moon et al 2006). Cette enzyme semble nécessaire au fonctionnement du chloroplaste, la délétion du gène correspondant induisant d'importantes perturbations du métabolisme, en particulier en conditions de stress (Perez-Ruiz et al 2006). Une autre enzyme appelée nucléorédoxine (Nrx) contient trois modules Trx, dont deux possèdent des sites actifs de séquence CPPC, et est présente chez les végétaux supérieurs. Elle est localisée dans le noyau, où elle pourrait réguler des facteurs de transcription (Laughner et al 1998). On peut noter que chez certains organismes, appartenant le plus fréquemment au monde bactérien, on retrouve aussi des motifs Trx dans des protéines de fusion naturelles, comme dans la protéine PilB de *Neisseria meningitidis*, dont le module MSR utilise le pouvoir réducteur fourni par le module Trx (Ranaivoson et al 2006). Les investigations pour comprendre le fonctionnement des protéines multi-modulaires et les mécanismes biochimiques sous-jacents sont un champ d'investigation récent et prometteur (Rouhier et al 2006b).

Chez le peuplier, notre modèle d'étude, il existe 5 gènes codant pour des isoformes de Trx cytosoliques, appelée Trx h1.1 et h1.2, h3, h4.1 et h4.2. La mitochondrie abrite elle aussi des isoformes spécifiques telles que la Trx h2, mise

en évidence récemment et interagissant notamment avec l'alternative oxydase (Article IV: Gelhaye et al 2004). Une Trx de type o a aussi été mise en évidence au niveau génomique mais n'est pas encore caractérisée chez le peuplier. Chez *Arabidopsis thaliana*, la Trx o1 est mitochondriale, alors que la Trx o2 ne possède pas d'extension N-terminale. La Trx h5 quand à elle, possède un peptide signal et semble être secrétée, comme cela a été montré pour la protéine homologue chez le tabac (Juarez-Diaz et al 2005). Au niveau chloroplastique, on trouve dans le génome du peuplier au moins 7 gènes codant pour des Trx de type m, un pour une Trx de type f, une pour une Trx de type x, et 2 pour des Trx y. De plus, trois gènes codant pour des protéines Trx-like au site actif CXXS sont présents dans le génome du peuplier, de même que d'autres gènes de Trx-like, homologues à celles *d'Arabidopsis thaliana* (Meyer et al 2005, Gelhaye, données non publiées). Les fonctions de ces Trx-like ne sont pas connues. La détermination du rôle physiologique de toutes ces isoformes est une question majeure des recherches actuelles chez les végétaux supérieurs, où le nombre d'isoformes est particulièrement élevé. Cette problématique se pose aussi pour la famille des glutarédoxines.

d- Les glutarédoxines (Grx) :

Les glutarédoxines appartiennent à la même famille d'oxydoréductases que les Trx. Ce sont aussi généralement des protéines de faible masse moléculaire, de 10 à 15 kDa, largement présentes chez tous les organismes. Le travail de recensement et d'inventaire réalisé chez les végétaux dont le génome est connu, *ie.* l'arabette, le peuplier, et le riz, indique que ces Grx forment des familles

multigéniques contenant de très nombreuses isoformes (Lemaire 2004, Rouhier et al 2004b, Rouhier et al 2006a). Chez les plantes supérieures, ces protéines se répartissent en trois groupes, suivant la séquence de leur site actif. Le groupe le plus important sur le plan du nombre d'isoformes est composé de protéines possédant un site actif de type CCx(C/S/G). On trouve aussi des Grx de type Cxx(C/S). Enfin, il existe des Grx possédant une cystéine conservée au niveau du site actif, de type CGFS. Les Grx utilisent le pouvoir réducteur du GSH pour réduire directement le déhydroascorbate (DHA) par exemple, mais aussi pour réduire de multiples protéines cibles telles qu'une Trx h4 (Gelhaye et al 2003) ou des Prx de type II (Rouhier et al 2001) chez les plantes supérieures. Les Grx sont aussi impliquées dans la glutathionylation de certaines protéines. Pour accomplir ces réactions de glutathionylation, seule une des cystéines des Grx est nécessaire. Récemment, il a été montré qu'une Trx f d'*Arabidopsis thaliana* était régulée par ce processus, le GSH se fixant à une cystéine située hors du site actif (Michelet et al 2005). Enfin, comme pour les Trx, des protéines contenant des modules Grx ont été mises en évidence par des analyses de séquences, mais leur fonction n'est pas toujours déterminée (Rouhier et al 2004b). Une exception notable est la caractérisation des fusions Prx-Grx présentes chez certaines bactéries telles que *Haemophilus influenzae* ou *Neisseria meningitidis* (Pauwels et al 2003, Rouhier et Jacquot, 2003). A l'instar des thiorédoxines, l'identification et l'étude des protéines multimodulaires avec un domaine glutarédoxine est un champ d'investigation en développement, notamment pour l'étude des relations structure-fonction chez ces enzymes (Rouhier et al 2006b).

D'autres propriétés remarquables ont été mises à jour par l'étude extensive des Grx de peuplier, avec notamment la présence d'un centre Fer-Soufre (Fe-S)

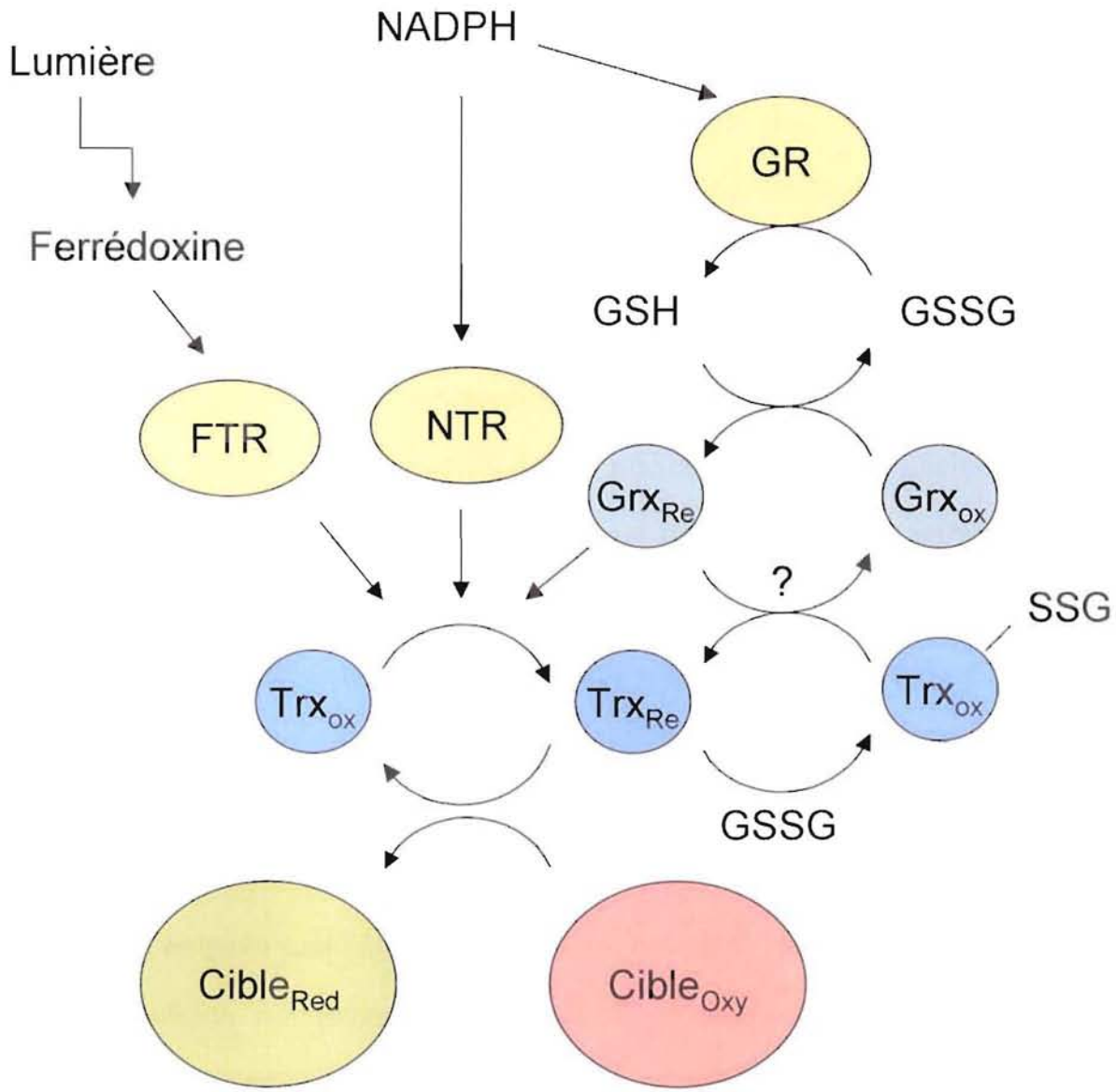

Figure 8. Schéma de principales voies de réduction des Trx dans les cellules végétales. Les réductases sont figurées en jaune. Le système dépendant de la lumière est localisé uniquement dans le chloroplaste. Le transfert d'électrons et protons s'effectue depuis le NADPH ou la Fd jusqu'aux différentes protéines cibles oxydées (d'après Gelhaye et al 2005).

chez l'une d'entre elles, dont le rôle reste inconnu (Feng et al 2006). Ce résultat est particulièrement intéressant puisqu'il a déjà été montré *in vitro* que certaines Grx de levure et d'*Escherichia coli* participent à l'incorporation de centres Fer-Soufre (FeS) dans les protéines (Achebach et al 2004, Molina-Navarro et al 2006).

Un schéma récapitulatif des systèmes réducteurs Trx et Grx-dépendants est présenté en figure 8.

e- Les glutarédoxines-like

En plus de l'identification de modules Trx ou Grx dans d'autres protéines, l'analyse des séquences génomiques permet de mettre à jour des gènes apparentés codant pour des protéines possédant un ou plusieurs motifs conservés de type CxxC. C'est le cas d'une classe de protéines encore non caractérisées et dénommées Grx-like. L'annotation automatique du génome du peuplier et d'autres génomes avait initialement classé ces protéines comme des Grx-like à cause d'une faible homologie de séquences, mais l'analyse détaillée des séquences et les connaissances sur les Grx ont permis par la suite de les dissocier de celles-ci. Elles ont pour principale caractéristique de posséder 4 motifs CxxC, regroupés sous la forme de deux séquences $CxxCx_7CxxC$ situées dans la partie C-terminale des protéines. Elles ont été regroupées en une famille distincte des Grx et leur caractérisation est en cours. L'analyse *in silico* de cette famille de protéines est l'objet de l'article III (Navrot et al 2007b).

Article III : Identification of a new family of plant proteins loosely related to glutaredoxins with four CxxC motives

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Nicolas Navrot, Eric Gelhaye, Jean-Pierre Jacquot and Nicolas Rouhier

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Cet article décrit une nouvelle famille de protéines, mises en évidence lors de travaux d'annotation du génome du peuplier. Ces protéines ont été appelées Grx-like du fait d'une faible homologie avec les Grx déjà connues. Elles sont aussi décrites comme riches en cystéine, ceci à cause de la présence de quatre motifs CxxC strictement conservés dans la région C-terminale. Les protéines contenant ces motifs sont regroupées au sein d'une famille multigénique qui n'existe quasiment que chez les plantes supérieures, seules quelques isoformes étant retrouvées chez les mammifères et les arthropodes. Trois sous-groupes ont été distingués selon la taille des protéines et la conservation et l'organisation des séquences adjacentes aux motifs CxxC. Ces protéines, de fonctions inconnues, pourraient appartenir aux systèmes oxydoréducteurs thiols-dépendants, et semblent être capables de fixer un ou plusieurs atomes de fer dans leur structure. L'expression des gènes codant ces protéines a été analysée, grâce aux banques d'EST, aux microarrays *d'Arabidopsis thaliana* disponibles en ligne et à des études de RT PCR semi-quantitatives.

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ORIGINAL PAPER

Identification of a new family of plant proteins loosely related to glutaredoxins with four CxxC motives

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Abstract The annotation of the recently released *Populus trichocarpa* genome, has allowed us to characterize extensively the multigenic families of the redoxin proteins. Proteins with two cysteines separated by two amino acids (CxxC motif) are often involved in redox reactions by promoting the formation, reduction or isomerization of disulfide bonds or by binding prosthetic groups or metals. We report here the presence of a new protein family in higher plants, constituted of 19 members in *Populus trichocarpa*, 15 in *Arabidopsis thaliana* and 17 in *Oryza sativa*. These proteins are almost specific to higher plants, with only two homologous genes found in mammals and arthropoda but none in other kingdoms. While these proteins were predicted as glutaredoxin-like proteins (GRL) in the automatic annotation procedure, they do not share the major conserved features of glutaredoxins but instead they display four conserved CxxC motives. A classification of these proteins, based on sequence similarity, gene structure and predicted cellular localization is proposed. The expression of these genes was also investigated by analyzing EST databases and *Arabidopsis* microarray results.

Keywords Genome · Glutaredoxin · Oxidoreductase · Plant

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Abbreviations

GRL	Glutaredoxin-like protein
GRX	Glutaredoxin
AU	Arbitrary unit

Introduction

Although one of the less abundant amino acids, cysteine is present in the catalytic site of many enzymes and frequently employed for redox functions, being involved in the binding of some cofactors and prosthetic groups or in thiol-disulfide exchanges. In addition to the well-known CxxC motif, two cysteinyl residues separated by two amino acids, recent analyses using new developing tools and softwares have identified similar roles for CxxS or CxxT motives (Fomenko and Gladyshev 2003, 2004; Ginalski et al. 2004). In the case of thiol-disulfide oxidoreductases, these motives are frequently found upstream of an α-helix (Fomenko and Gladyshev 2003; Coudevylle et al. 2005). Another important point is the nature of the two amino acids included in these motives. They were shown in many enzymes to be crucial to maintain low pKa values of the N-terminal cysteine but also to influence their redox potential (Grauschopf et al. 1995; Chivers et al. 1997; Moutevelis and Warwicker 2004). In addition to thiol disulfide oxidoreductases, the CxxC motif is also present in many thioredoxin or glutaredoxin-interacting proteins, in zinc-finger proteins, like transcription factors (Takatsuji 1998), as well as in many metal-containing proteins involved in electron transfer (ferredoxin, cytochromes, scaffold proteins, CCmH for

cytochrome assembly) (Meyer 2001; Meyer et al. 2005) or in some eukaryotic or prokaryotic repairing systems (methionine sulfoxide reductases B) (Vieira Dos Santos et al. 2005; Ranaivoson et al. 2006), just to name a few.

The sequencing of land plant genomes, coupled to microarray technology, has permitted to identify new gene families and to study their expression, but functional annotation of protein sequences is another current challenge. We report here the analysis of a new family of proteins that we discovered in silico during the annotation process of the *Populus trichocarpa* genome, released by the US Department of Energy Joint Genome Institute (http://genome.jgi-psf.org/Poptr1). Indeed, during the inventory and annotation of glutaredoxin (Grx) genes (Rouhier et al. 2006), we identified several additional genes, computer-tagged as glutaredoxin-like proteins (GRL) or as cysteine-rich glutaredoxins, due to a low homology to classical Grx. The motif enabling to classify them as GRL concerns few amino acids essentially present in the C-terminal part of the protein, but not the active site motif CxxC/S. After retrieving the gene and protein sequences, further investigations rather suggest that these proteins constitute an independent class of proteins that could be putative redox dependent enzymes based on the presence of repeated CxxC motives, somehow analogous to the nucleoredoxin or to the protein disulfide isomerase family. In order to obtain a more general view concerning the distribution of this protein family, homologous genes were searched in the various sequenced genomes. A complete classification of the plant proteins is proposed based on sequence and phylogenetic analyses.

Analysis of the GRL family in *Populus trichocarpa*

Using the sequences automatically annotated and additional blast searches, the entire gene family coding these related proteins is reported in *P. trichocarpa*. The main characteristic we looked for was the conservation of specific patterns in the C-terminal part that seem to be characteristic of this type of protein. We identified the GRL family as proteins constituted of eight strictly conserved cysteines, arranged in two $CxxCx_7CxxC$ clusters. Based on this motif, we have identified 19 putative GRL genes in the poplar genome, which are listed in Table 1. Based on the high homologies that several couple proteins display both in amino acid sequence and length, it is likely that seven of these sequences have been duplicated, indicating that there are in fact only 12 original sequences. As a

Table 1 Characteristics of the different glutaredoxin-like proteins (GRL) from *Populus trichocarpa* genome

Name	Gene model	Localization	TP length	Size (AA)	ESTs	At homolog	Os homolog	Number of exons
GRL1a	estExt_Genewise1_v1.C_LG_I7084	Chloroplastic	54	266	4	At5g13810	–	1
GRL1b	estExt_fgenesh4_pg.C_LG_III1668	Chloroplastic	54	259	4	At5g13810	–	1
GRL2a	eugene3.00011881	Chloroplastic	52	253	6	At5g58530	–	1
GRL2b	gw1.IX.2948.1	Chloroplastic	–	247	0	At5g58530	–	1
GRL3a	eugene3.00031141	Cytosolic	–	364	4	At1g64500	Os01g13480	1
GRL3b	gw1.I.8540.1	Cytosolic	–	362	1	At1g64500	Os01g13480	2
GRL4a	eugene3.00061459	Cytosolic	–	407	3	At5g03870	Os04g33680	1
GRL4b	gw1.XVI.2517.1	Cytosolic	–	412	0	At5g03870	Os04g33680	1
GRL5	estExt_fgenesh1_pm_v1.C_1550005	Secreted	25	112	7	At5g15802	Os03g30092	2
GRL6a	eugene3.00080131	Cytosolic	–	401	3	At5g01420	Os07g06600	1
GRL6b	gw1.X.3332.1	Cytosolic	–	379	0	At5g01420	Os07g06600	1
GRL7a	grail3.0030002601	Secreted	24	290	2	At5g06470	–	1
GRL7b	gw1.XVI.2146.1	Secreted	24	290	1	At5g06470	–	1
GRL8	gw1.I.5479.1	Chloroplastic	73	341	0	At4g10630	Os02g01200	1
GRL9a	gw1.XVI.1350.1	Cytosolic	–	412	4	At3g57070	Os03g24030	1
GRL9b	gw1.VI.1079.1	Cytosolic	–	413	4	At3g57070	Os03g24030	1
GRL10	gw1.XVII.1039.1	Mitochondrial	25	383	2	At3g28850	Os03g07470	2
GRL11	EstExt_fgenesh4_pg.C_LG_IX0141	Chloroplastic	20	162	7	At3g44020	Os01g70460.2	4
GRL12	Grail3.0041009401	Secreted	17	118	11	At1g22630	Os05g01530	4

Gene models and number of exons were found in the US Department of Energy Joint Genome database (http://www.jgi.doe.gov). Localization and transit peptide length were predicted using TargetP software (http://www.cbs.dtu.dk/services/TargetP). For GRL2b, the transit peptide length was omitted, since software prediction is probably not significant (5 amino acids length). *Arabidopsis* and rice homologs were found in the respective TIGR genomic databases (http://www.tigr.org)

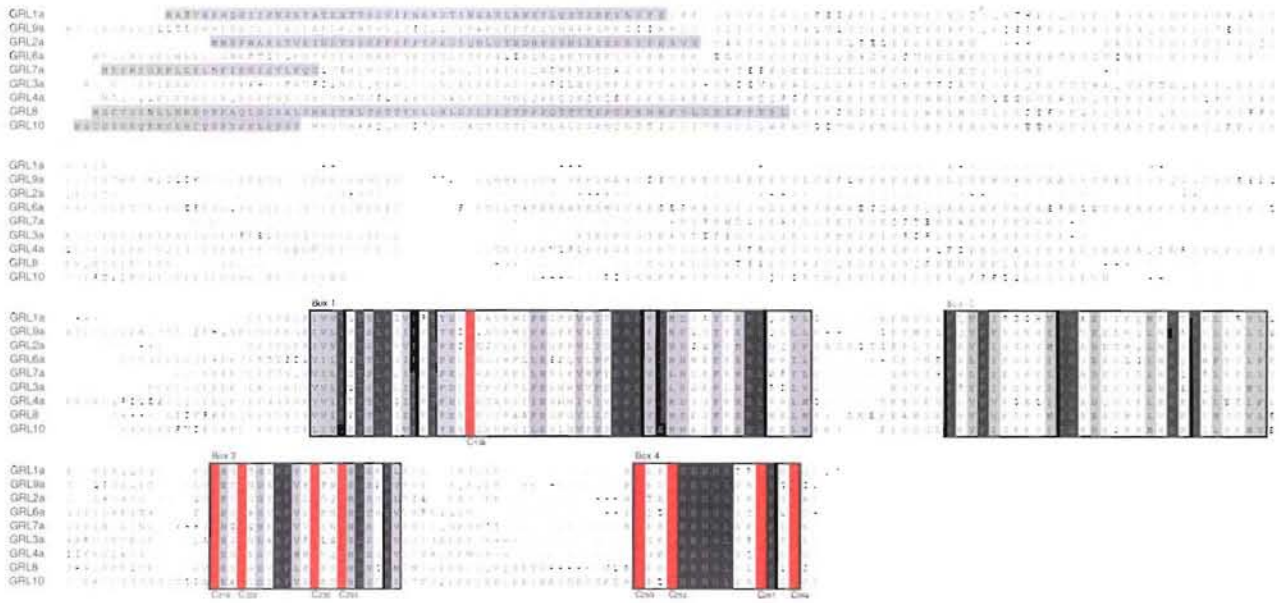

Fig. 1 Sequence alignment of type I poplar glutaredoxin-like proteins (GRLs). The alignment was performed using ClustalW. Close genes which are assumed to be duplicated are not indicated in this alignment. The main conserved regions are in boxes 1–4. Identical amino acids are figured on black background, similarity is figured on grey background. Predicted N-terminal signal peptides are on grey background. The nine conserved cysteines are in white font on red background and are numbered according to PtGRL 1a sequence

consequence 5 sequences (GRL 5, 8, 10 11 and 12) have not been duplicated in *P. trichocarpa*. The GRL amino acid sequences have been analyzed for the presence of putative N-terminal signal peptides and their characteristics are summarized in Table 1. We do not show here a complete sequence alignment of the whole poplar GRL family because the sequences of GRL5, 11 and 12 are very divergent outside the cysteine clusters and, in addition, they are shorter. These sequences have thus been omitted in the poplar sequence comparison shown in Fig. 1, but separate sequence comparisons are shown with their plant homologs in Fig. 2. Likewise, only 7 of the 14 genes derived from putative duplications are included in this sequence comparison.

All the GRL proteins, except GRL5, 11 and 12, form a subgroup that we call here type I GRLs (upper part of Fig. 3). Overall, the genes coding for type I GRLs are generally constituted of a single exon, except for isoforms 3b and 10, whose genes are encoded by two exons. We hypothesize that following the duplication event from GRL3, an intron has been acquired in GRL3b but not in GRL3a. The length of these proteins ranges from 247 to 413 amino acids, including an N-terminal putative signal peptide for some isoforms. According to software predictions, 6 isoforms are predicted to be chloroplastic (GRL 1a, 1b, 2a, 2b, 8), 2 to be secreted (GRL 7a, 7b) and 1 to be mitochondrial (GRL 10). The other isoforms are predicted to be devoid of signal peptide and thus to locate in the cytosol. The poor conservation in both homology and length in the N-terminal part of these proteins is striking, suggesting that these parts may not be essential to the protein function, but could give specificities to the different isoforms (Fig. 1). The only four regions shared without any gap and with substantial homology among the whole family are boxed in Fig. 1. In box 1, an interesting feature is the presence of a first conserved single cysteine (Cys 138 in GRL1a). The eight other conserved cysteines are arranged in two clusters in the C-terminal boxes 3 and 4, respectively, with the sequence $CxxCx_7CxxC$. The number of amino acids between the two clusters varies from 14 to 18 amino acids for most of the sequences, but in GRL8 and 10, the spacer region is 25 and 31 amino acid-long, respectively. In the seven amino acids between the two CxxC groups, the sequences share some homology. In the first cluster, an arginine and a phenylalanine at positions 226–227 are strictly conserved. Two other residues, a glycine and a lysine, are also conserved at positions 235 and 238. In the second cluster, the sequence NENGL starting at position 254 is strictly conserved in poplar, and strongly conserved in other organisms (see Fig. 4). A proline in position 262, adjacent to the first cysteine of the last CXXC motif, is conserved.

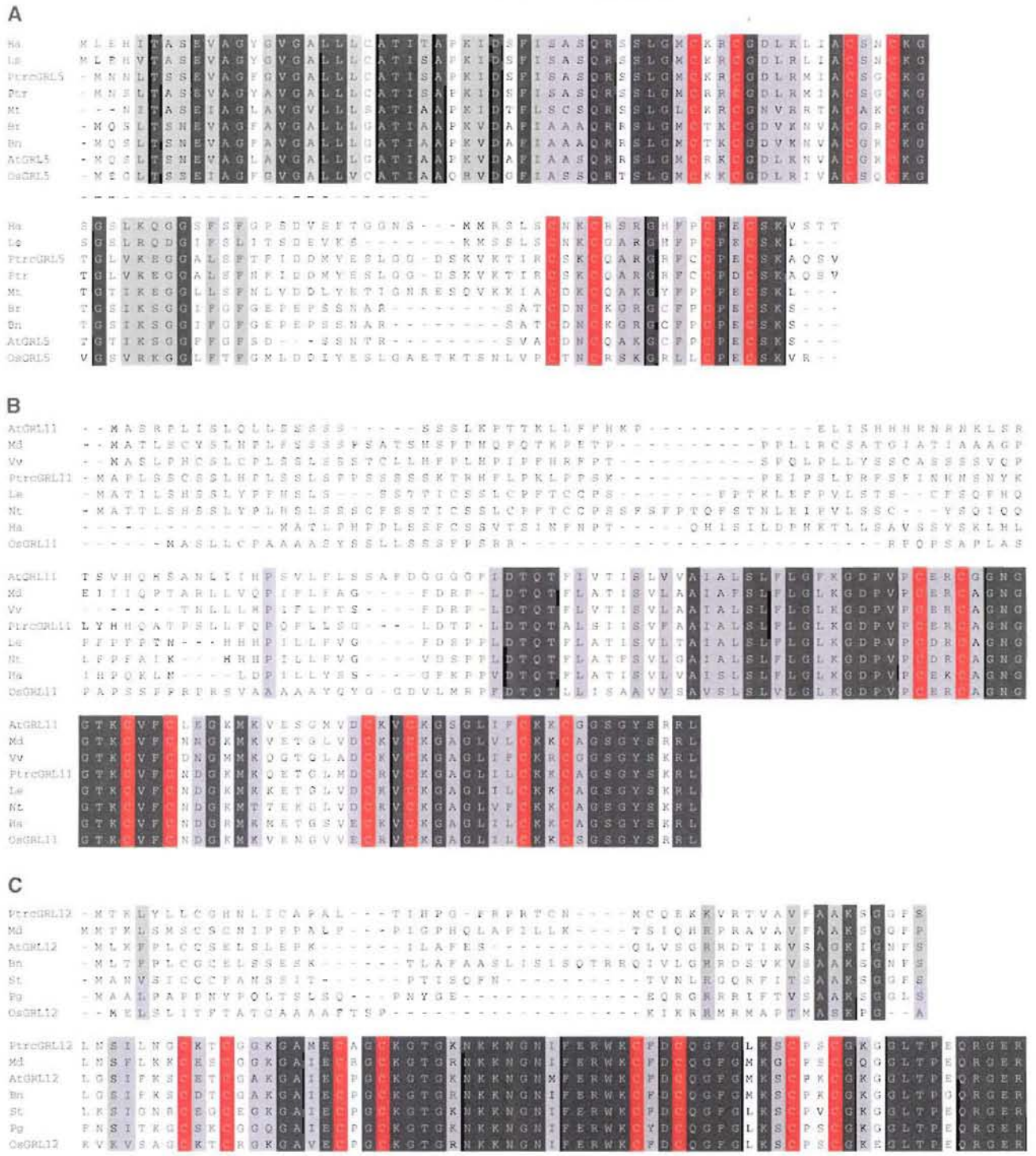

Fig. 2 Sequence alignments of type II and III glutaredoxin-like proteins (GRLs) from higher plants. The alignment was performed using ClustalW The GRL characteristic clustered cysteines are displayed on red background, identical residues on black background, and similarity on grey background. Gene models for *Populus trichocarpa* GRLs are listed in Table 1. (**A**) Poplar GRL 5 homologs: AtGRL 5, *Arabidopsis thaliana*, At5g15802; OsGRL 5, *Oryza sativa*, Os03g30092; Ha: *Helianthus annuus*, AJ828571; Ls: *Lactuca sativa*, DW084724; Ptr: *Populus tremula* × *tremuloides*, DT489588; Mt: *Medicago truncatula*, BE123933; Br: *Brassica rapa*, CX271064; Bn: *Brassica napus*, CD811743. Consensus signal peptide is underlined. (**B**) Poplar GRL 11 homologs: AtGRL 11, *Arabidopsis thaliana*, At3g44020; Md: *Malus domestica*; Vv: *Vitis vinifera*; Le: *Lycopersicon esculentum*; Nt: *Nicotiana tabacum*, EB429795; Ha: *Helianthus annuus*, DY925373; OsGRL 11, *Oryza sativa*, Os01g70460. (**C**) Poplar GRL12 homologs: AtGRL 12, *Arabidopsis thaliana*, At1g22630; Md: *Malus domestica*, EB150127; Bn: *Brassica napus*, CD820121; St: *Solanum tuberosum*, CV498905; Pg: *Panax ginseng*, DV553436; OsGRL 12, *Oryza sativa*, Os05g01530

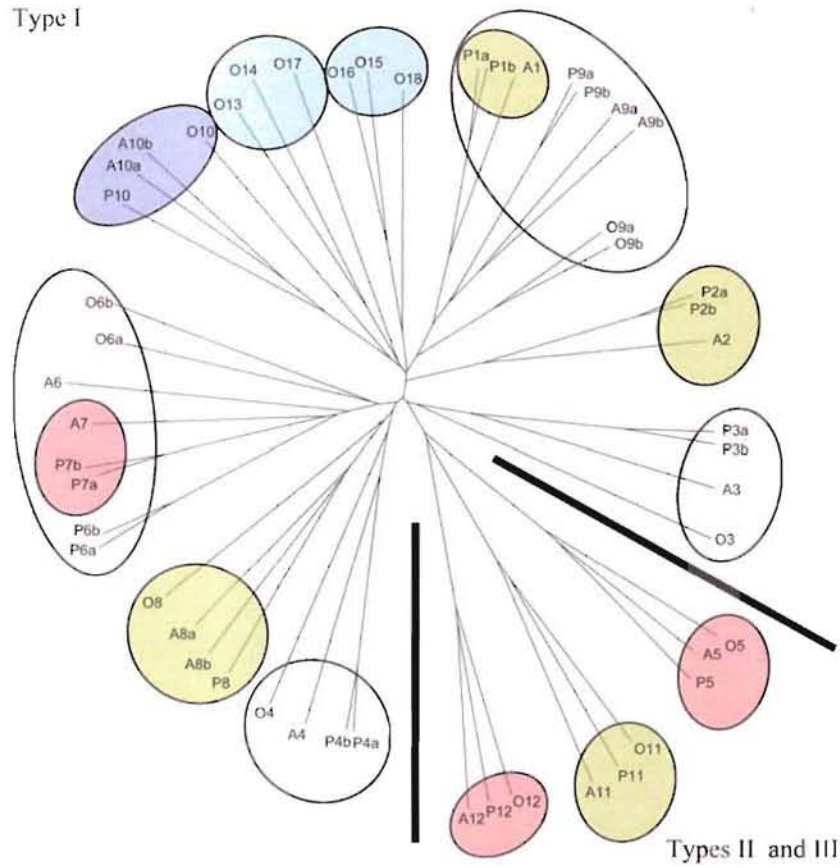

Fig. 3 Phylogenetic tree including the glutaredoxin-like protein (GRL) sequences from *Populus trichocarpa*, *Arabidopsis thaliana* and *Oryza sativa*. The tree was built using ClustalW and visualized using TreeView. Gene models numbers for poplar GRL (P1a to P12) are listed in Table 1. Other accession numbers are as follows: A1, At5g13810; A2, At5g58530; A3, At1g64500; A4, At5g03870; A5, At5g15802.1; A6, At5g01420; A7, At5g06470; A8a, At1g32760; A8b, At4g10630; A9a, At3g57070; A9b, At2g41330; A10a, At3g28850; A10b, At5g39865; A11, At3g44020; A12, At1g22630.1; O3, Os01g13480; O4, Os04g33680; O5, Os03g30092; O6a, Os07g06600; O6b, Os03g44650; O8, Os02g01200; O9a, Os03g24030; O9b, Os07g46570; O10, Os03g07470; O11, Os01g70460; O12, Os05g01530; O13, Os02g51370; O14, Os06g12190; O15, Os05g39450; O16, Os01g61350; O17, Os04g54860; O18, Os05g28530. The corresponding protein sequences are given as supplemental material. In green are the predicted chloroplastic isoforms, in red the secreted ones, in purple the mitochondrial ones, and in white the cytosolic ones. In blue are figured the two subgroups of rice-specific GRL proteins

The second type of GRL is represented by a single isoform, namely GRL 5. This isoform is shorter than the type I proteins with 112 amino acids length. It is encoded by a two- exon gene, and the corresponding protein is predicted to be secreted.

A third type includes isoforms GRL 11 and 12, which are also very different from the main GRL subgroup. Although coding for shorter proteins than those of type I, both genes are composed of four exons. GRL 11 is constituted of 162 amino acids and is predicted to be targeted to chloroplast, while GRL 12 (118 amino acids) is probably secreted. The mature proteins devoid of N-terminal extensions are constituted of 142 and 101 amino acids, respectively. Sequences of the type II and III are isolated subgroups, identified in the section located in the lower part of Fig. 3.

Homologs in other plants

As the genomes of two other model plants, *Arabidopsis thaliana* and *Oryza sativa* are available, we also performed an in-depth search for GRLs in these organisms using TIGR and MIPS databases (http://www.tigr.org) (Arabidopsis Genome Initiative 2000; International Rice Genome Sequencing Project 2005). A total of 15 and 17 homologous genes were found, respectively, in the *Arabidopsis* and rice genomes. In these genomes, the gene structure is similar to *P. trichocarpa* as all the genes encoding type I GRLs are constituted of a single exon, while the GRL 5, 11 and 12 genes contain 2, 4 and 4 exons, respectively. The only obvious exceptions, mentioned earlier, concerned GRL 3b and 10, which

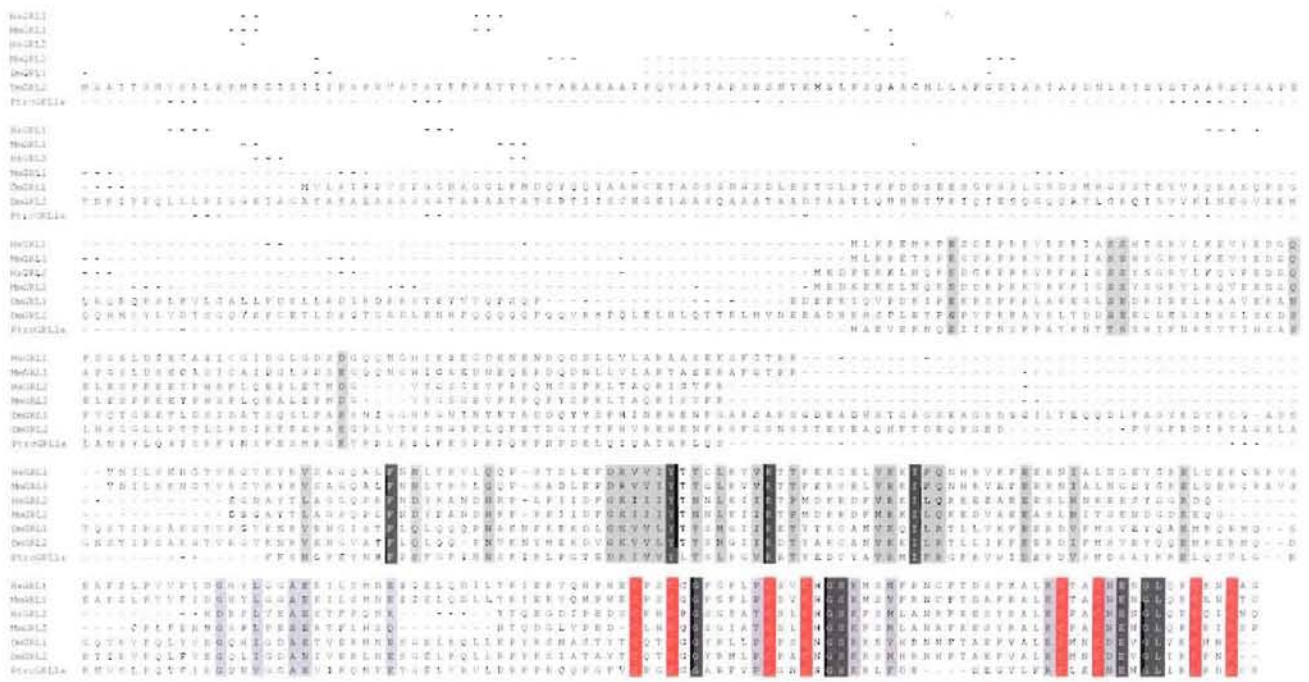

Fig. 4 Amino acid sequence comparison of glutaredoxin-like protein (GRL) from different kingdoms. The alignment was performed using ClustalW. The GRL characteristic clustered cysteines are displayed on red background, identical residues on black background, and functional homology on grey background. Genbank accession numbers are as follows: human HsGRL 1: XM_941109, HsGRL 2: XP_945052, mouse MmGRL1 XM_485620, MmGRL 2: NP_001028598, drosophila DmGRL 1: NP_731045, DmGRL 2: NP_726866, poplar PtrcGRL 1a: estExt_Genewise1_v1.C_LG_I7084

contain two exons in *P. trichocarpa*, but only one in *Arabidopsis* or rice. We did not find GLR homologs in the unicellular photosynthetic organism *Chlamydomonas reinhardtii*.

We built a phylogenetic tree using the GRL sequences from *Populus*, *Arabidopsis*, and *Oryza* (Fig. 3). In general, proteins which group into specific branches are homologous and possess the same predicted subcellular localization than the poplar proteins. Nevertheless, the distribution of the sequences in the various groups also depends on the organism considered as the sequences of *O. sativa* are generally more divergent, and they do not always group with poplar and *Arabidopsis* sequences. In addition, *O. sativa* possesses a specific set of additional GRLs compared with the two other organisms, these sequences were called OsGRL 13–18 (Fig. 3). On the contrary, no homolog of poplar GRL 1, 2 and 7 was found in *O. sativa*.

For the specific isoforms of type II and III GRL (GRL 5, 11 and 12), we found homologs in several higher plants (Fig. 2). The sequences are generally highly conserved in the C-terminal part, in the neighbourhood of the CxxC clusters. Nevertheless, some distinctions can be made. For the GRL 5 proteins, the putative secretion signal sequence is highly conserved, whereas the spacer region between the $CxxCx_7CxxC$ is poorly conserved (Fig. 2A). On the contrary, the N-terminal parts of GRL 11 and 12 are not conserved, whereas the C-terminal regions are (Fig. 2B, C). It is noticeable that poplar and Arabidopsis GRL 12 are predicted to be secreted, while all other sequences used in the alignment would rather be routed to plastids. All GRL 11 sequences are predicted to be chloroplastic.

Homologs in non-plant organisms

It seems that these proteins are also present in few other organisms, as we also found two isoforms belonging to the type I GRL in mammals (*Homo sapiens* and *Mus musculus*) and in arthropoda (*Drosophila melanogaster*). In Fig. 4, six isoforms from the organisms mentioned earlier are aligned with poplar GRL 1a. Although they belong to different kingdoms (i.e. mammals, arthropods and plants) and the length of some isoforms is very different (the Drosophila sequences are considerably longer), all these proteins share the eight clustered C-terminal cysteines. Apart from these 8 cysteines, some residues are also strictly conserved among these various

organisms. Thus, it would not be surprising to find in further studies that these residues are involved in protein activity or folding. Nevertheless, the ninth cysteine, corresponding to Cys138 in poplar type I GRLs, which was conserved in plants, is replaced by an arginine in two mammalian sequences (GRL 2 from *Homo sapiens* and *Mus musculus*). It is also noticeable that, except for poplar GRL 1a, the length of the spacer region between the two cysteine clusters is conserved. Finally, no protein equivalent to the shorter poplar GRLs (GRL 5, 11 or 12), was found by blasting the different organism databases available.

Expression studies

For *A. thaliana* genes, it is now possible, using microarray data and useful softwares such as Genevestigator, to follow the expression pattern of a specific gene family in different plant organs or in plants submitted to various environmental conditions (Zimmermann et al. 2004). We show here an expression profile for the different Arabidopsis GRL in the different plant organs (Fig. 5). The *A. thaliana* GRL (AtGRL) genes seem to be differentially expressed in several parts of the plant. AtGRL 1 is strongly expressed in all tissues analyzed. AtGRL 11 and 12 are expressed in differentiated green tissues, i.e. not in calli or roots. On the contrary, AtGRL 10a is expressed mostly in calli, roots and cell suspensions. When looking more carefully at these profiles, some genes are also very specifically expressed in particular organs and tissues. For example, AtGRL 1, 2 and 10a are strongly expressed (8079, 6192 and 12649 AU, respectively) in pollen (a subdivision of inflorescence) and AtGRL 3 (4088 AU) is very specific to cauline leaves (a subdivision of rosette) (data not shown). These informations have to be confirmed experimentally to become really physiologically relevant, but they can be helpful to understand the specificity or redundancy between these genes. For example, AtGRL 1 is strongly expressed in all plant parts and even more in pollen, unlike AtGRL 2 which has low levels of expression in all plant parts but in the pollen.

In a second step, we tried to evaluate the influence of growth conditions on the Arabidopsis GRLs transcript level using the Response Viewer tool of Genevestigator software. The different GRL transcript levels available were analyzed individually. AtGRL 1 and AtGRL 2 do not show particular induction or repression, with stress to control ratios between 0.37 and 2.76. On the contrary, AtGRL 3 transcription seems strongly influenced by the growth conditions. For example, it is repressed (ratio of 0.01–0.04) when the plant is in contact with pathogenic agents as *Myzus* or *Agrobacterium*. Different light qualities and anoxia also induce a strong expression of this gene, with a

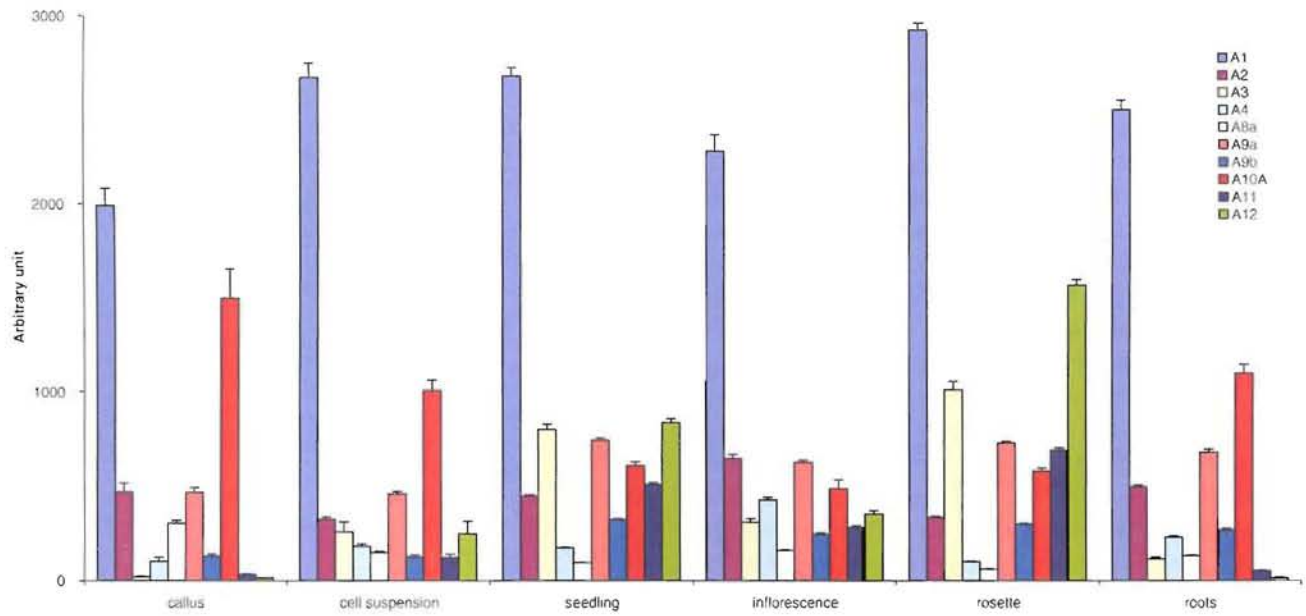

Fig. 5 Expression of the glutaredoxin-like protein (GRL) gene family in different *Arabidopsis thaliana* organs or tissues. Genevestigator GeneAtlas profiling was performed for the available genes based on the ATH1 chips (Affymetrix) available in the database. Some transcript levels are missing due to an absence of specific probe on these chips. Data are expressed in arbitrary unit corresponding to the expression values and standard errors according to the software settings. A1, At5g13810; A2, At5g58530; A3, At1g64500; A4, At5g03870; A8a, At1g32760; A9a, At3g57070; A9b, At2g41330; A10a, At3g28850; A11, At3g44020; A12, At1g22630

ratio between 5 and 13 in the first situation and a ratio of 8 in the second. AtGRL 4, AtGRL 8a are too weakly expressed to provide significant conclusions. AtGRL 9a and 9b are regulated in various conditions (ratio: 0.31–4.71) including pathogen infection, but their expression level is quite low (below 500 AU). The transcription of AtGRL 10a is slightly repressed in various light conditions, with a ratio down to 0.31, but more interestingly the expression of this gene is strongly induced (ratio of 17) by treatment with cycloheximide, an inhibitor of protein synthesis. Considering the shorter GRL isoforms, AtGRL 11 is poorly expressed and moderately regulated in stress conditions (ratio: 0.09–2.02). Finally, AtGRL 12 is likely to be regulated in various conditions, in particular it could be repressed upon pathogen infection, for example by *Agrobacterium* (ratio of 0.05). As a conclusion, even if experimental data are still required, it is likely that the GRL genes are not equally represented at the transcript level and that their transcription can be regulated according to the plant growth conditions. Thus, GRL proteins could potentially play a role in the plant development and in its response to changing conditions.

Concluding remarks

Software analyses presented the family of proteins described here as GRL, which is a quite teasing name, but in this case rather misleading. This genomic sequence analysis indicates that the GRL family is quite large, as it is constituted of three subgroups with at least 12 different isoforms in poplar. Compared to rice proteins, which are slightly divergent, poplar and Arabidopsis GRL families are more closely related, based on the phylogenetic tree and percentage identities, this being in agreement with the closer relationship of the two dicotyledonous species. This type of protein is likely to be widespread in the plant kingdom, with large GRL families in Arabidopsis and rice genomes (see sequences in Figs. 2, 3). In addition, EST sequences corresponding to these proteins are widely present in the databases and thus most of these genes are presumably expressed in plant cells. Nevertheless, their role is still unknown, and their characterization just started. An in silico analysis of expression data indicates that some of these proteins could be involved in the response to pathogen infection or to changing light conditions. From sequence analysis, a role of these proteins in redox processes is possible based on the presence of repeated CxxC motives. Nevertheless, the arrangement of the conserved cysteines also presents a low sequence homology with DnaJ chaperones and may be also a signature of zinc-finger domain. It is especially true for isoforms 5, 11 and 12, which share conserved residues with some DnaJ family members. They contain, respectively, 1, 3 and 2 DnaJ zinc-finger patterns in their sequence, i.e. amino acids CxxCxGxG (Miernyk 2001). On the other hand, their sequences differ considerably when other DnaJ characteristics are considered, and especially they do not possess the HPD peptide signature. Another putative function of these clusters may be to bind an iron-sulfur cluster, as is the case for some plant and mammals glutaredoxins (Lillig et al. 2005; Feng et al. 2006; Rouhier et al. unpublished results) or other metals such as the zinc ions bound to methionine sulfoxide reductase B using two CxxC motives (Kumar et al. 2002).

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3 Les protéines cibles des systèmes thiol-dépendants

Depuis leur découverte, il a été démontré que les Trx régulent ou servent de donneur de pouvoir réducteur à de nombreuses enzymes, incluant certaines protéines spécifiques du métabolisme végétal. Parmi les protéines particulièrement bien caractérisées figurent la ribonucléotide réductase (RNR), la fructose-bis-phosphatase (FBPase), la malate déshydrogénase à NADP (NADP-MDH) ou encore plus récemment les méthionine sulfoxyde réductases (MSR) et les péroxyrédoxines (Prx). Les thiorédoxines interagissent aussi avec des facteurs de transcription induisant la transcription de gènes impliqués dans les systèmes antioxydants, tels que le gène codant une MSR de type A chez la levure (Hanbauer et Moscovitz 2006). Les détails moléculaires de l'une de ces interactions Trx-facteur de transcription sont bien connus pour le système NF kappa B-Trx (Qin et al 1995).

L'inventaire des protéines interagissant avec les systèmes Trx ou Grx est devenu un sujet d'intenses recherches depuis quelques années, avec l'émergence de techniques permettant un traitement de masse des protéines, couplé à une identification relativement aisée. Ces techniques reposent notamment sur l'utilisation de mutants monocystéiniques, qui permettent de retenir des protéines potentiellement capables d'être réduites par les Trx (Balmer et al 2003). Un marquage des protéines par des marqueurs spécifiques des thiols est aussi couramment utilisé, en association avec une électrophorèse bidimensionnelle (Wong et al 2002). Enfin, des techniques utilisant du GSH radiomarqué ou biotinylé permettent d'identifier les protéines glutathionylées, qui sont des cibles potentielles des Grx (Ito et al 2003). Ces différentes techniques donnent des listes imposantes

de protéines, dont une petite partie était déjà connue. Des cibles spécifiques de certains organites comme le chloroplaste ou la mitochondrie (Balmer et al 2003, Balmer et al 2004, Millar et al 2005) ou de certains stades de développement, comme la germination (Wong et al 2003, 2004, Maeda et al 2004) ont été identifiées. D'autres travaux se sont intéressés à l'identification des protéines cibles de l'oxydation dans les cellules (Møller et Kristensen 2004). Ainsi, parmi ces cibles potentielles s'ouvrent de nouvelles pistes de recherche, avec des protéines non identifiées auparavant. C'est le cas par exemple de certaines kinases, comme les nucléoside diphosphate kinases (NDK), qui sont en cours d'étude au laboratoire. Si la régulation de ces enzymes de manière redox était avérée, elle constituerait un nouveau lien, en plus des interactions ROS-MAP kinases, entre les voies de régulation redox et celles des phosphorylations. Les cibles identifiées doivent ensuite faire l'objet d'analyses plus spécifiques, qui visent à confirmer la possibilité et la nature de ces interactions, par analyse biochimique, par co-immunoprécipitation, par complémentation fonctionnelle dans la levure, ou encore par des systèmes « double-hybride » dans la levure (Vignols et al 2005). On entre alors dans une phase d'études post-protéomiques, qui vont chercher à établir la réalité et les caractéristiques de l'interaction enzyme-cible (Verdoucq et al 1999). Les interactions entre différents groupes de peroxydases thiol-dépendantes et leurs réducteurs ont été particulièrement étudiées ces dernières années. Parmi ces peroxydases, on peut distinguer deux familles de protéines divergentes au niveau de leur structure primaire, d'une part les peroxyrédoxines (Prx), et d'autre part les glutathion peroxydases (Gpx).

a- Les peroxyrédoxines (Prx)

Les peroxyrédoxines sont des peroxydases non-hémiques de faible masse moléculaire, généralement autour de 20 kDa. Elles sont présentes chez la plupart des organismes, et chez les plantes, il en existe de nombreuses isoformes. Par exemple, il existe 10 gènes codant pour des Prx chez *Arabidopsis thaliana*, 9 chez le peuplier et 9 chez le riz (Dietz et al 2006, Rouhier et Jacquot 2005, Rouhier, données non publiées). Chez les plantes, elles sont groupées en 4 types, suivant leur séquence primaire et la position des cystéines conservées. Les Prx à une cystéine conservée (Prx 1-Cys) sont les seules à posséder une seule cystéine conservée. Les Prx des autres classes ont deux cystéines conservées. Parmi elles, on trouve les Prx 2-Cys, les Prx de type II et les Prx Q. Les différentes classes de Prx ont des mécanismes réactionnels spécifiques mais partant d'une étape commune, dans laquelle le substrat va réagir avec la cystéine conservée N-terminale, appelée cystéine catalytique ou peroxidasique, pour former un acide sulfénique, qui sera par la suite régénéré en thiol.

Les Prx à une cystéine conservée, ou Prx 1-Cys sont les moins bien caractérisées biochimiquement pour le moment. Elles sont principalement localisées dans le noyau des cellules, mais leur mécanisme de régénération n'a pas encore été démontrée expérimentalement. On suppose que l'acide sulfénique formé lors de la catalyse est directement attaqué par un composé ou une protéine possédant au moins un groupement thiol (GSH, Grx, Trx). L'influence de la glutathionylation de la cystéine catalytique sur la structure oligomérique d'une Prx II de peuplier, qui pourrait appartenir aux Prx 1-Cys, a par ailleurs été récemment démontrée (Noguera et al 2006).

Les Prx 2-Cys dites à 2 cystéines « classiques », fonctionnent sous forme d'homodimères. La cystéine C-terminale d'un monomère va réagir avec l'acide sulfénique du monomère associé pour former dans ce cas un pont disulfure intermoléculaire (König et al 2003). Deux isoformes de cette catégorie sont présentes dans les chloroplastes chez l'arabette et le peuplier.

Les Prx de type II sont les plus représentées en terme de gènes chez les plantes supérieures. Chez l'arabette, il en existe 6 isoformes, nommées Prx II A à F, mais l'une d'entre elle (Prx IIA) ne semble pas exprimée. Certaines Prx de type II sont réduites à la fois par des Trx et des Grx, c'est le cas de la Prx IIB de peuplier (Rouhier et al 2001, 2002), alors que la Prx IIB d'*A. thaliana* ne semble réduite que par des Grx (Brehelin et al 2003). D'autres, les Prx IIF de peuplier et d'*Arabidopsis thaliana*, semblent pouvoir en plus accepter directement le GSH comme réducteur (Gama et al sous presse, Finkemeier et al 2005). Toutefois, ces études ne testent généralement pas toutes les isoformes existantes et il est par conséquent très difficile d'aboutir à des conclusions fermes. La régénération de ces enzymes s'effectue vraisemblablement directement par réaction du groupement sulfényl (-SOH) soit avec les Trx, soit avec le glutathion, soit avec les Grx, sans passer par la formation d'un pont disulfure intramoléculaire. En effet, bien que très conservée, une deuxième cystéine située 25 acides aminés après la cystéine peroxidasique est absente chez certains organismes, ou n'intervient pas dans le mécanisme catalytique (Seo et al 2000). De plus, le remplacement de cette cystéine par une sérine dans une Prx végétale n'abolit pas complètement l'activité de la protéine (Rouhier et al 2002). Enfin, la distance observée entre les cystéines de la Prx IIB de peuplier, bien que résolue sous forme réduite, n'est pas en faveur d'une réaction possible entre les cystéines (même si des réarrangements structuraux sont

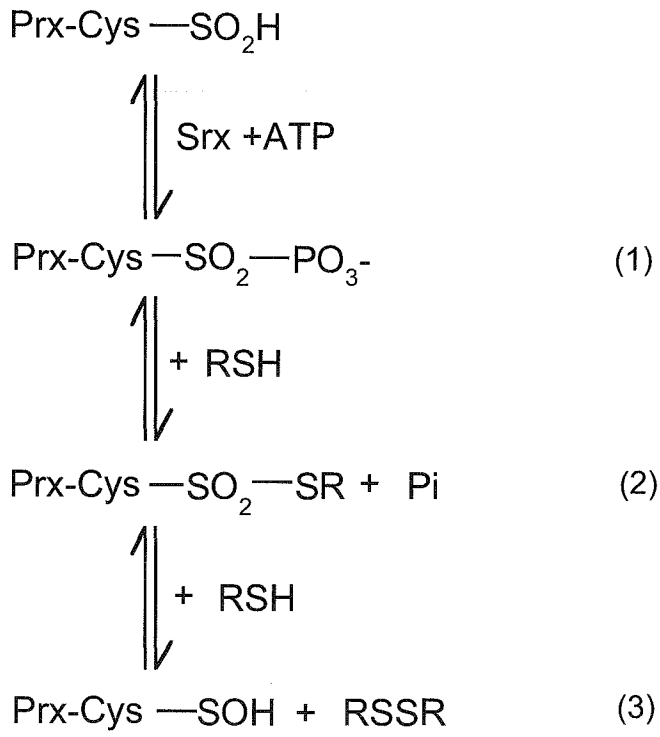

Figure 9: Schéma du mécanisme de réduction de la Prx superoxydée sous forme acide sulfinique en acide sulfénique par la sulfirédoxine (d'après Jeong et al 2006). La Srx ayant fixé un groupement phosphoryl va le transférer à la Prx pour former un ester phosphoryl sulfinique (1). Cet ester est ensuite converti en monoxyde disulfide par réaction avec un thiol en solution, qui va libérer un groupement phosphate (2). La réaction de ce monoxyde avec un second thiol en solution va aboutir à la formation d'un dithiol oxydé et de la Prx sous forme acide sulfénique (3), qui sera réduite ultérieurement par les voies thiol-dépendantes classiques.

évidemment possibles dans une forme oxydée). De ce point de vue, ces protéines devraient appartenir aux Prx à 1 cystéine conservée.

Enfin, il existe une forme atypique de Prx à deux cystéines, la Prx Q, qui est chloroplastique. Elle est attachée aux thylacoïdes et semble jouer un rôle dans la protection des photosystèmes (Lamkemeyer et al 2006). Pour leur mécanisme réactionnel, les Prx Q sont actives sous forme de monomères, et la seconde cystéine conservée va réagir avec la fonction SOH formé au niveau de la cystéine catalytique, générant un pont disulfure intramoléculaire, qui sera ensuite réduit spécifiquement par les Trx, mais pas le glutathion ou les glutarédoxines (Collin et al 2004, Rouhier et al 2004a).

De manière générale, les Trx, Grx ainsi que les protéines CDSP32 et cyclophilines peuvent réduire les ponts disulfures au sein des Prx, avec des affinités variables suivant les isoformes, donnant parfois une indication sur le donneur le plus probablement impliqué *in vivo* (Collin et al 2003).

Des études portant sur les Prx 2-Cys humaines ont montré que l'oxydation de Prx par une concentration élevée de peroxydes aboutit à l'inactivation de l'enzyme due à la formation d'un acide sulfinique au niveau de la cystéine catalytique de l'enzyme (Rabilloud et al 2002, Yang et al 2002). Cette inactivation fut d'abord considérée comme irréversible mais récemment de nouvelles enzymes ATP-dépendante, la sulfirédoxine (Srx) et la sestrine, ont été mises en évidence chez la levure, chez les mammifères ou encore dans les chloroplastes des plantes. Elle sont capables de réduire l'acide sulfinique des Prx en acide sulfénique, qui serait réduit par la suite via les Trx ou le GSH (Biteau et al 2003, Budanov et al 2004, Liu et al 2006). Le mécanisme de cette réaction a été décrit plus récemment pour l'isoforme humaine de la Srx (Jonsson et al 2005, Jeong et al 2006). Il est

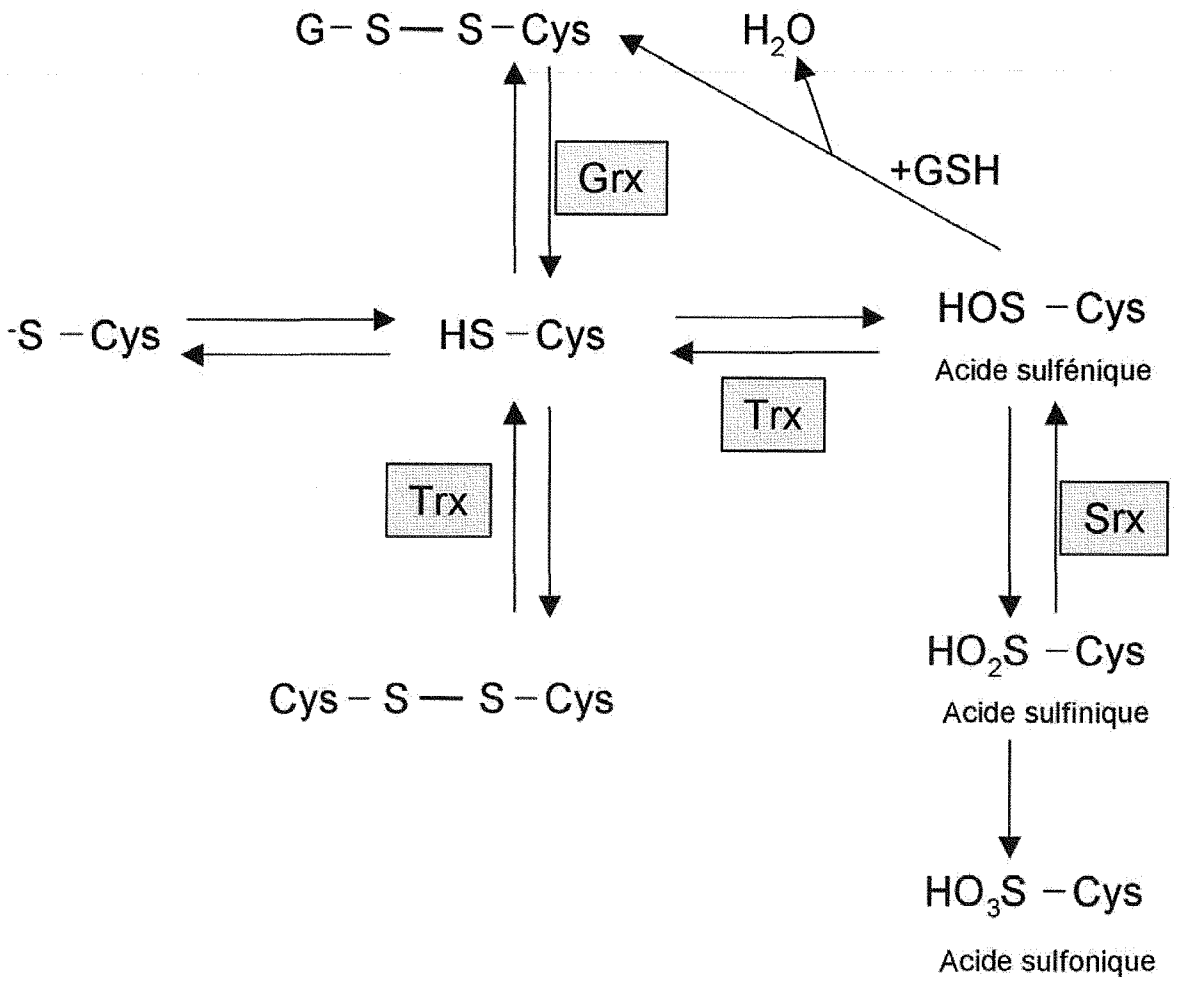

Figure 10: Schéma des principaux états d'oxydation de la cystéine. Les enzymes impliquées sont encadrées. Trx : thiorédoxine, Grx : glutarédoxine, Srx : sulfirédoxine. GS : groupement glutathionyle, GSH : glutathion.

détaillé dans la figure 9. En cas d'oxydation encore plus importante, la cystéine passe sous forme d'acide sulfonique, cette dernière réaction étant irréversible (Figure 10). Un rôle important des Prx pourrait être la régulation de la concentration en ROS dans la cellule, tant qu'elles restent sous forme catalytiquement active. Lorsque les concentrations en oxydants augmentent, les Prx seraient réversiblement inactivées, d'autres systèmes prenant alors éventuellement le relais. C'est cette sensibilité à l'oxydation qui permettrait aux Prx de jouer un rôle dans la transduction des signaux. Wood et al (2003) suggèrent que les Prx joueraient un rôle d'élimination des ROS jusqu'à une certaine concentration seuil, au-delà de laquelle elles seraient inactivées par suroxydation, permettant aux ROS d'activer différentes voies de réponses cellulaires, notamment via des facteurs de transcription. Cette théorie du « floodgate » est renforcée par la possibilité de réduction des acides sulfiniques formés sur les Prx *in vivo*, et donc la réactivation de celles-ci une fois un épisode de stress oxydatif aigü terminé (Georgiou et Masip 2003). Toutefois des questions demeurent concernant cette régulation, et chez *Schizosaccharomyces pombe*, ce système semble aussi être impliqué dans la régulation de la transcription de certains gènes en réponse à de faibles concentrations en H_2O_2 via la régulation de la protéine AP1 (Vivancos et al 2005).

b- Les glutathion peroxydases (Gpx)

Les Gpx chez les mammifères

Les glutathion peroxydases (Gpx) constituent la principale voie de détoxification des ROS chez les animaux (Maiorino et al 1990, 1991). Il en existe au

moins six isoformes, et quatre d'entre elles possèdent une sélénocystéine (Sec) dans leur site actif (Brigelius-Flohe 1999). Cet acide aminé atypique est identique à la cystéine mais il possède un atome de sélénium à la place d'un atome de soufre dans la chaîne latérale. Il est incorporé dans les protéines grâce à un codon UGA, combiné à une machinerie spécifique d'incorporation. Celle-ci est composée d'une séquence nucléotidique spécifique, nommée séquence d'insertion de la sélénocystéine (SECIS) qui va former une structure de type tige-boucle à proximité de la séquence codante sur l'ARNm correspondant à la protéine. Des protéines spécifiques vont ensuite reconnaître cette structure et incorporer la Sec au niveau du codon UGA (Low and Berry 1996). Cette machinerie a été mise en évidence chez les animaux, chez certains procaryotes ainsi que chez *Chlamydomonas reinhardtii* (Novoselov et al 2002). Pour le moment, aucune machinerie ni sélénoprotéines ne sont connues chez les végétaux supérieurs. La sélénocystéine a une très forte électronégativité et un pKa plus bas (5,2) que celui de la cystéine (8). La présence de cet acide aminé atypique va conférer aux Gpx à sélénium une plus grande efficacité catalytique pour la réduction des peroxydes. Ces enzymes ont fait l'objet d'intenses recherches dans le domaine médical ces vingt dernières années, et leur importance est cruciale en tant qu'antioxydants mais aussi en tant que catalyseurs de l'agrégation de protéines riches en cystéine au cours de la formation de la capsule mitochondriale des spermatozoïdes (Maiorino et al 2005). Ces enzymes ont aussi permis de mettre en évidence et d'évaluer la nécessité de l'apport en sélénium pour les animaux (Arthur 2000). Parmi les Gpx animales à sélénocystéine, les Gpx1 à 3 sont tétramériques, tandis que la Gpx4, aussi nommée Phospholipid Hydroperoxyde Gpx (PHGpx) est monomérique. Cette dernière enzyme a un rôle d'élimination des peroxydes, et notamment des

peroxydes de phospholipides au niveau des membranes (Thomas et al 1990). Il est à noter qu'en plus de l'activité GSH-dépendante, il a été montré que la Gpx3 humaine peut fonctionner en utilisant le pouvoir réducteur des systèmes Grx et Trx (Björnstedt et al 1994). Néanmoins, cette activité a fait l'objet d'assez peu d'études. La Gpx5, dépourvue de sélénocystéine, est localisée dans l'épididyme et les spermatozoïdes de mammifères (Drevet 2006). C'est une enzyme dimérique, qui possède une activité de réduction de l'H_2O_2 et joue un rôle important dans la protection de ces tissus en conditions de stress oxydant (Vernet et al 1996, 1999). D'autres protéines Gpx-like, ne contenant pas de sélénocystéine, ont aussi été identifiées, dans l'oeil de boeuf (Singh et Shichi 1998) ou encore dans le système olfactif de mammifères (Dear et al 1991).

Les recherches sur les Gpx, notamment en médecine, ont aussi permis d'identifier ces protéines chez de nombreux microorganismes, pathogènes ou non, et de caractériser leur fonctionnement enzymatique, avec pour objectif d'évaluer leur potentiel en tant que cibles thérapeutiques. Une isoforme de Gpx, similaire à la PHGpx humaine, a ainsi été caractérisée chez le pathogène *Schistosoma mansoni*, qui contient une sélénocystéine dans son site actif (Maiorino et al 1996). Par contre, chez *Plasmodium falciparum*, une protéine homologue à la PHGpx humaine contient une cystéine à la place de la sélénocystéine typique, et montre une activité de réduction de certains hydroperoxydes qui est Trx-dépendante (Sztajer et al 2001).

Les Gpx de bactéries et champignons

Il existe encore peu d'études portant sur les homologues des Gpx de mammifères chez les bactéries, ou chez les champignons, levure exceptée. Des recherches dans les génomes et les bases de données EST montrent que de nombreux genres bactériens, comme *Escherichia coli* possédent une Gpx, qui contient trois cystéines conservées en général, et deux chez certains genres (Yersinia, Xanthomonas par exemple). Les propriétés de ces enzymes et leur rôle sont encore peu connus. Chez les champignons, il existe aussi une Gpx, deux chez certaines espèces comme *Cryptococcus neoformans* (Missall et al 2005). Là aussi, très peu de travaux sur ces enzymes ot été publiés. Seules les Gpx de levure, au nombre de trois, ont été extensivement étudiées. Les Gpx bactériennes et fongiques sont plus proches de celles de la levure que des Gpx de mammifères.

Les Gpx de levure

Dans la levure, trois isoformes de Gpx ont été découvertes, et leurs séquences se rapprochent de celle de la PHGpx humaine (Inoue et al 1999, Avery et Avery 2001). Ces enzymes sont toutes des formes dépourvues de sélénocystéine, qui est remplacée par une cystéine conservée dans le site actif. L'expression des isoformes Gpx1 et Gpx2 est induite par un stress oxydant alors que Gpx3 est constitutivement et fortement exprimée. De plus, l'expression de Gpx2 est sous le contrôle du facteur de transcription Yap1 (Inoue et al 1999). Plus récemment, il a été montré que la Gpx2 de levure possède une activité de réduction de peroxydes importante lorsqu'elle utilise la Trx comme donneur d'électrons. En revanche, contrairement à ce qui est observé chez les Gpx animales à sélénocystéine, cette activité est très faible lorsque le GSH est utilisé (Tanaka et al

2005). Ceci a amené à reconsidérer ces protéines à cystéine, et à les ranger avec les Prx parmi les peroxydases Trx-dépendantes. Delaunay et al (2002) ont par ailleurs montré que Gpx3 participe à la transduction du signal en cas de stress oxydant. Sous forme oxydée, Gpx3 va induire la formation d'un pont disulfure intramoléculaire dans le facteur de transcription Yap1, le rendant actif et capable de s'accumuler dans le noyau de la cellule où il va induire l'expression de certains gènes.

Les Gpx d'organismes photosynthétiques

Unicellulaires

Les Gpx sont aussi présentes chez les organismes photosynthétiques, des cyanobactéries jusqu'aux plantes supérieures. Chez le protiste photosynthétique *Euglena gracilis*, une Gpx, dépourvue de sélénium, a été mise en évidence (Overbaugh et al 1985). Chez la cyanobactérie *Synechocystis* PCC 6803, deux Gpx ont été isolées. Elles ne contiennent pas de sélénocystéine, et réduisent différents types de peroxydes dont des peroxydes de phospholipides. Ces enzymes ne sont pas capables d'utiliser le GSH comme réducteur mais, de manière surprenante, elles sont efficacement réduites par le NADPH (Gaber et al 2001). Il a aussi été montré que la transcription des gènes codant pour ces deux Gpx est induite en conditions de stress, comme une forte lumière ou une salinité élevée (Gaber et al 2004). Chez l'algue unicellulaire *Chlamydomonas reinhardtii*, une isoforme de Gpx a été isolée, et il a été montré que cette protéine est une isoforme contenant une sélénocystéine dans son site actif (Shigeoka et al 1991, Fu et al 2002). La présence

d'isoformes à cystéine est aussi vraisemblable, comme cela a été montré chez l'espèce halotolérante *Chlamydomonas* W80 (Takeda et al 2003), et confirmé par l'analyse du génome de *C. reinhardtii* (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html). Il semble cependant que l'existence de sélénoprotéines chez les organismes photosynthétiques se limite à ces seules algues vertes.

Plantes supérieures

A partir des informations disponibles, notamment la présence d'activité glutathion peroxydase dans des tissus végétaux (Drotar et al 1985), et au vu de l'importance des Gpx chez les mammifères, des équipes de chercheurs en biologie végétale ont recherché des homologues de ces protéines chez les plantes supérieures. Dès 1992, Criqui et al mettaient en évidence un gène homologue aux Gpx animales et son expression dans des protoplastes ainsi que différents organes de tabac. D'autres gènes homologues de l'isoforme PHGpx humaine furent par la suite mis en évidence chez différentes plantes, comme chez le riz (Li et al 2000, Kang et al 2003). En 1995, une Gpx végétale, nommée Cit-SAP (Citrus Salt Associated Protein) a été purifiée pour la première fois à partir de cultures de cellules de *Citrus sinensis* tolérantes au stress salin, et son activité de réduction des peroxydes démontrée *in vitro* (Beeor-Tzahar et al 1995). Cette activité était d'autant plus délicate à mettre en évidence qu'il était déjà connu que certaines glutathion-S-transférases (GST) de plantes peuvent aussi montrer une activité de ce type (Bartling et al 1993). La faible activité GSH-dépendante de cette Gpx végétale, expliquée alors par la présence d'une cystéine classique dans le site actif, laissait

planer un doute sur l'implication de cette famille de protéines dans l'élimination des peroxydes *in vivo* (Eshdat et al 1997). Les résultats des études au niveau de la transcription des gènes de Gpx végétales ont montré que l'expression de ces gènes est induite par de nombreux types de stress différents, notamment stress salin chez *Citrus sinensis* (Beeor-Tzahar et al 1995), stress mécanique au niveau des tiges de tomate (Depège et al 1998), stress oxydant chez l'orge (Churin et al 1999), stress métallique chez *Arabidopsis thaliana* (Sugimoto et Sakamoto 1997), stress biotique chez le tournesol (Roeckel-Drevet et al 1998). L'influence de la lumière, des kinases, ainsi que de substances de type hormone (acide jasmonique, acide abcissique), ou encore l'interaction avec un pathogène (*Magnaporthe grisae*) jouent sur le taux de transcrits d'une Gpx de riz (Agrawal et al 2002). Des influences semblables (kinases, hormones), ainsi que d'autres dues à l'infection par un champignon pathogène (*Plasmopara*) ou à l'oxyde nitrique ont aussi été mises en évidence chez le tournesol (Herbette et al 2003). Ainsi, l'expression de ces gènes de Gpx végétales est induite en conditions de stress et ces protéines ont certainement un rôle dans la défense cellulaire contre le stress oxydant (Rodriguez-Milla et al 2003). Il a été montré que la surexpression de gènes de Gpx dans la levure ou dans des cellules de tabac augmente leur tolérance au stress oxydant causé par différents agents (Chen et al 2004, Yoshimura et al 2004).

Grâce à la production de protéines recombinantes, d'autres études ont consisté à tester *in vitro* l'efficacité de nombreux réducteurs sur ces protéines. Herbette et al (2002) ont montré que deux Gpx de tomate et de tournesol pouvaient réduire des peroxydes de nature variée en utilisant le GSH, mais aussi la Trx d'*E. coli*, cette activité Trx-dépendante étant d'ailleurs plus importante que l'activité GSH-dépendante. Jung et al (2002) ont caractérisé une enzyme de type Gpx chez

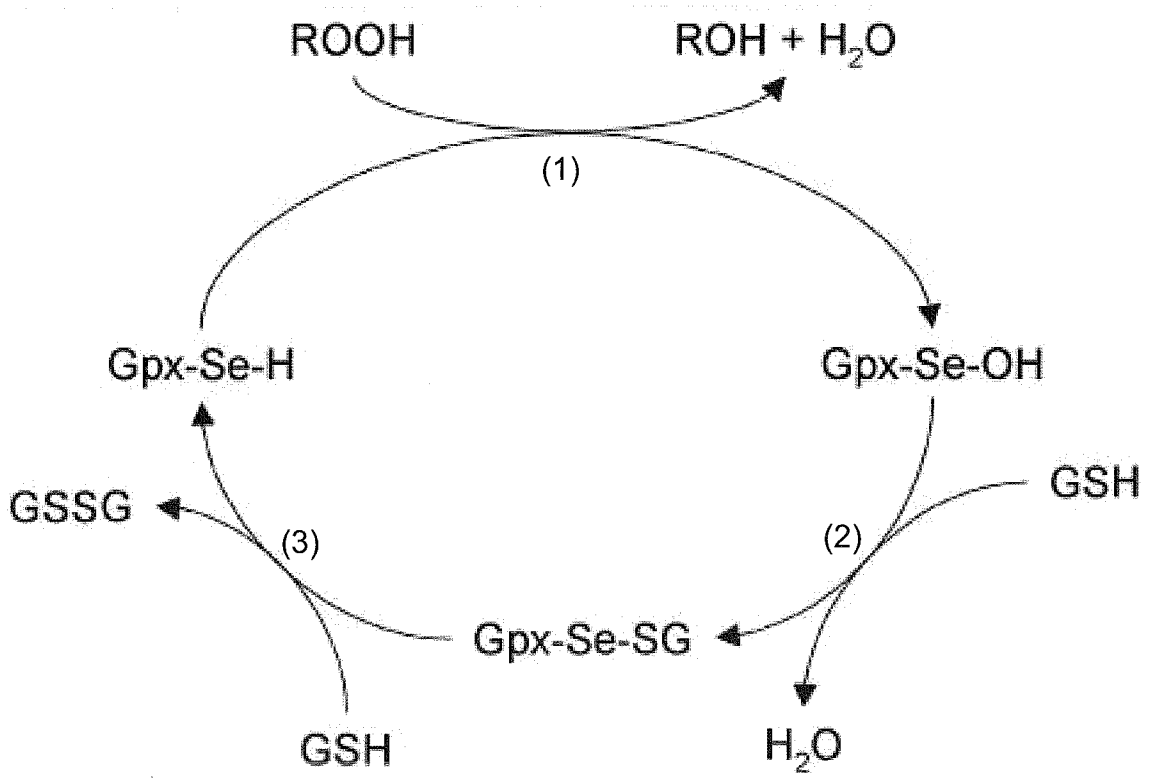

Figure 11. Mécanisme réactionnel de la Gpx humaine, d'après Prabhakar et al (2005). Lors de la réaction (1), le peroxyde ROOH est réduit en alcool correspondant ROH, avec formation d'un acide sélénénique au niveau de la cystéine catalytique de la Gpx. La protéine va ensuite fixer une molécule de glutathion réduit GSH, pour former un sélénosulfide (2). dans l'étape (3), une deuxième molécule de GSH va attaquer le sélénosulfide pour régénérer la Gpx sous forme réduite et active. Globalement, deux molécules de GSH sont consommées et deux molécules d'eau produites par cycle de l'enzyme.

le chou, et ont montré que son activité était strictement dépendante de la Trx d'*E. coli in vitro*, et faisait vraisemblablement intervenir les 3 cystéines conservées présentes chez les Gpx végétales. Ainsi, le mécanisme biochimique de la réduction des peroxydes par les Gpx végétales serait différent de celui des Gpx animales (Figure 11), déjà connu et récemment réexaminé en détail (Prabakhar et al 2005). Cela a été montré indirectement lors du remplacement dans une Gpx de *Citrus sinensis* de la cystéine conservée chez les plantes par la sélénocystéine typique des Gpx animales. Cette substitution a induit une augmentation de l'activité enzymatique, mais elle reste très inférieure à l'activité des enzymes animales (Hazebrouck et al 2000).

La croissance exponentielle du nombre de séquences nucléotidiques disponibles, sous la forme d'ESTs d'abord, puis de génomes complets, a donné de nouveaux moyens d'analyse des gènes présents chez les plantes, aboutissant, comme pour de nombreuses autres enzymes (Trx, Grx, Apx), à la découverte de familles multigéniques. Ainsi, chez *Arabidopsis thaliana*, une famille de sept gènes a été mise en évidence par des recherches *in silico* (Rodriguez-Milla et al 2003). Un huitième gène a ensuite été ajouté à cet inventaire (Rouhier & Jacquot 2005). L'expression de ces différents gènes est variable selon l'isoforme considérée, et selon l'organe ou le tissu végétal. Cette expression est également influencée par les conditions environnementales, ou l'action d'hormones (Rodriguez-Milla et al 2003, cette étude).

L'analyse des séquences protéiques par des logiciels de prédiction indique que les Gpx végétales sont susceptibles d'être présentes dans différents compartiments cellulaires (Herbette et al 2004). Une Gpx de tomate est localisée

dans le cytoplasme ainsi qu'au niveau de la paroi. Des études d'import dans des organites isolés ont depuis confirmé la présence de Gpx dans les chloroplastes de pois (Mullineaux et al 1998). De même, l'expression dans des cellules de tabac de fusion Gpx-GFP ont mis en évidence une localisation d'enzymes de peuplier dans les mitochondries et chloroplastes (cette étude).

IV Mon travail de recherche

Mon travail de thèse s'inscrit dans la thématique de l'étude des systèmes antioxydants redox-dépendants chez le peuplier, qui est développée au sein du laboratoire. En particulier, le groupe s'est efforcé depuis plusieurs années d'étudier le système thiorédoxine chez le peuplier, à travers l'étude biochimique et physiologique des Trx et de leur cibles. Le développement des programmes de séquençages d'EST puis du génome de peuplier a encore élargi ce champ d'investigations. Par exemple, de nouvelles isoformes, comme les Trx h4 et h5, ou encore de nouvelles cibles identifiées comme potentiellement intéressantes, telles que les nucléoside diphosphate kinases, ou encore les métallothionéines, ont pu être obtenues et au moins en partie caractérisées durant ma thèse. Des résultats, préliminaires ou partiels ont été obtenu pour certaines enzymes, mais ne sont pas détaillés dans ce manuscrit.

Le plus grande part de mon travail, a été consacrée à la caractérisation de la famille des Gpx chez le peuplier, en lien avec les autres composants des systèmes de détoxication redox cellulaire: Trx, Grx et Prx. L'implication des Gpx dans les mécanismes de défense cellulaire contre le stress oxydant dans les cellules

végétales semble très probable. Cependant, si les données sur l'expression des gènes sont nombreuses et abondent dans ce sens, les études portant sur l'expression et l'activité des protéines sont rares. Nous avons donc axé l'étude de ces enzymes sur la caractérisation de leur propriétés biochimiques et l'étude de leur expression, comparée à celle des autres membres de la famille des Prx.

RESULTATS

Le travail présenté dans ce manuscrit concerne tout d'abord l'étude des thiorédoxines de peuplier. Ces travaux ont donné lieu à deux articles publiés et un article en préparation présentés ici, dans lesquels les investigations portent sur les interactions entre Trx et cibles.

Le premier d'entre eux, l'article IV : Gelhaye E et al. (2004) A specific form of thioredoxin h occurs in plant mitochondria and regulates the alternative oxidase, décrit la caractérisation d'une isoforme de Trx mitochondriale de peuplier, la Trx h2.

Les interactions entre le système Trx et l'ascorbate peroxydase ont aussi été étudiées, et font l'objet de l'article V : Gelhaye E et al (2006), Ascorbate peroxidase–thioredoxin interaction. Il apparaît que le système Trx peut entraîner une inactivation de l'Apx, et il semble donc que les deux systèmes puissent interagir, notamment lors d'un stress oxydant.

Une isoforme particulière de Trx, la Trx h4, a fait l'objet d'études biochimiques, qui sont présentées ici sous la forme d'un article en préparation (Article VI : Gelhaye E (en préparation) Catalytic Mechanism of a Glutaredoxin-dependent Thioredoxin from Poplar), et d'études structurales qui sont en cours au laboratoire de cristallographie LCM3B de Nancy.

Nous avons aussi entrepris la caractérisation d'une isoforme de Trx h, la Trx h5, qui pourrait être secrétée par la cellule végétale, compte tenu de la présence d'une séquence d'adressage pour les voies de sécrétion et de l'homologie avec une Trx de type h secrétée *in vitro* chez le tabac (Juarez-Diaz et al 2005). La forme recombinante mature de cette protéine a été produite dans *E. coli* sans son extension N-terminale. Elle possède une activité classique de thiorédoxine, mais

semble aussi posséder une activité peroxydase, en réduisant notamment l'H_2O_2. Elle est un donneur d'électrons pour différentes Gpx de peuplier, dont une isoforme aussi potentiellement secrétée. Des études de localisation de cette protéine, par fusion de la séquence d'adressage avec une protéine fluorescente (GFP) ont été entreprises.

La deuxième partie, et la plus importante part de mon travail, a été de caractériser une famille de protéines cibles des Trx, les glutathion peroxydases (Gpx), chez le peuplier. Au commencement de ma thèse, ces enzymes étaient encore peu caractérisées et leurs relations avec les autres systèmes redox-dépendants (Trx, Grx) faisaient l'objet d'un nombre très limité d'études.

Le travail de recensement des gènes codant pour ces protéines, leur clonage, expression et purification, sont inclus dans l'article VII: Navrot N et al (2006) Plant glutathione peroxidases are functional peroxiredoxins distributed in several subcellular compartments and regulated during biotic and abiotic stresses. Des études biochimiques, concernant les caractéristiques et le mécanisme réactionnel de ces enzymes, ainsi que des études d'expression dans des conditions environnementales variées ont permis d'évaluer et de discuter les capacités et les rôles potentiels de ces Gpx dans la réponse et l'adaptation de la plante à son environnement. Nous avons ainsi pu établir que ces Gpx possèdent une activité Trx-dépendante et peuvent être regroupées dans la famille des Prx végétales.

La structure de la Gpx5 de peuplier sous forme oxydée et réduite a aussi été résolue, en collaboration avec le laboratoire de cristallographie LCM3B de Nancy et fait l'objet d'un article en préparation (Koh et al, en préparation). Ce travail a permis d'établir que la Gpx5 de peuplier existe sous forme de dimère non-covalent, stabilisé par des interactions hydrophobes. Le mécanisme catalytique proposé suite aux

études biochimiques et de mutagénèse réalisée sur la Gpx3.2 est aussi en accord avec la présence dans la structure de la forme oxydée de l'enzyme d'un pont disulfure entre les cystéines homologues aux Cys107 et Cys155 de la Gpx3.2.

Article IV : A specific form of thioredoxin h occurs in plant mitochondria and regulates the alternative oxidase.

Gelhaye E, Rouhier N, Gerard J, Jolivet Y, Gualberto J, Navrot N, Ohlsson PI, Wingsle G, Hirasawa M, Knaff DB, Wang H, Dizengremel P, Meyer Y, Jacquot JP.

Proc Natl Acad Sci U S A. 2004 Oct 5;101(40):14545-50. Epub 2004 Sep 22.

Cet article décrit une nouvelle isoforme de Trx mitochondriale chez le peuplier. En plus des Trx de type o, une Trx de type h, la Trx h2, est importée dans la mitochondrie chez le peuplier. Sa localisation a été démontrée par des études d'expression (Western blot), ainsi que par microscopie, à l'aide d'une fusion entre la séquence d'adressage de Trxh2 et la GFP, une protéine fluorescente. Cette Trx h2 est capable de réduire l'alternative oxydase (Aox), une enzyme mitochondriale, pour la rendre active. Elle n'est cependant pas capable de réduire efficacement une glutathion peroxydase mitochondriale (Gpx3) de peuplier. La Trx o d'*A. thaliana* n'est pas capable non plus de réduire la Gpx3. Cette étude met en évidence une Trx de type h mitochondriale qui peut être glutathionylée et décrit son interaction avec différentes protéines cibles de ce compartiment. La présence de cette isoforme de Trx dans la mitochondrie renforce l'idée d'un réseau complexe de protéines redox-dépendantes dans la mitochondrie végétale.

A specific form of thioredoxin *h* occurs in plant mitochondria and regulates the alternative oxidase

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The plant mitochondrial thioredoxin (Trx) system has been described as containing an NADPH-dependent Trx reductase and Trx *o*. In addition to the mitochondrial isoform, Trx *o*, plants are known to contain several chloroplastic Trx isoforms and the cytosolic Trx *h* isoforms. We report here the presence in plant mitochondria of a Trx isoform (PtTrx*h*2) belonging to the Trx *h* group. Western blot analyses with mitochondrial proteins isolated from both poplar and GFP fusion constructs indicate that PtTrx*h*2 is targeted to plant mitochondria. The recombinant protein, PtTrx*h*2, has been shown to be reduced efficiently by the mitochondrial Trx reductase AtNTRA. PtTrx*h*2 is also able to reduce alternative oxidase homodimers and to allow its activation by pyruvate. In contrast, neither PtTrx*h*2 nor AtTrx*o*1 exhibits activity with several poplar glutathione peroxidases and especially a putative mitochondrial isoform. Incubation of PtTrx*h*2 with glutathione disulfide led to the formation of glutathionylated Trx, identified by mass spectrometry. The formation of a glutathione adduct increases the redox potential of PtTrx*h*2 from −290 to −225 mV. In addition to Trx *o*, this study shows that Trx *h* could also be present in mitochondria. This previously unrecognized complexity is not unexpected, considering the multiple redox-regulated processes found in plant mitochondria.

poplar | glutathione peroxidase | glutathionylation | redox potential

Thioredoxins (Trxs) are small proteins involved in cellular redox regulation. These ubiquitous proteins, which are present in all organisms from prokaryotes to eukaryotes, display a particularly large diversity in photosynthetic organisms (1, 2). For example, at least 21 genes encoding Trxs have been detected in the fully sequenced genome of *Arabidopsis thaliana* (3). In plants, several Trxs are found: the chloroplastic Trxs *f*, *m*, *x*, *y*, and CDSP32, the mitochondrial Trxs *o*, and the Trxs *h*, which previously have been thought to be present exclusively in the cytosol. The chloroplastic Trxs, which are nuclear-encoded, are reduced by ferredoxin in a reaction catalyzed by a heterodimeric ferredoxin–Trx reductase (2). Recently, a mitochondrial system involving a NADPH-Trx reductase (NTR), NTRA, and at least one Trx *o* has been detected in *A. thaliana* (4).

The members of the third Trx group, called Trxs *h*, possess at least one specific characteristic that allows their identification: the presence of a conserved Trp residue (W16 in poplar Trx *h*1) that produces a unique characteristic shoulder at 290 nm in the UV-region absorbance spectrum (5). Based on primary structure analysis, Trxs *h* could be classified into three subgroups (3, 6). Members of subgroups I and II are reduced by NTR, whereas members of subgroup III are dependent on the glutathione/glutaredoxin system (7). In addition, all members of the second group contain N-terminal extensions (3). The significance of these extensions remains unclear, given that prediction programs, such as TARGETP (www.cbs.dtu.dk/services/TargetP) or PSORT (http://psort.ims.u-tokyo.ac.jp), suggest that a majority of these proteins should be cytosolic (3).

A poplar Trx, called PtTrx*h*2, belonging to subgroup II, was characterized recently (8). The recombinant protein has been produced in *Escherichia coli*. In this case, the N-terminal extension was cleaved in the *E. coli* cells, with the first 19 amino acids missing, suggesting the presence of a putative cleavage site in the N-terminal extension of the protein.

We report here that PtTrx*h*2 is associated with mitochondria. The redox properties of PtTrx*h*2 were investigated by examining its interaction with two potential mitochondrial Trx targets, alternative oxidase (AOX) and glutathione peroxidase (Gpx). In addition, we report that this Trx displays a potential site for glutathionylation and that formation of the glutathione adduct alters its redox potential.

Materials and Methods

GFP Experiments. For *in vivo* intracellular localization, the cDNA sequences of the putative transit peptide and the first putative α-helix of PtTrx*h*2 were cloned between the *Nco*I and *Bam*HI sites of plasmid pCK-GFP3A, which allows the expression of protein–enhanced-GFP fusions under the control of a 35S promoter. The following oligonucleotides were used to obtain a chimeric protein containing the first 65 amino acids of PtTrx*h*2 connected to GFP: forward oligonucleotide, 5′-CCCCCATG-GCTAATTTTGATCACTCCAATGGA-3′; reverse oligonucleotide, 5′-CCCCGGATCCGCCGTGAATTCAATCAC-3′. The resultant plasmid was transfected in tobacco leaves by bombardment, and images were obtained with a Zeiss LSM510 confocal microscope. The pCK-mRFP plasmid, which harbors the DsRED1 gene (Clontech), which codes for the red fluorescent protein fused to the mitochondrial yeast COXIV presequence, was used as a mitochondrial marker. It was cotransfected with the tested construction.

Mitochondria Purification. Mitochondria, isolated from 13-day-old soybean (*Glycine max*) cotyledons, were purified as described in ref. 9. For poplar (*Populus tremula* × *Populus alba*, clone 717-1-B4, Institut National de la Recherche Agronomique Orléans, Olivet Cedex, France), growing leaves from 6-month-old cuttings were used. The leaves (60 g) were washed with cold distilled water, cut, and placed in 300 ml of extraction medium [30 mM Mops buffer, pH 7.4/0.4 M mannitol/1% (wt/vol)

Abbreviations: AOX, alternative oxidase; Gpx, glutathione peroxidase; GSSG, oxidized glutathione; Trx, thioredoxin; NTR, NADPH-Trx reductase.

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BSA/3 mM cysteine/4 mM DTT/1.25 mM EGTA/2.5 mM $MgCl_2$/0.4% (wt/vol) polyvinylpyrrolidone/0.4% (wt/vol) phenol/chloroform/polyvinylpolypyrrolidone]. After two rounds of homogenization at a low speed for 2 s with a Waring blender, the homogenate was rapidly filtered through two layers of Miracloth (100 μm). The leaf fragments retained by the net were homogenized again (three times for 2 s) with 200 ml of the extraction medium and filtered as described above. The two filtrates were pooled and subjected to differential centrifugations as described in ref. 9. Mitochondria were resuspended in washing medium [0.4 mM mannitol/10 mM Mops buffer, pH 7.2/0.5% BSA (wt/vol)] and purified as described in ref. 10. Mitochondria were washed twice in 30 ml of washing medium devoid of BSA. The final pellet, suspended to $\approx$1.5 μg of protein per μl of washing medium, was stored at -80°C.

Western Blot Analysis. Polyclonal antibodies (IgGs) against poplar PtTrx*h*2 and poplar chloroplastic methionine sulfoxide reductase A were raised in rabbits and purified from the serum by affinity chromatography involving covalently linking proteins to Sepharose columns by using a technique similar to the one described for type II peroxiredoxin in ref. 11. Antibodies against glycine cleavage system subunits P and T were gifts from J. Bourguignon (Centre Nationale de la Recherche Scientifique, Grenoble, France) (12).

Protein extraction from leaves, stems, and roots was carried out as described in ref. 11. Western blots were performed by using polyvinylidene difluoride membranes (Millipore) and the Immun-Star Goat Anti-Rabbit Detection kit (Bio-Rad).

Cloning of Processed PtTrx*h*2. Because the full-length recombinant protein is processed in *E. coli* (8), the ORF of poplar Trx*h*2. in which the nucleotide sequence encoding the first 19 amino acids was deleted, was cloned by PCR, by using the previously described plasmid pET-popTrx*h*-2L (8) as a template, into the expression plasmid pET-3d between the two restriction sites *Nco*I and *Bam*HI with the following oligonucleotides: forward, 5′-CCCC*CATGG*CTAATTTTGATCACTCCAATGGA-3′; reverse, 5′-CCCCC*GGATCC*TTATATCATCCCATGCTTC-TCAAT-3′. In this construction, the recombinant protein starts with the sequence MANFDH.

Expression and Purification of the Recombinant Proteins. For PtTrx*h*2 production, *E. coli* strain BL21(DE3) was cotransformed with the helper plasmid pSBET and the recombinant plasmid (13). Production and purification of the recombinant protein were performed as described in ref. 8.

The procedures for the expression and purification of AtNTRA, AtNTRB, AtTrxo1, PtTrx*h*1, PtTrx*h*3, and PtPrxQ are described in refs. 4 and 14–17.

Trx Activity. The reduction of Trx by NADPH and the recombinant *A. thaliana* NTRA or -B was followed at 412 nm by using 5,5′-dithiobis(2-nitrobenzoic acid) as a substrate (4).

AOX Reduction and Activity. The reduction of soybean AOX by PtTrx*h*2 was performed as follows. PtTrx*h*2 was reduced by incubation with 10 mM DTT for 30 min and then dialyzed to remove DTT. The absence of detectable DTT in the protein preparation was checked by using 5,5′-dithiobis(2-nitrobenzoic acid). Purified frozen/thawed soybean mitochondria samples containing 2.5, 5, or 10 μg of total protein were incubated for 30 min with 150 μM PtTrx*h*2 or 40 mM DTT before being subjected to nonreductive SDS/PAGE. AOX immunodetection also was performed by using monoclonal antibodies [a generous gift from T. E. Elthon (University of Nebraska, Lincoln) to Y.J.] raised against the *Sauromatum guttatum* AOX (18).

To test the AOX activity, mitochondrial oxygen consumption was measured with a Clark-type O_2-electrode (Rank Brothers, Cambridge, U.K.). Purified soybean mitochondria were oxidized with 10 mM diamide, washed, and frozen, allowing membranes to break down. The mitochondria then were suspended in 1 ml of reaction medium containing 0.3 M sucrose, 5 mM KH_2PO_4, 10 mM *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (pH 7.0), 10 mM NaCl, 2 mM $MgSO_4$, and 0.1% (wt/vol) BSA at 25°C. PtTrx*h*2 (500 μM) was reduced in 30 mM Tris·HCl (pH 8)/1 mM EDTA buffer by using the NADPH/AtNTRB system described in ref. 8. Subsequent additions were made as described in *Results*.

Gpx Activity. The Gpx activity was measured by following H_2O_2 reduction. The reaction medium had the following composition: 50 mM Tris·HCl, pH 8.0/500 μM DTT/500 μM H_2O_2 with varying amounts of PtTrxs and PtGpxs. After 1 min of incubation at ambient temperature, the reaction was started by adding H_2O_2. For each time point, 50 μl of the reaction mixture was added to 950 μl of FOX2 reagent as described in ref. 17. The absorbance at 560 nm was read after a 1-h incubation.

Glutathionylation Experiments. The reaction mixture (50 μl) containing 30 mM Tris·HCl (pH 8), 1 mM DTT, and 50 μg of poplar Trx *h*1, *h*2, or *h*3 (concentration of $\approx$80 μM) was incubated 10 min before adding 5 mM oxidized glutathione (GSSG). The excess of GSSG was removed by extensive dialysis (1/25,000), and the resultant glutathionylated protein was concentrated by ultrafiltration.

Redox Potential Determination. Titration of Trx *h*2 redox potential was determined by using the monobromobimane method described in ref. 19. PtTrx*h*2 redox potential also was determined by using atNTRB-catalyzed poising by direct reduction by $NADP^+$/NADPH (20). PtTrx*h*2 and GSSG-treated PtTrx*h*2 (10–40 μM) were mixed with 108 μM NADPH in 500 μl. Then, 85 nM AtNTRB was added, and the NADPH oxidized was determined at 340 nm against an identical blank without NADPH. To determine the redox potential, the experiment was continued by adding 1 mM $NADP^+$ to reverse the equilibrium of the reaction. The concentration of the $NADP^+$ stock solution was checked by reading the *A* at 260 nm, assuming that ε = 15.3 $mM^{-1}\cdot cm^{-1}$. From the equilibrium concentrations, redox potentials were calculated by using the Nernst equation (20).

Electrospray MS. A Micromass Q-Tof Ultima (Waters) hybrid tandem mass spectrometer was used for the acquisition of the electrospray ionization mass spectra. This instrument is equipped with a nanoflow-electrospray source. The samples were infused into the mass spectrometer by using nanoflow capillaries (Proxeon Biosystems, Odense, Denmark). The needle voltage was $\approx$1,800 V, and the collision energy was 10 eV for the MS analyses. Samples for flow injection analyses were diluted 1:20 with a solution of 50:50 acetonitrile and 0.1% formic acid. Data analysis was accomplished with a MassLynx data system and TRANSFORM deconvolution software supplied by the manufacturer (Waters).

Results

Mitochondrial Localization of PtTrx*h*2. Two sets of experiments aimed at investigating the subcellular localization of PtTrx*h*2 were undertaken. First, a transient expression experiment was carried out by using a chimeric protein obtained by fusing the putative transit peptide of PtTrx*h*2 to GFP. After bombardment of tobacco leaf epidermis, the protein–GFP fusion was found only in mitochondria (Fig. 1*A*).

The mitochondrial localization was further demonstrated by using specific antibodies together with mitochondria purified from poplar leaves (Fig. 1*B*). The quality of the mitochondrial

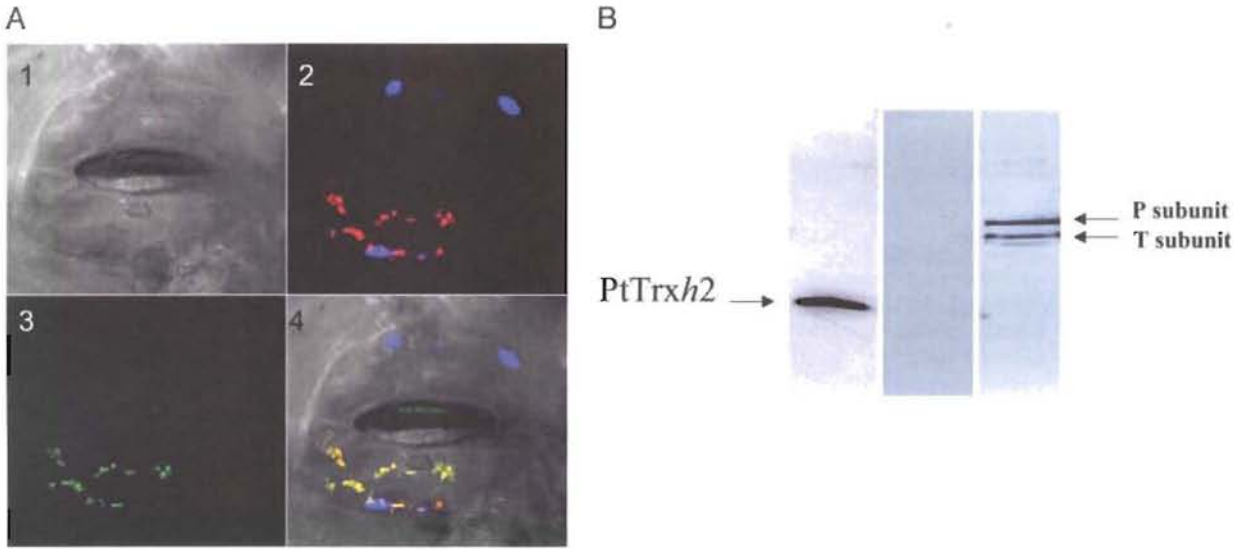

Fig. 1. Mitochondrial localization of PtTrx*h*2. (*A*) Mitochondrial localization of the PtTrx*h*2 fused to enhanced GFP in *N. tabacum* stomata. (Image 1) Image of stomata from *Nicotiana benthamiana* under visible light. Only the lower guard cell was transfected. (Image 2) Autofluorescence of chlorophyll (blue) and fluorescence of the mitochondrial marker (red). (Image 3) Fluorescence of the fusion protein (green). (Image 4) Merged images. (*B*) Immunodetection of PtTrx*h*2 (*Left*), methionine sulfoxide reductase A (*Center*), and glycine decarboxylase complex subunits P and T (*Right*) in poplar purified mitochondria.

preparation was checked by using antibodies recognizing both chloroplastic and cytosolic methionine sulfoxide reductases (N.R., unpublished data). In this case, no signal could be detected by using the mitochondrial preparation (Fig. 1*B Center*), whereas a strong signal could be detected in whole poplar leaf extracts (data not shown). The PtTrx*h*2 signal intensity (Fig. 1*B Left*) was similar to that obtained by using antibodies raised against glycine decarboxylase complex subunits (Fig. 1*B Right*) used as mitochondrial markers.

PtTrx*h*2 Belongs to a Specific Subgroup of Trxs *h*. Trx *h* have been classified into three different subgroups based on primary structure analysis (3). Subgroup II includes Trxs exhibiting an N-terminal extension (6). A phylogenic tree has been constructed by using various Trxs *h* belonging to subgroup II (Fig. 2). These Trxs could be further divided into three subclasses: one group containing proteins related to AtTrx*h*2 called IIA; one group containing proteins from cereals, which cluster separately (IIB); and a third group (IIC) that contains proteins related to AtTrx*h*8 and also includes PtTrx*h*2. Prediction of subcellular localization of each protein was performed by using a combination of two programs, TARGETP and IPSORT. With the exception of AtTrx*h*7, PtTrx*h*2-related members all are predicted to possess a peptide signal. In contrast, AtTrx*h*2-related sequences are mainly predicted to be cytosolic. However, it should be pointed out that soybean Trxs, which have been shown to be anchored to plasma membranes (21), are predicted to be chloroplastic proteins by these programs. These analyses suggest that mitochondrial Trxs *h* could constitute a quite homogeneous subclass in Trx *h* subgroup II.

Expression of PtTrx*h*2. The expression of PtTrx*h*2 in plant organs was investigated by recording the number of ESTs coding for this protein among all of the poplar ESTs present in the GenBank database. Around 80 ESTs are present of a total 210 detected for the whole Trx *h* group. For comparison, only six ESTs encoding Trx *o* have been found in the database. The *PtTrxh2* gene thus appears to be transcribed at high levels in leaves (≈69 hits) and at low levels in roots (5 hits) and petioles (5 hits). Trx *h*2 also was followed at the protein level in roots, stems, and leaves. The protein is present in similar detectable amounts in all parts of the plant (data not shown).

atNTRA and atNTRB. The presence of NADPH Trx reductase in *A. thaliana* mitochondria is demonstrated in ref. 4. The activity of PtTrx*h*2 has been monitored in the presence of both cytosolic (AtNTRB) and mitochondrial (AtNTRA) NADPH Trx *A. thali-*

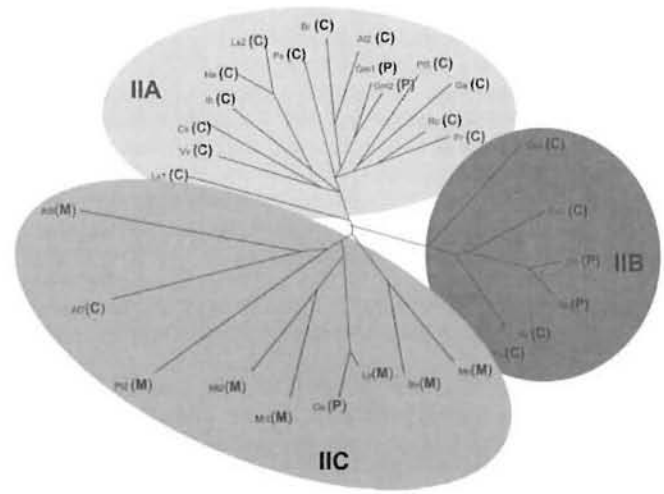

Fig. 2. Phylogenetic tree of various Trxs *h* belonging to the subgroup II. The CLUSTALW software was used to generate the phylogenetic tree of the following Trxs (GenBank accession numbers in parentheses): At2, *A. thaliana* Trx*h*2 (S58123); At7, *A. thaliana* Trx*h*7 (AAD39316); At8, *A. thaliana* Trx*h*8 (AAG52561); Bv, *Beta vulgaris* (BQ489103); Br, *Brassica rapa* (AF352030); Cs, *Citrus sinensis* (CF837405); Ca, *Capsicum annuum* (CA523711); Ga, *Gossypium arboretum* (BG440056); Gm1, *G. max* (AW620807); Gm2, *G. max* (BM188930); Ha, *Helianthus annus* (TC13569); Hv, *Hordeum vulgare* (BI960260); Ib, *Ipomoea batatas* (AY344228); Mc, *Mesembryanthemum crystallinum* (BE033809); Mt1, *Medicago truncatula* (AW560796); Mt2, *M. truncatula* (AW686237); Ls1, *Lactucca sativa* (BU008396); Ls2, *L. sativa* (TC9551); Ly, *Lycopersicon esculentum* (BG126499); Pr, *Prunus armeniaca* (CB818939); Os1; *Oryza sativa* (AK062383); Os2, *O. sativa* (CB681257); Ps, *Pisum sativum* (AY170651); Pt2, *Populus trichocarpa* Trx*h*2 (AF483266); Pt5, *P. trichocarpa* Trx*h*5 (BU869308); Rc, *Rosa chinensis* (BI978567); Sb, *Sorghum bicolor* (AW747151); Ts, *Triticum aestivum* (CD914485); Vv, *Vitis vinifera* (CF513794); Zm, *Zea mays* (AY104013). The subcellular prediction (C, cytosol; M, mitochondria; P, plastid) was performed by using IPSORT (http://hypothesiscreator.net/iPSORT).

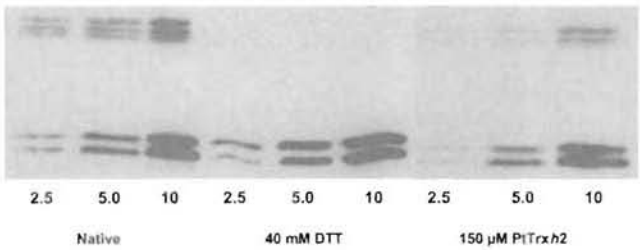

Fig. 3. AOX reduction. Purified and frozen/thawed soybean mitochondria (2.5, 5, and 10 μg of proteins) were mixed with reduced 150 μM PtTrx*h*2 or 40 mM DTT and incubated for 30 min before being subjected to SDS/PAGE. AOX dimers and monomers were immunodetected.

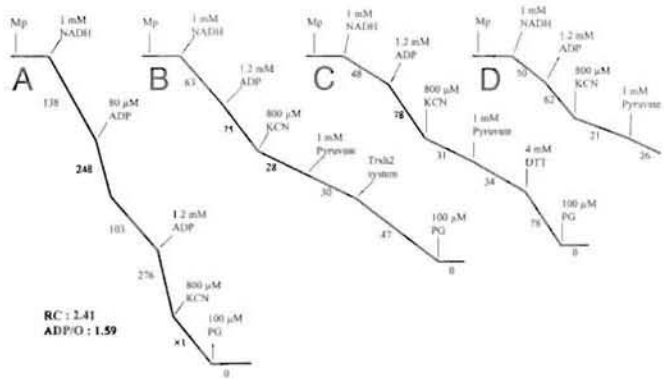

Fig. 4. AOX activation. NADH oxidation by mitochondria isolated from soybean cotyledons. (*A*) Purified mitochondria in respiration medium. (*B–D*) Purified soybean mitochondria were incubated for 30 min with 10 mM diamide and washed two times, and the oxygen uptake was measured in sucrose-depleted respiration medium. Where indicated, the following additions were made to the oxygen electrode chamber: 35 μM NTR reduced PtTrx*h*2 (*B*). PtTrx*h*2 (875 μM) was previously reduced during 15 min in the presence of atNTRB (12.5 μM) and NADPH (2.5 mM). As control, either 4 mM DTT (*C*) or only NADPH/NTR (*D*) was added. Numbers on the traces represent nmol O_2 per min per mg of protein.

ana reductases (data not shown). The observed activity when using 5,5′-dithiobis(2-nitrobenzoic acid) as an electron acceptor was similar regardless of which NTR was used, suggesting that PtTrx*h*2 could be reduced by NTR in mitochondria.

In additional experiments, SDS/PAGE performed without reductant addition showed that PtTrx*h*2 is present mainly as homodimers, which could be reduced by DTT (data not shown). The thiol content of the preparation was determined by using the 5,5′-dithiobis(2-nitrobenzoic acid) method. As expected, nearly three thiols are titrated for the reduced protein and nearly no SH group (0.03 SH per protein) is present in the oxidized protein, suggesting that Cys-29 is involved in a disulfide bridge, possibly linking two monomers together.

AOX Reduction. AOX, a potential mitochondrial Trx target, has been shown to be redox-regulated (22). Western blot experiments allowing the detection of oxidized AOX dimers as well as reduced AOX monomers were performed by using PtTrx*h*2-treated or untreated soybean mitochondria (Fig. 3). As a control, similar experiments were carried out by using DTT as reductant. A significant reduction of inactive dimers to monomers by PtTrx*h*2 was observed, suggesting that Trx could regulate AOX dimerization and possibly its activity in mitochondria.

To confirm this hypothesis, the activity of AOX also was monitored in the presence or absence of NTR-reduced PtTrx*h*2 by using purified soybean mitochondria (Fig. 4). Purified mitochondria presented a high phosphorylative efficiency and a cyanide resistance estimated at 30% of the state-3 rate when NADH was used as substrate (Fig. 4*A*). The cyanide-insensitive respiration was fully inhibited by propylgalate, a specific inhibitor of AOX. To study the effect of PtTrx*h*2, mitochondria were oxidized by using diamide (3 mM), and oxygen uptake was recorded after a 2-min incubation in the electrode medium devoid of sucrose. Under these conditions, the subsequent addition of pyruvate poorly stimulated the cyanide-insensitive respiration (Fig. 4*B*), confirming that the AOX protein needs to be reduced to be activated by this effector. The addition of NTR-reduced PtTrx*h*2 enhanced the recorded AOX activity (Fig. 4*B*). A similar effect was obtained with DTT (Fig. 4*C*). Moreover, the addition of the reductant system of Trxs, depleted of Trx*h*2, poorly modified the oxygen uptake (Fig. 4*D*). Similar experiments performed with AtTrx*o*1 and the cytosolic PtTrx*h*1 indicated that these isoforms also could activate AOX (data not shown).

Gpx Activity. Despite their classification, plant Gpxs use Trxs for peroxide reduction (23). The ability of PtTrx*h*2 to reduce poplar Gpxs *in vitro* has been investigated. A representative experiment involving the putative mitochondrial PtGpx3 is shown in Fig. 5. Surprisingly, PtTrx*h*2 remained completely inactive in this test, in contrast to the cytosolic PtTrx*h*1 and PtTrx*h*3. Similar results were obtained with the other tested Gpx isoforms, PtGpx1 and PtGpx5 (data not shown). The mitochondrial AtTrx*o*1 was also unable to reduce the different isoforms of PtGpx (data not shown).

Glutathionylation of PtTrx*h*2. To investigate the susceptibility of PtTrx*h*2 to glutathionylation, the protein was reduced by DTT, incubated with excess GSSG, and analyzed by quadrupole time-of-flight. Reduced recombinant PtTrx*h*2 had the expected molecular mass of 14,056.875 Da. Quadrupole time-of-flight analysis of PtTrxh2 that had been treated with GSSG showed the formation of a peak with a mass of 14,361.125 Da, compatible with the addition of one glutathione molecule (305.5 Da). A representative analysis of untreated PtTrx*h*2 and GSSG-treated PtTrx*h*2 is shown in Fig. 6. When the GSSG-treated PtTrx*h*2 was rereduced by using 10 mM DTT, a peak at 14,059 was obtained (data not shown), as expected for reductive cleavage of the adduct. PtTrx*h*1 and PtTrx*h*3, two other poplar Trxs *h*, also were tested. In both cases, no glutathione addition was detected on these Trxs in these experimental conditions.

To investigate the effect of glutathionylation on the enzymatic activity of PtTrx*h*2, the time courses of reduction and reoxida-

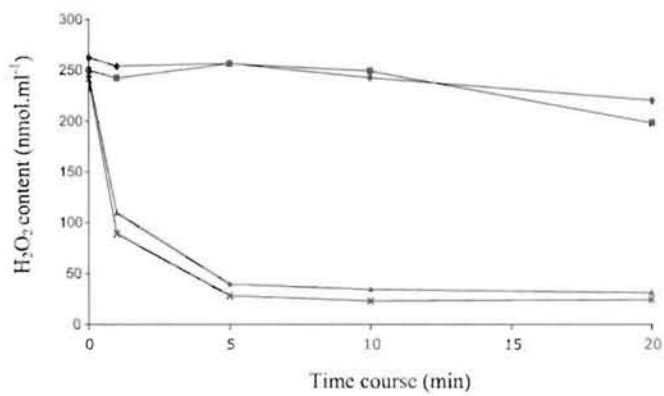

Fig. 5. Gpx activity. The reduction of H_2O_2 by poplar Gpx 3 (PtGpx3) was measured by following the disappearance of 500 μM H_2O_2 with 500 μM DTT or 20 μM of different Trx isoforms and 2 μM Gpx3 in a typical ferrous oxidation–xylenol orange (FOX) assay. ♦, minus Trx; ■, 20 μM PtTrx*h*2; ▲, 20 μM PtTrx*h*1; ×, 20 μM PtTrx*h*3.

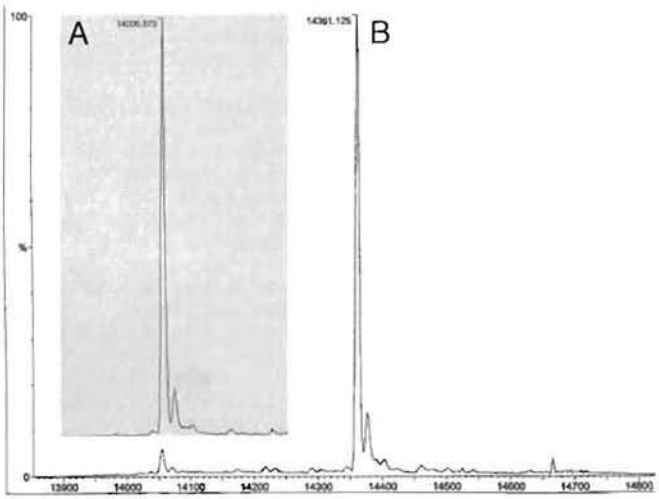

Fig. 6. Glutathionylation of PtTrx*h*2. The deconvoluted spectra of native PtTrx*h*2 (*A*) and PtTrx*h*2 incubated with 5 mM GSSG (*B*) are shown.

tion of disulfides in GSSG-treated PtTrx*h*2 and PtTrx*h*2 in the presence of 85 nM AtNTRB are compared in Fig. 7. For PtTrx*h*2, the amount of oxidized NADPH determined in the first part of the curves indicates that the three cysteines are reduced (≈1.5 oxidized NADPH per protein). In contrast, the amount of oxidized NADPH corresponds to only one disulfide bridge in GSSG-treated PtTrx*h*2 (≈1 oxidized NADPH per protein). From equilibrium constants, redox potentials of −287 ± 15 mV for PtTrx*h*2 and −225 ± 15 mV for GSSG-treated PtTrx*h*2 were calculated by using a value of −320 mV for the couple NADP , NADPH at pH 7.0 and 20°C. The oxidation-reduction midpoint potential (E_m) of PtTrx*h*2 also was measured by using the monobromobimane titration method. The E_m value is approximately −292 mV ± 10 mV at pH 7.0, a value similar to that found with the NTR method and for other Trxs *h* (24, 25).

Discussion

In poplar, as well as in other plants, the Trx *h* group can be divided into three distinct subgroups. The main characteristic of subgroup II members is the presence of an amino acid N-terminal extension. Nevertheless, it had been thought that these isoforms were localized in the cytosol (2, 3). In this study, we demonstrate that at least one

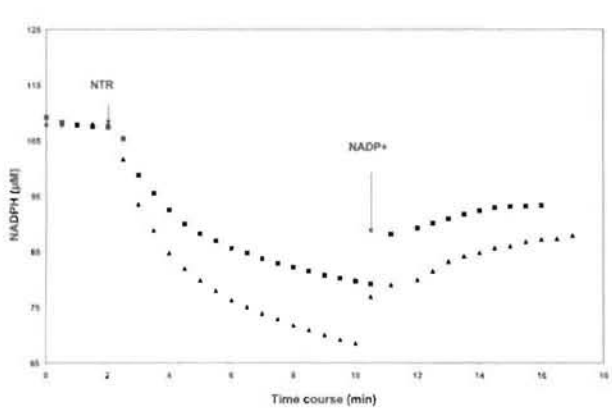

Fig. 7. Determination of redox potential of PtTrx*h*2 and glutathionylated PtTrx*h*2. Reduction of disulfides in 26.6 μM PtTrx*h*2 (▲) and 26 μM GSSG-treated PtTrx*h*2 (■) was started by the addition of 85-nm Trx reductase B from *A. thaliana*. When the reaction was stopped, NADP⁺ was added to a final concentration of 1.0 mM. The formation of NADPH was followed from the increase at 340 nm.

poplar Trx *h* (PtTrx*h*2) belonging to this subgroup is associated with mitochondria. This mitochondrial localization is in agreement with previous evidence for the presence of Trxs and particularly Trxs *h* in plant mitochondria (26, 27). This mitochondrial localization of PtTrx*h*2 raises the possibility of subcellular organelle localization for other Trxs *h* with N-terminal extensions. The phylogenic tree presented in this study raises the possibility that mitochondrial Trx*h*s could constitute a distinct subdivision of the Trx*h* subgroup II. However, the physiological significance of the N-terminal extensions found in other proteins of this subgroup remains to be elucidated.

The mitochondrial localization of PtTrx*h*2 increases the complexity of the Trx system in this organelle, because it was recently shown that Trx *o* and NTRA are also localized in mitochondria (4). In poplar, EST database analysis indicated the presence of at least one mitochondrial Trx *o* (7). The presence of at least two distinct isoforms of Trx in mitochondria raises the question of the specificity and also the function of these Trxs in this organelle, both isoforms being reduced by NTR. EST database analysis suggested that PtTrx*h*2 may be expressed to a significantly larger extent than PtTrx*o*, suggesting a major role for this isoform in poplar.

The functions of Trx in plant mitochondria remain largely unknown. Nevertheless, recent data collected by using proteomic approaches suggest the presence of at least 50 potential Trx-linked proteins involved in different fundamental processes (28). Among these potential targets, Gpx has been identified. Interestingly, PtTrx*h*2 and atTrx*o*1 are not able to serve as electron donors for different isoforms of poplar Gpx, whereas these Gpxs are reduced by either PtTrx*h*1 or PtTrx*h*3, two putative cytosolic isoforms. Among the tested PtGpxs, PtGpx3 is predicted to be targeted to mitochondria and could be classified as part of a mitochondrial Gpx subgroup including AtGpx6 (29). The mitochondrial potato homolog of AtGpx6 has been found to be a potential target of Trx (28). Because the E_m value of PtTrx*h*2 is quite similar to those of other Trxs (24) and that PtTrx*h*2 is able to interact with nonmitochondrial proteins (6, 17), it is likely that structural features, rather than thermodynamic factors, are responsible for the absence of interactions between PtTrx*h*2 and PtGpxs. The details of this Trx/target interaction specificity thus require further investigation. Taken together, these data suggest that additional Trxs could exist in mitochondria and that the Trx system of this organelle may be as complex as that found in chloroplasts. It is also possible that other unidentified reductants may be present in mitochondria.

Another potential target of mitochondrial Trxs is AOX. Besides the cytochrome *c* pathway, plant mitochondria have an alternative respiratory pathway involving the homodimeric AOX. AOX plays a role in decreasing the formation of mitochondrial reactive oxygen species (30–32). A redox regulation of AOX has been identified. When the subunits are linked by a disulfide bond, the enzyme is inactive (22). In contrast, when the disulfide is reduced, monomers could be activated by α-keto acids (33, 34). The reductive activation of AOX dimers by PtTrx*h*2 and AtTrx*o*1 described in this study suggests that Trxs could play a major role in the activation of AOX and also in reactive oxygen species detoxification.

Another characteristic of PtTrx*h*2 is its ability to be glutathionylated. This property may be unique for PtTrx*h*2, because two other poplar Trxs, PtTrx*h*1 and PtTrx*h*3, are not glutathionylated. Data on glutathionylation processes in plants are scarce. In contrast, this phenomenon is well known in mammalian systems. For instance, glutathionylation of human Trx has been shown to reduce its enzymatic activity (35). In this study, we have shown that glutathionylation significantly alters the PtTrx*h*2 redox potential, suggesting that glutathione adduct could also modulate its enzymatic activity. The physiological significance of this posttranslational modification of PtTrx*h*2 remains to be elucidated and deserves further investigation.

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Article V : Ascorbate peroxidase–thioredoxin interaction.

Eric Gelhaye, Nicolas Navrot, Isabel K. Macdonald, Nicolas Rouhier, Emma Lloyd Raven, Jean-Pierre Jacquot

Photosynthesis Research, accepté.

Cet article décrit la mise en évidence d'interactions entre les systèmes thiol-dépendants et les peroxydases dépendantes de l'ascorbate chez les végétaux supérieurs. Nous avons montré que l'ascorbate peroxydase de pois est capable d'oxyder le GSH ou encore une Trx de peuplier. La réaction avec ces composés contenant des thiols va aboutir à la consommation d'O_2 couplée à une production d'H_2O_2 et à l'inactivation de la peroxydase. Cette inactivation n'est pas uniquement due à l'action d'H_2O_2 mais aussi à l'action de radicaux formés par l'oxydation des groupements thiols du GSH, de la Trx ou encore du DTT. Il est donc possible qu'Apx et Trx (ou GSH) puissent interagir *in vivo*, particulièrement en cas de stress oxydant, quand le substrat réduit de l'Apx, l'ascorbate, pourrait être présent en quantité plus faible, et en présence d'H_2O_2. Cette interaction entre des protéines présentes dans le cytosol, pourrait aussi avoir lieu dans d'autres compartiments (le chloroplaste notamment), mais sa signification et son rôle physiologique restent à déterminer.

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REGULAR PAPER

Ascorbate peroxidase–thioredoxin interaction

Eric Gelhaye · Nicolas Navrot · Isabel K. Macdonald · Nicolas Rouhier · Emma Lloyd Raven · Jean-Pierre Jacquot

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Abstract Proteomics data have suggested ascorbate peroxidase (APX) to be a potential thioredoxin-interacting protein. Using recombinant enzymes, we observed that incubation of pea cytosolic APX with reduced poplar thioredoxins *h* drastically inactivated the peroxidase. A similar inactivation is induced by reduced glutathione and dithiothreitol, whereas diamide and oxidized glutathione have no effect. Oxygen consumption measurements, modifications of the APX visible spectrum and protection by hydrogen peroxide scavenging enzymes suggest that APX oxidizes thiols leading to the generation of thiyl radicals. These radicals can in turn react with thiyl anions to produce the disulfide radical anions, which are responsible for oxygen reduction and subsequent hydrogen peroxide production. The APX inactivation is not due solely to hydrogen peroxide since fluorimetry indicates that the environment of the APX tryptophan residues is dramatically modified only in the presence of thiol groups. The physiological implications of this interaction are discussed.

Keywords Thioredoxins · Ascorbate peroxidase · Glutathione · Inactivation · Thiyl radical

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Abbreviations

APX	Ascorbate peroxidase
NTR	NADH thioredoxin reductase
NTS	NADPH–thioredoxin system
TRX	Thioredoxin

Introduction

The regulation of the cellular redox state is crucial for plant development and adaptation to environmental stimuli. Several redox metabolites are involved in this regulation. Among them, ascorbate plays a major role, particularly as a substrate for ascorbate peroxidase (APX), an enzyme converting hydrogen peroxide to water. APX has been identified in many higher plants and constitutes a family of isoenzymes distributed in different subcellular compartments (Shigeoka et al. 2002). In *Arabidopsis thaliana*, three APX isozymes are present in chloroplasts: a thylakoid-bound APX, a lumenal APX, and a stromal APX, the latter having been recently shown to be targeted both to the stroma and mitochondria (Chew et al. 2003). The cytosol contains one cytosolic APX (APX1), and an additional APX (APX2) that is inducible essentially under extreme light or heat stress conditions (Karpinski et al. 1999; Panchuk et al. 2002). APXs contribute together with other enzymes to the control of hydrogen peroxide concentration, a signaling molecule involved in the regulation of key biological processes, such as programmed cell death, abiotic stress response and

pathogen defense (Gechev and Hille 2005; Schutzendubel and Polle 2002; Overmyer et al. 2003). Cytosolic APX1 has been shown to play a key role in protecting chloroplasts against oxidative stress during photosynthesis, suggesting a cross-compartment protection mediated by this enzyme (Davletova et al. 2005).

Ascorbate peroxidase which belongs to the class I heme peroxidases has been extensively studied as a peroxidase model and the 3D structure of the APX-ascorbate complex has been solved (Sharp et al. 2003a). APX, as other peroxidases, is able to catalyze the oxidation of non-physiological, aromatic substrates, at rates comparable to those recorded with ascorbate (Sharp et al. 2003b). Furthermore, thiol-containing compounds such as glutathione have been found to be good substrates for horseradish peroxidase (Burner and Obinger 1997) and lactoperoxidase (Guo et al. 2004).

Besides ascorbate, thiol-depending pathways, and especially thioredoxins, also play a major role in the cellular redox control (Gelhaye et al. 2005; Buchanan and Balmer 2005). Thioredoxins are small ubiquitous disulfide oxidoreductases with two redox active cysteines in the catalytic site. The development of proteomic tools has led to identify several putative thioredoxin-interacting proteins (Balmer et al. 2003; 2004; Wong et al. 2004; Yamazaki et al. 2004). Among these, cytosolic ascorbate peroxidase has been postulated to interact with thioredoxins *h* (Marchand et al. 2004; Yamazaki et al. 2004). Thioredoxins *h* constitute a subfamily of thioredoxins, essentially located in the cytosol (Meyer et al. 2002; Gelhaye et al. 2004a); however, at least one isoform has been found in mitochondria (Gelhaye et al. 2004b). Thioredoxins *h* are usually reduced by a flavoprotein called NADPH Thioredoxin Reductase (NTR), which has also been found both in the cytosol and in mitochondria (Florencio et al. 1988; Laloi et al. 2001). The putative interaction between ascorbate peroxidase and thioredoxin could be very important in the understanding of the global cellular response to oxidative stress. Indeed, APX is known to play a major role in the regulation of the cellular redox state (Davletova et al. 2005) and the relationship between this enzyme and the thioredoxin system remains largely unknown.

The aim of this study was to investigate potential interactions between thioredoxins and APX. Using recombinant proteins of cytosolic origin, we demonstrate that the thioredoxin system (NTR/Trx) as well as glutathione are oxidized by pea cytosolic APX, this process leading to an inactivation of the peroxidase.

Materials and methods

Enzymes

APX was expressed and purified according to published procedures (Metcalfe et al. 2004). Poplar thioredoxin *h*1(PtTrxh1), thioredoxin *h*2 (PtTrxh2), type II peroxiredoxin (PtPrxII) and methione sulfoxide reductase A (PtMsrA) have been prepared as described previously (Behm and Jacquot 2000; Gelhaye et al. 2002; Gelhaye et al. 2003). NADPH-thioredoxin reductase B (NTR) from *A. thaliana* has been isolated as described in Jacquot et al. (1994).

Time dependence of inactivation

The inactivation of APX by the NADPH-Thioredoxin system (NTS) was followed by incubating APX (2 μM) with NADPH (2.2 mM), NTR (9.75 μM) and PtTrxh1 (10.5 or 100 μM) in 50 mM phosphate buffer, pH 7.0. The reaction was initiated by the addition of APX. At the appropriate times 10 μl aliquots were removed and their enzymatic activities were determined in the ascorbate peroxidase assay (500 μM ascorbate, 100 μM H_2O_2 in 50 mM phosphate buffer, pH 7.0).

The assays determining the influence of different compounds on APX activity have been performed as follows. APX (6.6 μM) was incubated with the tested compound during 30 min and 5 μl aliquots were removed and their residual activity was determined using the ascorbate assay (500 μM ascorbate, 100 μM H_2O_2 in 50 mM phosphate buffer, pH 7). The relative activity of APX control (without addition) was defined as 100.

Kinetic parameters have been determined using APX (13 μM) pre-incubated during 20 min with NTS1 (PtTrxh1 100 μM) as described above. The resulting mixture has been dialyzed over-night against 50 mM phosphate buffer, pH 7.0 in order to remove NADPH and activity measured using 28 nM of APX. As a control, APX has been submitted to the same treatment but without NTR addition.

APX activity assay

APX activity was determined in assays containing 100 μM hydrogen peroxide and either ascorbate or guaiacol (a substrate of APX) in 50 mM phosphate buffer, pH 7.0. For ascorbate, the activity was followed as the decrease in A_{290} due to the consumption of ascorbate (ϵ_{290} = 2800 M^{-1} cm^{-1}). The activity with guaiacol was determined at 470 nm (ϵ_{470} = 26600 M^{-1} cm^{-1}). The

reactions were initiated with hydrogen peroxide (100 μM).

Alternatively, H_2O_2 disappearance was followed directly. The reaction mixture (500 μl) contained 50 mM phosphate buffer pH 7.0, 500 μM ascorbate, 30 nM APX. The reaction was started by adding 1 mM H_2O_2. After the given incubation times, 2.5 μl was mixed with 1000 μl of FOX1 (ferrous oxidation in xylenol orange) reagent (Wolff et al. 1994). The absorbance was then read at 560 nm after 1-h incubation.

DTNB reduction

The reduction of thioredoxin by NADPH and recombinant NTR from *A. thaliana* was followed at 412 nm using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as a substrate (Jacquot et al. 1994).

Oxygen consumption assay

Oxygen consumption measurements were made with a Clark-type oxygen electrode filled with 1 ml of 50 mM phosphate buffer. The reagents were added in the following order: first, 50 mM phosphate buffer (pH 7.0) containing either GSH (1 or 10 mM) or NTS1 [NADPH (2.2 mM), PtTrxh1 (35 μM) and NTR (0.8 μM)], then after 5 min base-line measurement, reactions were initiated with 9.5 μM of APX. All experiments were done at room temperature.

Fluorescence measurements

Apx (9.5 μM) in 50 mM Tris buffer pH 7.0 was excited at 280 nm and emission spectra were recorded with a Cary eclipse fluorescence spectrophotometer (Varian). Similar experiments were also performed by adding GSH (10 mM) or H_2O_2 (10 mM), emission spectra being recorded after 10 min.

Results

Inactivation of APX by the thioredoxin system

In order to test the effect of reduced compounds (e.g. thioredoxin or glutathione) on APX, we have devised an experimental setup that includes two consecutive phases. The APX is first preincubated with the desired compound in an Eppendorf tube for a given time, and then an aliquot of APX is used for determining its activity in a spectrophotomoter. The decrease in activity due to inactivation by NTS1 (NADPH/NTR/PtTrx*h*1) was followed over time, with ascorbate as a substrate. APX inactivation requires reduced NTR and is enhanced in presence of thioredoxin (Fig. 1). Control experiments, where NADPH or NTR were omitted did not induce any inactivation, confirming the need for reduced thiol compounds. The NTS1-treated APX exhibited much lower catalytic rates with either ascorbate or guaiacol than untreated APX (Fig. 2). Steady-state oxidation of guaiacol by APX obeys Michaelis–Menten kinetics (Lad et al. 2002). The NTS1 incubation of APX leads to a strong decrease of k_{cat} from 1410 to 350 min^{-1}, whereas no significant changes in K_m values were observed (around 10 ± 4 mM). Steady state oxidation of ascorbate by APX did not obey Michaelis–Menten kinetics and the data were fitted to the Hill equation (Lad et al. 2002), leading to a k_{cat} value of 9400 ± 1600 min^{-1} for the untreated enzyme. Nevertheless, after NTS1 treatment, the obtained data could be fitted to Michaelis–Menten equation, the k_{cat} value being dramatically decreased (k_{cat} = 2200 ± 374 min^{-1}).

The NTS1-inactivation of APX was also confirmed when measuring H_2O_2 disappearance instead of ascorbate oxidation (Fig. 3), showing that the observed inactivation does not result from a competitive reduction of H_2O_2 by NTS1. We have observed many times in separate experiments, that the NTS system is unable to reduce H_2O_2 by itself.

After incubation with APX, the NTS1 system remained fully active when tested in the DTNB assay suggesting that NTR and Trx are not irreversibly

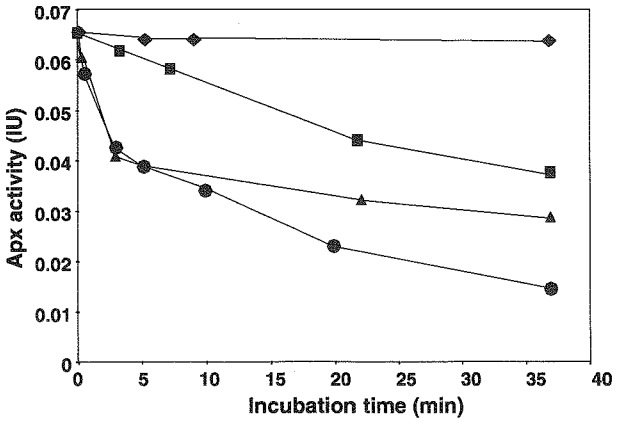

Fig. 1 Inactivation of APX by NADPH Thioredoxin System. Apx (2 μM) has been incubated with; ♦, NADPH (2.2 mM); ■, NADPH and NTR (9.75 μM); ▲, NADPH, NTR and PtTrxh1 (10.5 μM) and; ●, NADPH, NTR and PtTrxh1 (100 μM) in phosphate buffer 50 mM, pH 7. Aliquots were assayed at the specified time for residual activity with ascorbate (ascorbate 500 μM and H_2O_2 100 μM)

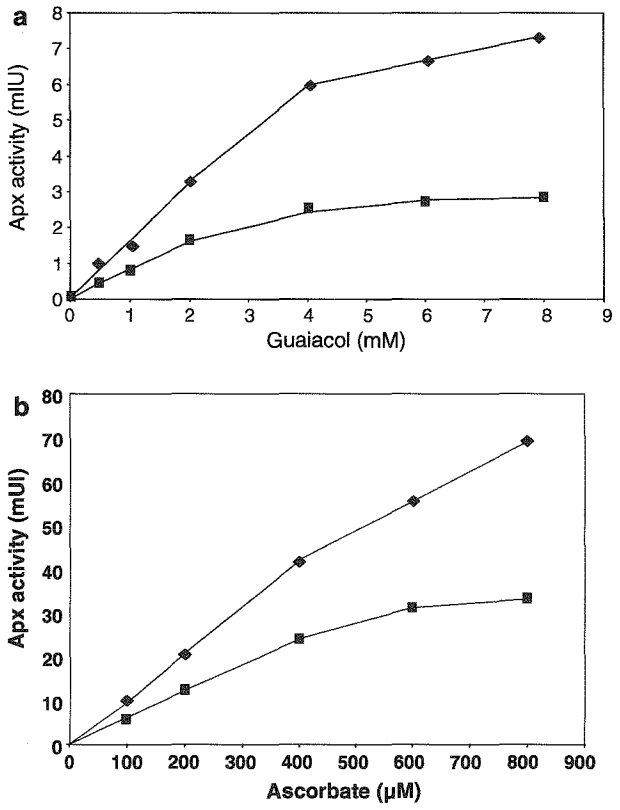

Fig. 2 Steady-state activity of NTS inactivated APX. Apx (13.3 μM) was incubated with 100 μM PtTrxh1, 6.5 μM NTR et 0.4 mM NADPH during 30 min, dialyzed in order to remove NADPH and the activity of the treated sample (■) was assayed in comparison with a control (♦) incubated 100 μM PtTrxh1, 6.5 μM NTR and also dialyzed. The activity has been tested using 30 nM of APX with 100 μM H_2O_2 varying the ascorbate (**A**) or guaiacol (**B**) concentrations

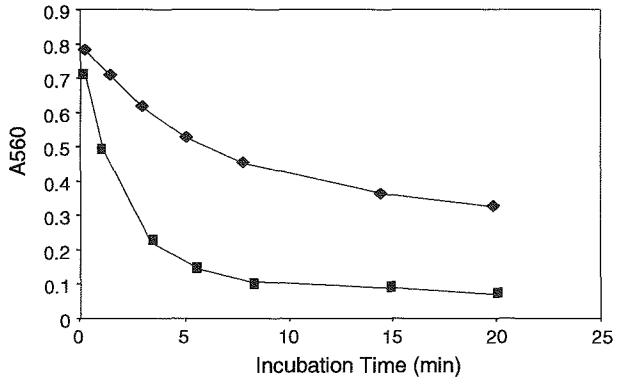

Fig. 3 Hydrogen peroxide disappearance in presence of NTS-inactivated APX. Apx (13.3 μM) was incubated with 100 μM PtTrxh1, 6.5 μM NTR et 0.4 mM NADPH during 30 min, dialyzed in order to remove NADPH and the activity of the (♦) treated sample was assayed in comparison with a control (■) incubated 100 μM PtTrxh1, 6.5 μM NTR and also dialyzed. The activity has been tested using 30 nM of APX, 1 mM H_2O_2 and 600 μM ascorbate

inactivated during the interaction with APX (data not shown).

Effects of various thiol compounds on APX

APX has been preincubated during 30 min with various compounds and its relative residual activity determined using ascorbate as substrate (Fig. 4). A strong inhibition of APX activity was observed using glutathione (1 mM) or DTT (1 mM). PtTrx*h*2 is a mitochondrial thioredoxin, which differs from PtTrx*h*1 by at least the catalytic site, WCGPC and WCPPC, respectively. The observed APX inactivation is similar with both thioredoxins and requires reduced forms of the tested compounds. In all cases, the APX-inactivation is prevented in the presence of ascorbate (1 mM) or guaiacol (9 mM).

APX-inactivation and peroxidases

Nitrites, which have been shown to be electron donors for heme peroxidases (Guo et al. 2004) are able to prevent at least partially APX inactivation by NTS1 (Fig. 5).

Two other proteins containing thiols in their catalytic site have been tested, PtMsrA (Gelhaye et al. 2003) and PtPrxII (Rouhier et al. 2001). PtMsrA is a methionine sulfoxide reductase, whereas PtPrxII is a type II peroxiredoxin catalyzing the reduction of hydrogen peroxide. Both proteins require thioredoxin as a reductant. The APX inactivation by NTS1 is

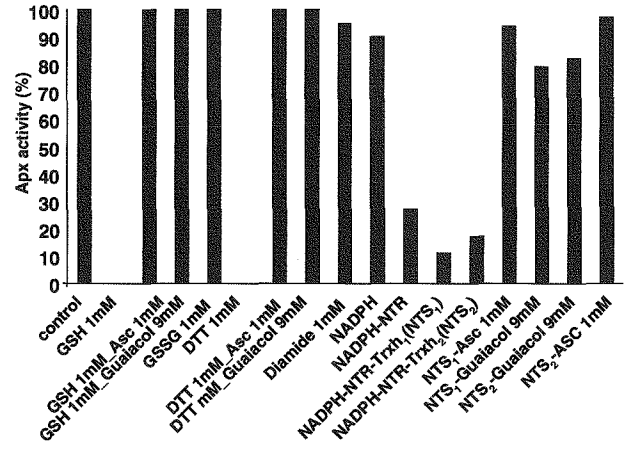

Fig. 4 Effect of various thiol compounds on APX activity. Apx (6.6 μM) has been incubated during 30 min in presence of various compounds. *NTS1*: NADPH 180 μM, PtTrxh1 35 μM, NTR 0.8 μM; *NTS2*: NADPH 180 μM, PtTrx*h*2 35 μM, NTR 0.8 μM. An aliquot (5 μl) has been assayed for activity in presence of 600 μM ascorbate and 100 μM H_2O_2, the control (100%) exhibiting an activity of 53 mUI

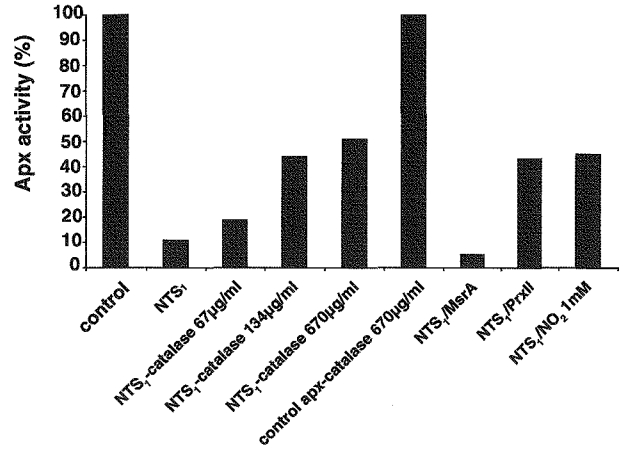

Fig. 5 APX inactivation and peroxidase. Apx (6.6 μM) has been incubated during 30 min in presence of *NTS1*: NADPH 180 μM, PtTrxh1 35 μM, NTR 0.8 μM; with or without catalase (67, 134 and 670 μg/ml), PtMsrA (2 μM), PtPrxII (2 μM), sodium nitrites (1 mM). An aliquot (5 μl) has been assayed for activity in presence of 600 μM ascorbate and 100 μM H_2O_2

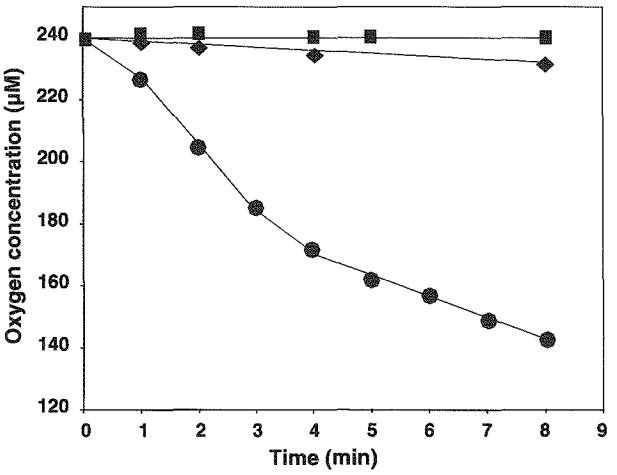

Fig. 6 Oxygen consumption during APX inactivation. APX (14, 5 μM) has been incubated: ■, alone as control; ◆, with 1 mM GSH; ●, with 10 mM GSH in 50 mM phosphate buffer, pH 7.0

enhanced in presence of PtMsrA, while the presence of PtPrxII prevents, at least partially, APX inactivation. In complementary experiments, the effect of various concentrations of catalase has been tested showing that this enzyme can also prevent to some extent the NTS1-induced APX inactivation. Taken together, these data suggest that APX-inactivation process could be linked to hydrogen peroxide formation.

Oxygen consumption

The formation of hydrogen peroxide is consistent with a reaction of thiols with APX generating highly reactive thiyl radical intermediates which can react with oxygen to product hydrogen peroxide according to the following reactions with glutathione as example (Guo et al. 2004):

$$GSH \xrightarrow{APX} GS^{\bullet}$$

$$GS^{-} + GS^{\bullet} \rightarrow GSSG^{\bullet -}$$

$$GSSG^{\bullet -} + O_2 \rightarrow GSSG + O_2^{\bullet -}$$

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^{+} \rightarrow H_2O_2 + O_2$$

$$O_2^{\bullet -} + GSH + H^{+} \rightarrow H_2O_2 + GS^{\bullet}$$

Oxygen consumption experiments have also been performed (Fig. 6). Oxygen consumption is detected in presence of APX (14.5 μM) and GSH 1 mM, whereas APX alone does not exhibit oxygen consumption activity. Oxygen consumption was greatly accelerated when the GSH concentration was increased to 10 mM. A weak oxygen consumption was also detected in presence of APX and NTS1 (data not shown).

Effect of thiols on the spectrum of APX

When GSH (1 mM) was added to APX, the APX spectrum was strongly modified in the Soret region with a maximal absorption peak at a wavelength of 414 nm (410 nm for the untreated APX) (Fig. 7). Furthermore, the spectrum was also altered in the 500–700 nm region. These modifications are similar to those obtained in presence of NTS1 (data not shown), suggesting that APX was able to oxidize sulfhydryl groups.

Effect of thiols on Trp fluorescence

The fluorescence emission spectrum of APX (excitation at 280 nm) was dramatically modified in the

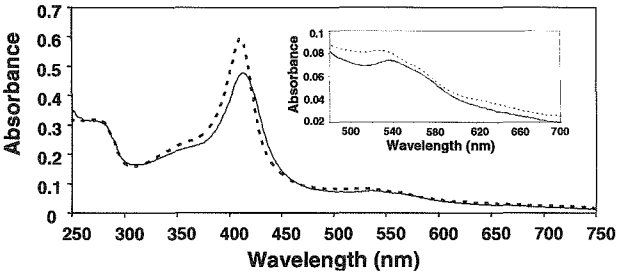

Fig. 7 Effects of glutathione on spectral changes in APX. The reaction was started by adding 1 mM GSH to 6 μM APX and spectra have been recorded immediately (dotted line) and after 30 min of incubation (solid line). The graph in the inset corresponds to the visible part of spectra

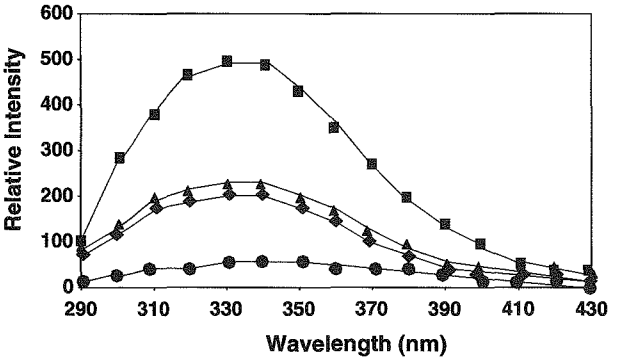

Fig. 8 Effects of glutathione on Trp fluorescence emission spectra. Apx (9.5 μM) in 30 mM Tris–HCl, pH 7.0, was exited at 280 nm (▲), and in presence of either (■) 10 mM GSH or (♦) 10 mM H_2O_2. As control, (●) 10 mM GSH in 30 mM Tris–HCl, pH 7.0 was also used

presence of 10 mM GSH, suggesting that the Trp environment is altered during interaction between GSH and APX. Interestingly, addition of hydrogen peroxide (10 mM) had no effect on Trp fluorescence (Fig. 8). A similar increase in fluorescence emission was observed when APX was previously treated with hydrogen peroxide (10 mM) before GSH addition (data not shown).

Discussion

Cytosolic APX has been found as a potential target of both thioredoxin (Yamazaki et al. 2004; Marchand et al. 2004) and glutaredoxin (Rouhier et al. 2005). These results from three different laboratories have been obtained using two different approaches. In the first case, affinity columns using monocysteinic mutants of either thioredoxin or glutaredoxin have been used, allowing the formation of stable mixed disulfides between the potential targets and the mutants. The trapped targets were then eluted with DTT (Yamazaki et al. 2004; Rouhier et al. 2005) and characterized. In the second case, APX has been detected after direct reduction of the targets by enzymatically reduced Trx followed by labelling of unmasked thiols (Marchand et al. 2004).

Alignment of primary sequences of plant cytosolic APX shows the presence of only one conserved cysteine, Cys32. The role of this cysteine in the APX catalytic mechanism has been extensively studied (Mandelman et al. 1998; Lad et al. 2002). Although Cys32 is located in the vicinity of the ascorbate binding site, modification of this residue with Ellman reagent does not affect oxidation of aromatic substrates as guaiacol. In addition, the C32S mutant exhibits similar activity with guaiacol but, in contrast, there is a dramatic decrease of ascorbate oxidation. All these data suggest that this residue is located close to the ascorbate-binding site confirmed crystallographically (Sharp et al. 2003a) with the binding of aromatic substrates such as guaiacol occurring at a different site (Mandelman et al. 1998; Lad et al. 2002; Sharp et al. 2004).

In this study, we show that thioredoxins as well as reduced glutathione are able to inactivate APX in well defined conditions. Taken together the obtained data suggest that APX is able to oxidize thiol compounds leading to the formation of thiyl radicals. Concerning thioredoxins, the presence of two cysteinyl residues at the catalytic site leads to the formation of a disulfide bridge as shown previously (Goldman et al. 1995). Similar mechanisms could also be proposed for NTR. This process is in agreement with the fact that the NTS enzymatic capacity is not lowered after incubation with APX. Similarly, reduced glutathione could be oxidized by APX as shown previously for other peroxidases (Guo et al. 2004). It is well known that the highly reactive thiyl radical intermediates can react with thiyl anions to produce the disulfide radical anion, which is responsible for oxygen reduction and consequent hydrogen peroxide production (Guo et al. 2004). Detection of oxygen consumption in presence of thiols is consistent with this hypothesis whereas APX exhibits no catalase-like activity (this work, Hiner et al. 2001). Another piece of evidence supporting this hypothesis is that catalase and peroxiredoxin prevent at least partially the inactivation. Finally, the modification of the APX UV/visible spectrum in presence of thiols is consistent with the oxidation of sulfhydryl groups, leading to thiyl radical formation and finally hydrogen peroxide generation. In this context, ascorbate and guaiacol can prevent APX inactivation probably reacting both with APX as substrates and with thiyl radicals to prevent hydrogen peroxide formation. Nitrites, as electron donors, are also able to partially protect APX against inactivation allowing a reduction of APX (Guo et al. 2004).

Inactivation of cytosolic APX has been shown in presence of hydrogen peroxide as sole substrate. In these conditions, the formation of a protein radical has been detected (Hiner et al. 2000; Hiner et al. 2001). By analogy with another peroxidase (Ccp), it has been suggested that a tryptophan residue could be involved (Trp171), the electron transfer from the heme leading to the formation of a Trp radical. Nevertheless, the inactivation of APX in presence of thiols cannot be explained only by the generation of hydrogen peroxide. The Trp fluorescence is indeed dramatically modified in thiol-inactivated APX, whereas no change has been detected in H_2O_2- treated APX (Fig. 8).

The observed inactivation of APX in presence of sulfhydryl components is probably not due simply to an alteration of APX Cys32. Indeed, in the tested conditions, incubation of APX with sulfhydryl groups altered both guaiacol and ascorbate oxidation whereas Cys32 is only involved in the ascorbate binding site (Mandelman et al. 1998; Lad et al. 2002; Sharp et al. 2003a, b, 2004).

Taken together, these data suggest that thiyl radicals induce large changes in APX (particularly in the vicinity of Trp residues) leading to the inactivation of the enzyme. The exact location of these changes is not defined and remains to be determined. Furthermore, it is not clear if these changes are reversible or not. We suggest that the thioredoxin-dependent APX inactivation leads to the exposure of a buried cysteine (probably the conserved Cys32) explaining the fact that APX was found as a potential thioredoxin target, again this hypothesis needs to be confirmed.

The obtained results raise interesting questions about the physiological significance of this possible interaction between APX and thiols. Clearly, APX is able to oxidize exposed protein cysteine residues as shown for thioredoxin and PtMsrA as well as reduced glutathione. From a physiological point of view, this phenomenon could occur when the ascorbate pool decreases and in presence of hydrogen peroxide. In these conditions, APX could play a role in thiol oxidation generating thiyl radical, which finally could lead to glutathionylation. This hypothesis needs further experiments to be confirmed.

This study highlights unexpected interactions between ascorbate peroxidase and thioredoxin that can result in a reductant-linked inactivation of APX. These reactions illustrate that the delicate interplay between the various cellular reducing systems can result in unwanted consequences. In this study, for convenience, we have used recombinant proteins that are normally targeted to the cytosol. Given the functional homology between cytosolic APX and its chloroplast counterparts, and also between thioredoxins of the various compartments, we expect that the APX-thioredoxin interaction could also take place in other compartments than the cytosol, and notably in the chloroplasts. Given the importance of APX in the protection of chloroplasts against reactive oxygen species, this leads to suggest that there should be some type of mechanisms that could prevent this negative effect. One possibility that immediately comes to mind is that a temporal/topological segregation of these systems should be able to prevent the putative damage linked to this reaction.

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Article VI : Catalytic Mechanism of a Glutaredoxin-dependent Thioredoxin from Poplar.

Eric Gelhaye, Nicolas Rouhier, Masakazu Hirasawa, Nicolas Navrot, David B. Knaff, Gunnar Wingsle and Jean-Pierre Jacquot

En préparation.

Cet article décrit la caractérisation d'une isoforme particulière de Trx chez le peuplier. Cette isoforme n'est pas réduite par la voie classique de la NADPH-thiorédoxine réductase, mais elle obtient son pouvoir réducteur des Grx. La Trx h4 présente la particularité de comporter une cystéine conservée en position N-terminale, supplémentaire à celles du site actif. L'étude biochimique des mutants cystéiniques de cette protéine a permis de proposer un mécanisme catalytique pour cette enzyme: l'attaque nucléophile se fait sur la cystéine catalytique typique des Trx (Cys 58). Un pont disulfure se forme ensuite entre Cys58 et Cys61. Ce pont disulfure est alors converti en un pont disulfure entre Cys61 et Cys4, qui est ensuite réduit par la Grx en présence de GSH et GR. La Trx h4 de peuplier est de plus capable de réduire une MSR de type A de peuplier, mais pas les différentes isoformes de Gpx testées. Il s'agit de la première étude montrant la réduction spécifique d'une Trx par une Grx. La glutathionylation de cette Trx est aussi discutée.

Catalytic Mechanism of a Glutaredoxin-dependent Thioredoxin from Poplar

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Running Title: Glutaredoxin-dependent thioredoxin

Summary

A poplar thioredoxin of the *h* type (PtTrx*h*4) has recently been shown to accept electrons from reduced glutaredoxin (Grx). Unlike other thioredoxins *h* characterized so far, NADPH:thioredoxin reductase is not able to catalyze reduction of PtTrx*h*4. PtTrx*h*4 contains three cysteines, one localized in a N-terminal extension (Cys-4) and two (Cys-58 and Cys-61) in the usual thioredoxin active site (WCGPC). Mutants in which each cysteine was replaced by a serine were constructed to investigate the mechanism of the Grx-dependent reduction and the properties of the mutants suggest that all three cysteines are involved in catalysis. Mutation of Cys-58 demonstrates that it is responsible for the initial nucleophilic attack during the catalytic cycle. The observation that the C4S mutant is inactive in presence of Grx but fully active when dithiothreitol is used as reductant suggests that Cys-4 is required for the interaction of PtTrx*h*4 with Grx. Thiol titrations and redox potential determinations showed that two intramolecular disulfide bridges involving Cys-58 can be formed, linking it to either Cys-61 or Cys-4. Overall the results support a three-step reaction mechanism: 1) Cys-58 attacks the sulfur atom of a target protein, 2) the heterodisulfide is then reduced via a double displacement mechanism involving formation of a disulfide bond between Cys-58 and Cys-61, followed by formation of a disulfide bond between Cys-4 and Cys-58, which liberates Cys-61, and 3) the disulfide bridge between Cys-4 and Cys-58 is then reduced either by a bicysteinic glutaredoxin or through a monothiol pathway involving a monocysteinic glutaredoxin.

Introduction

Thioredoxins are small molecular weight proteins found in all organisms from prokaryotes to higher eukaryotes. They are involved in many cellular processes, dealing primarily with cell redox regulation. In plants, numerous isoforms have been reported. For example, at least 20 genes coding for thioredoxins are present in the completely sequenced genome of *Arabidopsis thaliana* (1). The thioredoxins *f*, *m*, *x* and *y* are present in chloroplasts (2, 3), whereas the thioredoxins *o* are localized in mitochondria (4). The thioredoxins *h* constitute a large disparate group including cytosolic and mitochondrial thioredoxins (1, 5, 6, 7). Thioredoxins *h* have been divided in three distinct subgroups in a classification based on protein primary structure and position of the introns (1). Members of the first and second groups are reduced by NADPH in a reaction catalyzed by NADPH-Thioredoxin Reductase (NTR). NTR is a homodimeric flavoprotein, with each subunit containing one NADPH-binding domain and one flavin adenine dinucleotide (FAD)-binding molecule. Members of the first and second thioredoxins *h* subgroups contain a conserved WC[G/P]PC catalytic site. The first cysteine is the one involved in the nucleophilic attack on disulfide bridge present in target proteins, leading to the formation of a disulfide bridge between the target protein and thioredoxin. This intermolecular disulfide is then reduced by the second cysteine, leading to the release of reduced target protein and oxidized thioredoxin.

It is only recently that members of plant thioredoxin *h* subgroup 3 have been detected. Poplar is among the plants in which this type of thioredoxin has been detected (8, 9). A poplar thioredoxin, PtTrx*h*4, contains the usual catalytic site WCGPC but differs from previously characterized thioredoxins *h* in the reduction pathway used. Indeed, this isoform is not reduced *in vitro* by NTR but by glutaredoxins (8). This unique feature raised several questions about the mechanisms of reactions involving PtTrx*h*4. For example, as most characterized

Trxs have a redox midpoint potential of ca -290 mV while Grxs are more electropositive (ca -200 mV), questions about the thermodynamic favorability of reduction of a Trx-like molecule by Grx naturally arise.

The unique properties of PtTrx*h*4 are analyzed in this paper and it is demonstrated that, in contrast to other thioredoxins, three cysteines rather than two are involved in the catalytic mechanism. Two of these cysteines are present in the usual thioredoxin catalytic site ($WC_{58}GPC_{61}$), whereas the third is localized in the N-terminal extension (Cys-4). The existence of alternative disulfide bridges inside the protein has also been demonstrated. From these data and kinetic experiments, a catalytic mechanism has been proposed for this thioredoxin isoform.

Experimental procedures

Cloning and mutations of PtTrxh4

The procedures for cDNA isolation and its subsequent cloning are described in (8). The PtTrxh4 mutants C4S, C58S, and C61S were generated by PCR using the cloning and mutagenic oligonucleotides shown below (*Nco*I and *Bam*H1 sites are underlined and mutagenic bases are in bold): PtTrx*h*4 direct, 5'- CCCCCCATGGGACTTTGCTTGGAT -3'; and PtTrx*h*4 reverse, 5' CCCCGGATCCTCATTTGTCACTAGGGGGCAA 3'; PtTrx*h*4C4S direct, 5'-CCCCCCATGGGACTT**AGC**TTGGATAAGCAT-3'; PtTrx*h*4C58S direct, 5'-TTCAGTGCAACATGGAGTGG**TCC**TTGTAGACAG-3'; PtTrx*h*4C58S reverse, 5'-CTGTCTACAA**GGA**CCACTCCATGTTGCACTGAA-3'; PtTrx*h*4C61S direct, 5'-ACATGGTGTGGTCCT**AGT**AGACAGATTGCACCG-3'; PtTrx*h*4C61S reverse, 5'-CGGTGCAATCTGTCT**ACT**AGGACCACACCATGT-3'.

The mutated PCR products that contained the restriction sites were in turn cloned into the expression plasmid pET-3d, yielding the constructions pET PtTrx*h*4C4S, pET PtTrx*h*4C58S and pET PtTrx*h*4C61S. The mutations of the recombinant plasmids were verified by DNA sequencing.

Expression and purification of the recombinant proteins

All procedures for the expression and purification of *Arabidopsis thaliana* NADPH Trx reductase B, poplar PrxQ, and Grx (WT and mutants) are described elsewhere (10, 11, 12, 13). For the expression of PtTrx*h*4, the recombinant plasmids were used to transform *Escherichia coli* strain BL21(DE3), which was also co-transformed with the plasmid helper pSBET as described in (14). All purification steps are similar to that one described in (8).

Thiol Content Titration

The thiol content of each protein preparation was measured using the DTNB procedure as described in (12).

Glutathionylation experiments

The reaction mixture (50 µL) containing 30 mM Tris-HCl, pH 8.0, 1 mM DTT and 50 µg poplar thioredoxin (concentration *ca* 80 µM) was incubated for 10 minutes before adding 5 mM oxidized glutathione.

Electrospray Mass Spectrometry

A Micromass Q-Tof Ultima (Waters Micromass MS Technologies) hybrid tandem mass spectrometer was used for the acquisition of the electrospray ionization (ESI) mass spectra. This instrument is 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 the collision energy was 10 eV for the MS analyses. Samples for flow injection analyses were diluted 1:20 with a solution of 50:50 acetonitrile:0.1% formic acid. Data analysis was accomplished with a MassLynx data system and Transform deconvolution software supplied by the manufacturer (Waters Micromass MS Technologies).

Redox potential determination.

Oxidation-reduction titrations were carried out as described previously using the fluorescence of the monobromobimane (mBBr)-modified form of the reduced protein to monitor the extent of the protein's reduction (15, 16). Ambient potentials (E_h) were established using mixtures of

oxidized glutathione (GSSG) and reduced glutathione (GSH) and the PtTRX*h*4 samples were incubated at these defined E_h values for 2 hours to allow redox equilibrium to be attained, E_m value was shown to be independent of and the total [GSSG plus GSH] concentration present in the redox equilibration buffer over the range from 2 to 5 mM. The oxidation-reduction midpoint potential (E_m) was calculated by fitting the data to the Nernst Equation for a two-electron process as described previously (17).

PrxQ activity measurement

The reduction of H_2O_2 by poplar PrxQ in the presence of PtTrx*h*4 was followed spectrophotometrically, using a Cary 50 spectrophotometer, by monitoring the decrease in absorbance arising from the oxidation of NADPH in a coupled enzyme assay system. The reaction mixture (500 µL) contained 30 mM Tris-HCl pH 8.0, 1 mM EDTA, 200 µM NADPH, 1 mM GSH, 0.5 IU glutathione reductase, 6 µM Grx, 16 µM PtTrx*h4* WT and mutants, 500 µM H_2O_2 and 2 µM PtPrxQ.

Alternatively, H_2O_2 disappearence was followed directly. The reaction mixture (100 µL) contained 30 mM Tris HCl pH 7.0, 500 µM DTT, 4 µM PtPrxQ and 36 µM PtTrx*h*4. The reaction was started by adding 500 µM H_2O_2. After given incubation times, 5 µL were mixed with 495 µL of FOX1 (ferrous oxidation in xylenol orange) reagent (18). The absorbance was then read at 560 nm after one-hour incubation.

Results

PtTrx*h*4 belongs to the third subgroup of thioredoxin *h* and exhibits the usual thioredoxin WCGPC catalytic site. Apart from cysteinyl residues found in the catalytic site, members of this subgroup exhibit one other conserved cysteine (Fig. 1). This residue is found in a N-terminal extension, another feature of the third subgroup members. Sequence analysis using several localization prediction programs suggest that the N-terminus extension does not correspond to a signal peptide and that this protein is probably cytosolic. In order to investigate the putative role of the three conserved cysteines in the catalytic mechanism of PtTrx*h*4, site-directed mutagenesis has been used in order to produce the following recombinant proteins in *E.coli*: PtTrx*h*4C4S, PtTrx*h*4C58S and PtTrx*h*4C61S.

Thiol content determination

The thiol content of all protein preparations was estimated using the DTNB method. All titrations were performed on enzymes that were free from reductant and after the addition of SDS (see "Experimental procedures"). Consequently, all thiols are titrated regardless of whether or not they were accessible in the native protein. A summary of these data is shown in Table 1. Nearly three thiols per protein are titrated for the reduced WT protein, and approximately one SH group is present in samples of the WT protein in the absence of an added reductant. Under reducing conditions, the thiol contents of the C4S, C58S and C61S mutants were all close to 2SH/mol in good agreement with the expected theoretical values. In the absence of an added reductant, C61S is fully oxidized while C4S and C58S mutants are only partially oxidized (i.e., thiol content values lower than 1 SH/mol were measured).

SDS-PAGE analysis

SDS-PAGE was performed either in presence or in absence of reductant (Fig. 2). In absence of DTT, WT and C61S dimers were detected but monomers were the most abundant forms observed for both proteins. Only the monomer was observed for the C4S mutant, suggesting that an intramolecular disulfide bridge is formed between Cys-58 and Cys-61. In the case of the C58S mutant, oligomeric forms of the protein were present in high abundance, suggesting the presence of intermolecular disulfide bridges involving Cys-4 and/or Cys-61. The fact that both cysteines were fully oxidized in the C61S mutant protein (Table 1) suggests that an intramolecular disulfide bridge between Cys-4 and Cys-58 could be also present in the wild-type protein. The dimer formation observed with the C61S mutant suggests that Cys-58 and/or Cys-4 could be also involved in intermolecular disulfide bridges.

Redox potential determination

The redox titrations of wild-type PtTrx*h*4 and of its three C/S variants, have been carried out at pH 7.0. Each result represents the average of at least two determinations and the average deviations suggest that the experimental uncertainty in E_m is between 5 and 10 mV. Fig. 3A shows the results of a redox titration of PtTrx*h*4 WT over the potential range from −50 to −250 mV. The data give a good fit to the curve expected for a single two-electron process with an E_m value of -165 ± 10 mV. The results obtained for the redox titration of PtTrx*h*4C61S give a good fit to the curve expected for a single two-electron process with an E_m value of −178 ± 10 mV (data not shown). Data from redox titrations of PtTrx*h*4C4S (Fig. 3B) and PtTrx*h*4C58S (data not shown) could not be fitted to the Nernst Equation for a single two-electron process but did give good fits for the sum of the Nernst Equation for two separate two-electron processes. In the case of PtTrx*h*4C4S, the E_m values for the two

components are −140 ± 10 mV and -200 ± 10 mV and for PtTrx*h*4C58S the E_m values of the two components are -130 ± 10 mV and -180 mV.

Glutathionylation

To investigate the susceptibility of PtTrx*h*4 to glutathionylation, the protein was reduced by DTT, incubated with large excess GSSG, and analyzed by quadrupole time-of-flight mass spectrometry. It showed the formation of a low-amplitude peak with a mass of 15,761.00 Da in addition to the peak corresponding to the oxidized recombinant PtTrx*h*4 (15,455.125 Da). This additional peak is compatible with the addition of one glutathione molecule (305.5 Da). A representative analysis of GSSG-treated PtTrx*h*4 is shown in Fig. 4. When the GSSG-treated PtTrx*h*4 was re-reduced with 10 mM DTT, a single peak at 15,459 was obtained (data not shown) as expected for reductive cleavage of the adduct. The susceptibility of PtTrx*h*4C4S, PtTrx*h*4C58S and PtTrx*h*4C61S to glutathionylation was also tested. In these conditions, a large-amplitude additional peak (around 90 % of the total preparation) corresponding to a glutathione adduct was detected with PtTrx*h*4C58S. PtTrx*h*4C61S was glutathionylated to a lesser extent (around 35 % of the total preparation), while PtTrx*h*4C4S showed no detectable glutathionylation (data not shown).

PrxQ activity

The activity of the prepared recombinant proteins was tested using a non-physiological PrxQ-based system involving PtPrxQ, GSH, and PtGrx (8). The activity was followed measuring the NADPH oxidation at 340 nm. Both the C4S and C58S mutants are totally inactive in this system whereas the mutant C61S retains some of the activity exhibited by the WT protein (Fig. 5). Taking into account the data reported from previously characterized thioredoxins, Cys-58 is probably the catalytic residue. As sequence homology comparisons suggest that

Cys58 and Cys61 are the active-site residues (with Cys 58 making the initial nucleophilic attack characteristic of thioredoxins), it is not surprising that converting either of these cysteine residues to serine results in loss of activity. The most interesting feature of PtTrx*h*4, based on the observed total loss of activity for the C4S mutant, is that three cysteines seem to be involved in catalytic mechanism. The interactions between Trxs and PrxQ were also investigated measuring H_2O_2 disappearance in presence of DTT (Fig. 6). Again, the mutant C58S is totally inactive in this system, consistent with the hypothesis that Cys-58 is the catalytic cysteine. The C61S mutant exhibited an activity very much lower than that detected with the wild type protein, showing the importance of Cys-61 in target protein reduction by PtTrx*h*4. In contrast to the results obtained when Grx was used as the reductant, the C4S mutant is fully active when DTT is the donor.

Interaction with PtGrx

The interaction between PtTrx*h*4 and several PtGrx mutants have been investigated using the PrxQ system (Fig. 7). The C27S mutant, in which the catalytic cysteine has been changed to serine, is not active. In this system, mutations of residues surrounding the Grx catalytic cysteines (Y52A and Y55F) altered the reduction efficiency of Grx (Rouhier et al., 2002) and thus the PtTrx*h*4 reduction by these mutants. In contrast, the C30S mutant was always able to reduce PtTrx*h*4, indicating that monocysteinic Grx's are able to interact with this kind of thioredoxin.

Interaction with NTR

It was previously shown that AtNTRB is not able to catalyze the reduction of WT PtTrx*h*4 using NADPH (8). We have now extended this observation by demonstrating (data not

shown) that none of the three PtTrx*h*4 mutants generated in this study, i.e. C4S, C58S and C61S, are also not reduced by NADPH in the presence of AtNTRB.

Discussion

The thioredoxin PtTrx*h*4 described here is the only characterized thioredoxin that is reduced by glutaredoxins. In contrast, all the other thioredoxins characterized to date have been shown to accept electrons either from NTR or FTR (6). It was thus of interest to examine the reasons for the unique properties of this thioredoxin. Three conserved cysteines are present in PtTrx*h*4, two found in the usual thioredoxin active site (WCGPC) and one localized in the first part of the protein (Cys-4). The results presented here clearly indicate that Cys-58 is the catalytic cysteine. The replacement of Cys-4 by serine leads to a fully active protein in presence of DTT. The replacement of Cys-61 by serine does reduce the catalytic efficiency of the enzyme by approximately 75%, regardless of which reduction system is used (Grx or DTT). However, the C61S protein does retain a significant portion of the activity exhibited by the WT protein, behaviour that is strikingly different from that of the C58S mutant, which is completely inactive.

Thiol titrations of the C4S and C61S mutants indicate that two intramolecular disulfide bridges involving Cys-58 could be present in PtTrx*h*4 (Cys-4/Cys-58 and Cys-58/Cys-61). The replacement of Cys-58 by a serine led to an oligomerization of the resulting protein under non-reducing conditions showing the presence of intermolecular disulfide bridge involving Cys-4 and/or Cys-61. Taken together, these results suggest that at least three different disulfide bridges could exist in PtTrx*h*4WT. Oxidation-reduction titrations of both the C4S and C58S mutants of PtTrx*h*4 revealed the presence of two two-electron components. In contrast, only one two-electron component was detected in the WT protein and its C61S variant. The two-electron nature of these redox couples, the E_h range over which they titrate and fact that the experiments rely on a thiol-specific reagent, mBBr, to monitor the course of the titrations leave little doubt that the components being titrated are dithiol/disulfide couples. It is not yet possible to provide a unique assignment of each E_m component in these two-

component titrations because of the multiplicity of possible disulfides that exist. For example, given the observation that WT PtTrx*h*4 and its C/S variants can form disulfide-linked dimers and oligomers makes it possible that one component in a two-component titration may arise from an intramolecular disulfide and the other from an intermolecular disulfide. The presence of glutathione in the redox equilibration buffers (necessitated by the E_h range over which the protein disulfides become reduced) further complicates the situation and leads to the possibility that some of the redox-active components detected in the titrations involve glutathionylated forms of the protein. As only a single redox couple is detected in titrations of the Cys-61 mutant, then either the magnitude of mBBr fluorescence changes associated with titration of a glutathionylated derivative are too small to detect (consistent with the low amplitude of glutathionylation observed for this mutant in mass spectrometry assays) or the E_m values of the glutathionylated and unmodified protein are too close to each other to be resolved. The low level of glutathionylation observed for the C61S may arise from a reduction of oxidized glutathione by the catalytic Cys-58, followed by a partial reduction of this disulfide bridge by Cys-4 in the presence of oxidized glutathione.

The C58S mutant of PtTrx*h*4 to glutathionylation occurs to a much greater extent than is the case for the C61S variant. This may well be due to the fact that the absence of Cys-58, the strongest nucleophile in the protein, allows the glutathionylated adduct formed at Cys-61 to accumulate because there is no nucleophile present to cleave the adduct. In the case of the C4S mutant, no glutathione adduct could be observed. It is possible that any mixed disulfide formed between glutathione and either active-site cysteine might be unstable in this mutant (7, 19, 20), as was shown previously using a synthetic peptide containing the C[G/P]PC motif (19).

The apparent E_m value of -165 mV measured for WT PtTrx*h*4 at pH 7.0 in single-component redox titrations may represent an over-simplified picture of the situation and may in fact

reflect the presence of more than one disulfide bridge with the E_m values of different components being too close to one another to be resolved as separate components (simulations suggest that the E_m values would have to differ). Setting aside these possible complications and assuming that the component with an E_m value of -165 mV at pH 7.0 seen in titrations of WT PtTrx*h*4 can be attributed either entirely or in large part to the active-site disulfide, it is obvious that this value is much more positive than E_m values in the -280 mV to -310 mV range observed for other thioredoxins from oxygenic photosynthetic organisms that have been characterized to date (6, 7, 21). However an E_m value of -165 mV is fully compatible with a thermodynamically favourable complete reduction of PrTrx*h*4 by glutaredoxins (22).

The mutant C4S is fully active in presence of DTT but remains inactive in presence of GSH alone or in presence of GSH/Grx. These data demonstrate that Grx is not able to reduce the disulfide bridge present between Cys-58 and Cys-61. However, it is not clear whether this absence of reduction of this mutated PrTrx*h*4 by Grx arises because the reduction is thermodynamically unfavourable or because of some kinetic (rather than thermodynamic) barrier. In contrast, the observation that C61S remains partially active in presence of Grx suggests that reduced Grx is able to reduce the disulfide bridge linking Cys-58 and Cys-4.

A scheme for the reaction of reduced PtTrx*h*4 with an oxidized target protein (e.g., PrxQ) is shown in Fig. 8. As is the case for other thioredoxins, catalysis proceeds through the formation of a heterodimer between the target protein and PtTrx*h*4 involving the catalytic Cys-58. The heterodisulfide is then cleaved by Cys-61, leading to the release of the reduced target protein and the formation of a disulfide bridge between Cys-58 and Cys-61. This disulfide bond is then cleaved by Cys-4 leading to the formation of a disulfide between Cys-4 and Cys-58. The reduced form of the protein can then be regenerated in a reaction with reduced Grx. The fact that both a WT Grx and a GrxC30S mutant are active in PtTrx*h*4

reduction clearly demonstrates that monocysteinic glutaredoxins are able to reduce PtTrx*h*4. In this mechanism, the thiolate of Grx initiates a nucleophilic attack on the thioredoxin mixed disulfide. A new disulfide between Grx and one thioredoxin cysteine is formed, which could then be reduced by GSH or another monocysteinic Grx leading to the release of the reduced Trx (12).

The catalytic mechanism proposed for PtTrx*h*4 is related to the one proposed previously for methionine sulfoxide reductase A, a mechanism that involves three cysteines and the formation of two successive intramolecular disulfide bridges during catalysis before the regeneration of the enzyme by thioredoxins. (23). This kind of mechanism requires that the three cysteines are spatially close enough in order to allow the disulfide formations. PtTrx*h*4 exhibits a N-terminal extension containing Cys-4 that is not present in other thioredoxins *h* (1, 3, 6). While it is possible to speculate that this extension allows the interaction of Cys-4 with the catalytic Cys-58 (there is certainly no current evidence that argues against this possibility), a three-dimensional structure of PtTrx*h*4 is needed to confirm this hypothesis.

Orthologs of PtTrx*h*4 are also present in different plants. Based on amino acid sequence comparisons, it seems quite reasonable to assume that these proteins should, like PtTrx*h*4, be able to use Grx as an electron donor. This assumption is consistent with the report that the *Phalaris cerelescens* ortholog is not reduced by NTR (9). It has been shown that ribonucleotide reductase cannot use monocysteinic Grx as donor whereas other reactions catalyzed by Grx require either bicysteinic or monocysteinic Grxs (22). Clearly, both versions are able to promote the activity of PtTrx*h*4. Interestingly, there are many naturally occurring monocysteinic Grx in plants. It will be of high interest to compare the relative efficiencies of the corresponding proteins for PtTrx*h*4 catalysis.

The physiological role of this particular kind of glutaredoxin-reducible thioredoxins remains to be elucidated (the interaction of PtTrx*h*4 with the chloroplastic PrxQ used in this study is a

non-physiological reaction). Although PtTrx*h*4 is unable to reduce several isoforms of poplar glutathione peroxidases (N. Navrot, unpublished), it is able to reduce a cytosolic methionine sulfoxide reductase A and a Prx II from poplar (8). In looking for other possible targets, it will be necessary to keep in mind the fact that the E_m value of PtTrx*h*4 is approximately 130 mV more positive than that of most thioredoxins and thus thermodynamic considerations will limit the number of protein disulfides that PtTrx*h*4 is capable of reducing.

Another characteristic of PtTrx*h*4 is its ability to be glutathionylated. This property is not unique to PtTrx*h*4, as human thioredoxin (19) and at least one other poplar thioredoxin, PtTrx*h*2 have been shown to be glutathionylated. In the case of poplar Trxh2, glutathionylation produces a significant shift in the E_m value of the protein (7). In the case of PtTrx*h*4, glutathionylation could be a way to protect the enzyme against oxidative damage (23). However, it is not yet clear if the protein forms glutathione adducts *in vivo*.

PtTrx*h*4 is the first example of a thioredoxin reduced by glutaredoxin. This observation forces one to consider the possibility that a direct interconnection between these two major redox regulating systems may well be physiologically important. Further investigations are needed to understand the physiological role of this unique thioredoxin and to explore its possible role as an important link between different pools of regulatory reductants.

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Fig. 1. Multiple alignment of related sequences to poplar Trx*h*4. This comparison was realized using CLUSTALW software. Accession numbers of the sequences aligned with PtTrx*h*4 are as follows: At9: *Arabidopsis thaliana* (At3g08710); Gm : *Glycine max* (CA799351) ; Hb : *Hordeum bulbosum* (AF159385) ; Hv : *Hordeum vulgare* (AF435815) ; Lp : *Lolium perenne* (159387) ; Ls : *Lactuca sativa* (TC9851) ; Mc : *Mesembryanthemum crystallinum* (CA838461) ; Os : *Oryza sativa* (AF435817) ; Pc : *Phalaris coerulescens* (AF159388) ; Pt : *Populus tricocharpa* (BU867450) ; Sb : *Sorghum Bicolor* (TC72759) ; Sc : *Secale cereale* (AF159386) ; Ta : *Triticum aestivum* (AF438359) ; Vv : *Vitis vinifera* (CB004453) ; Zm : *Zea mays* (AF435816). The asterisk corresponds to strict identity, the colon corresponds to functional homology, and the period corresponds to structural homology.

Fig. 2. Analysis of purified recombinant proteins by SDS-PAGE. 14% SDS-PAGE under both reducing and nonreducing conditions showing PtTrx*h*4WT, PtTrx*h*4C4S, PtTrx*h*4C58S and PtTrx*h*4C61S.

Fig. 3. Oxidation-reduction titrations of wild-type PtTrxh4 (A) and its C4S variant at pH 7.0 (B). Both proteins at a concentration of 68 µg/ml were titrated in 100 mM MOPS buffer (pH 7.0) at a total glutathione concentration of 2 mM with a redox equilibration time of 2h.

Fig. 4. Glutathionylation of PtTrx*h*4. The deconvoluted spectrum of PtTrx*h*4 incubated with 5 mM GSSG are shown. Two peaks are detected corresponding to: A, oxidized protein; B, glutathionylated protein.

Fig. 5. Activity of PtPrxQ in presence of PtGrx and PtTrx*h*4. PtPrxQ (2 μM) was incubated with either PtTrx*h*4WT or the different mutants (16 μM) in presence of the GSH/Grx system (200 μM NADPH, 1 mM GSH, 0.5 IU glutathione reductase, 6 μM PtGrx, 500 μM H_2O_2). NADPH oxidation was measured at 340 nm as absorbance change per min.

Fig. 6. Activity of PtPrxQ in presence of DTT and PtTrx*h*4. The reduction of H_2O_2 by PtPrxQ (5 μM) was measured by following the disappearance of 500 μM H_2O_2 using 1 mM DTT in presence of either PtTrxh4WT or the different mutants (30 μM).

Fig. 7. Activity of PtPrxQ in presence of PtTrx*h*4 and various catalytic site mutants of PtGrx. PtPrxQ (2 μM) was incubated with PtTrx*h*4WT (16μM) and PtGrx (6 μM) in presence of 200 μM NADPH, 1 mM GSH, 0.5 IU glutathione reductase and 500 μM H_2O_2. NADPH oxidation was measured at 340 nm as absorbance change per min.

Fig. 8. Proposed mechanism for Grx-dependent PtTrx*h*4 catalysis

Table 1. Thiol content of PtTrx*h*4 under oxiding or reducing conditions. Thiols were titrated using the reduction of DTNB as described under "Experimental procedures." Data are expressed in mol SH/mol enzyme. The S.D. is typically ± 0.2 SH/ mol SH.

	Native conditions	Reducing conditions
PtTrx*h*4 WT	0.75	2.88
PtTrx*h*4 C4S	0.93	1.64
PtTrx*h*4 C58S	0.64	1.93
PtTrx*h*4 C61S	0.05	1.55

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Sec             -------MGGCVG--KGR-SIVEEKLDFKGGNVHVITTKEDWDQKIEEANKDGKIVVANF
Ta              -------MGGCVG--KDR-SIVEEKLDFKGGNVHVITTKEDWDQKIEEANKDGKIVVANL
Hv              -------MGGCVG--KGR-GVVEEKLDFKGGNVHVITTKEDWDQKIEEANKDGKIVVANF
Hb              -------MGGCVG--KDR-SIVEDKLDFKGGNVHVITTKEDWDQKVAEANKDGKIVVANF
Lp              -------MGGCVG--KDR-SIVEDKLDFKGGNVHVITTKEDWDQKVAEANKDGKIVVANF
Pc              -------MGGCVG--KDR-GIVEDKLDFKGGNVHVITTKEDWDQKIAEANKDGKIVVANF
Os              -------MGGCVG--KGRRHIEEDKLDFKGGNVHVITSKEDWDRKIEEANKDGKIVVANF
Zm              -------MGGCAG--KVR-RDDEEKLDFKGGNVHIITSNEGWDQKIAEANRDGKTVVANF
Sc              MGLKRLMTCCCCI--AKQHADDEDKIDFGGGNVHVVTSKEDWDQKIAEANKDGKIVVANF
Ls              -------MGICFSSTHNDGDESDHNAEFAGGNVTLVSSKDAWDQKLSEAKKDHKIVIANF
Vv              -------MGQCFMKHHNDDDDSDHNAAFASGNVHLITTKENWEEKLAEASKDGKIVIANF
Gm              -------MGSCVSKNKARDNDSDHNVDFAAGNVKLITTKEAWDQYLEEARRDGKIVIANF
Mc              -------MGLCLSKNSADGGESSH-LELAGGNVHLVTTKEAWEGKLSQASREGKIVIANF
Pt4             -------MGLCLAKRNHDADDDEPHIELAGGNVHLITTKERWDQKLSEASRDGKIVLANF
At9             -------MGSCVSKGKGD-DDSVHNVEFSGGNVHLITTKESWDDKLAEADRDGKIVVANF
                       *                 : .*** :::::: *:  : :* :: * *:**:

Sec             SASWCGPCRVVAPVYAGMSKTYPQLMFLTIDVDDLMDFSSTWDIRATPTFFFLKNGQQID
Ta              SASWCGPCRVIAPVYAEMSKTYPQLMFLTIDVDDLMDFSSTWDIRATPTFFFLKNGQQIE
Hv              SASWCGPCRVIAPVYAEMSKTYPQLMFLTIDVDDLMDFSSTWDIRATPTFFFLKNGQQID
Hb              SASWCGPCRVIAPVYAEMSKTYPQLMFLTIDVDDLMDFGSTWDIRATPTFFFLKNGQQID
Lp              SASWCGPCRVIAPVYAEMSKTYPQLMFLTIDVDDLMDFSSTWDIRATPTFFFLKNGQLID
Pc              SASWCGPCRVIAPVYAEMSKTYPQLMFLTIDVDDLVDFSSTWDIRATPTFFFLKNGQQID
Os              SASWCGPCRVIAPIYAEMSKTYPQLMFLTIDVDDLMDFSSSWDIRAKPTFFFIKNEKQVD
Zm              SASWCGPCRVIAPVYAEMSKTYPQLMFLTIDVDDLMDFSSSWDIRATPTFFFLKNGQQID
Sc              SASWCGPCRVISPVYAEMSQTYPQLMFLTIDVDELMEFSSSWDIRATPTFFFLKNGQQVD
Ls              SASWCGPCRMIAPYYIELSEKHPSLMFLSVDVDELTDFSTQWDIKATPTFFFLRNGEQFD
Vv              SATWCGPCKMIAPFYCELSEAHPSLMFLTVDVDELSEFSSSWDIKATPTFFFLRDGQQVD
Gm              SAAWCGPCKMIAPYYCELSEKYTSMMFLVVDVDELTDFSTSWDIKATPTFFFLKDGQQLD
Mc              SAGWCGPCKMIAPYYSELSEKYPCLTFVTVDVDELIDLSTSYDIKATPTFFFLKDGQQID
Pt4             SARWCGPCKQIAPYYIELSENYPSLMFLVIDVDELSDFSASWEIKATPTFFFLRDGQQVD
At9             SATWCGPCKIVAPFFIELSEKHSSLMFLLVDVDELSDFSSSWDIKATPTFFFLKNGQQIG
                ** *****: ::* :  :*: :. : *: :***:* ::.: ::*:*.*****::: : .

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Hv              KLVGANKPELEKKVQALGDGS-----------
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Lp              KLVGANRPELEKKVQAIGDGS-----------
Pc              KLVGANKPELEKKVQALGDGS-----------
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Zm              KLVGANKPELEKKVLAAADASTS---------
Sc              KLVGANKPELEKKVAAIAGASSQADAASSKTV
Ls              KLVGANKPELLKKINAIVDSEPPRRAV-----
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Gm              KLVGANKPELQKKIVAINDSLPEYKQ------
Mc              KLVGSNRPELLKKIIAISDSQGKCPDQ-----
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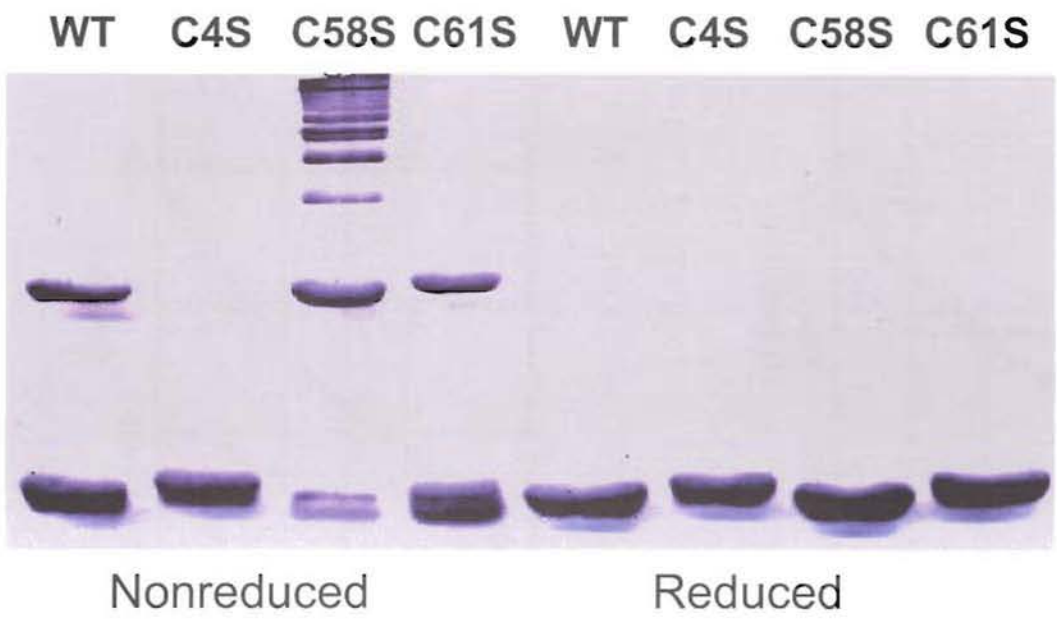
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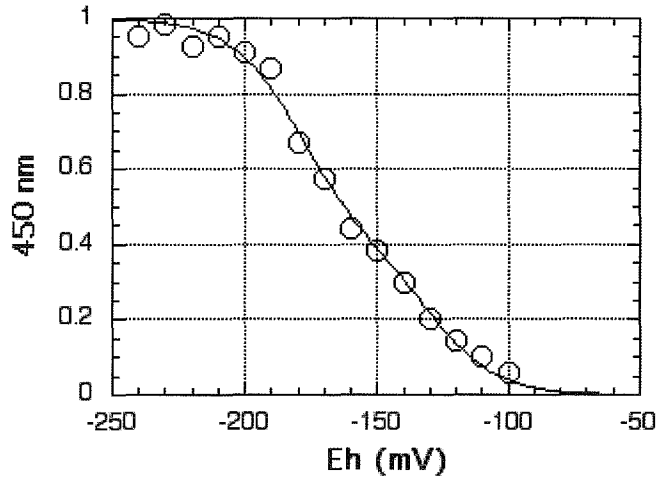
Reduced

A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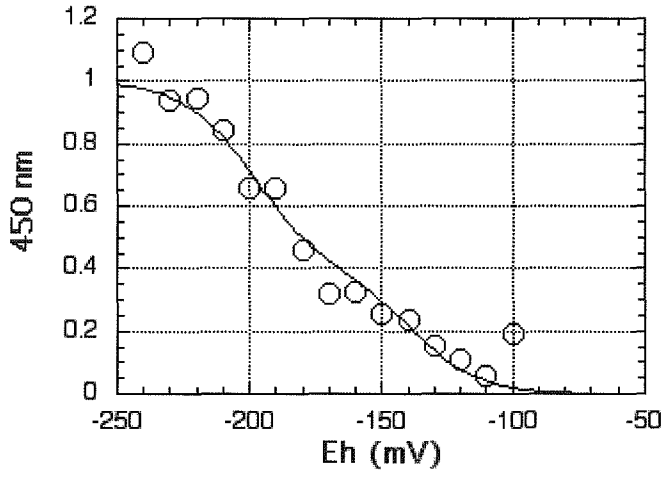
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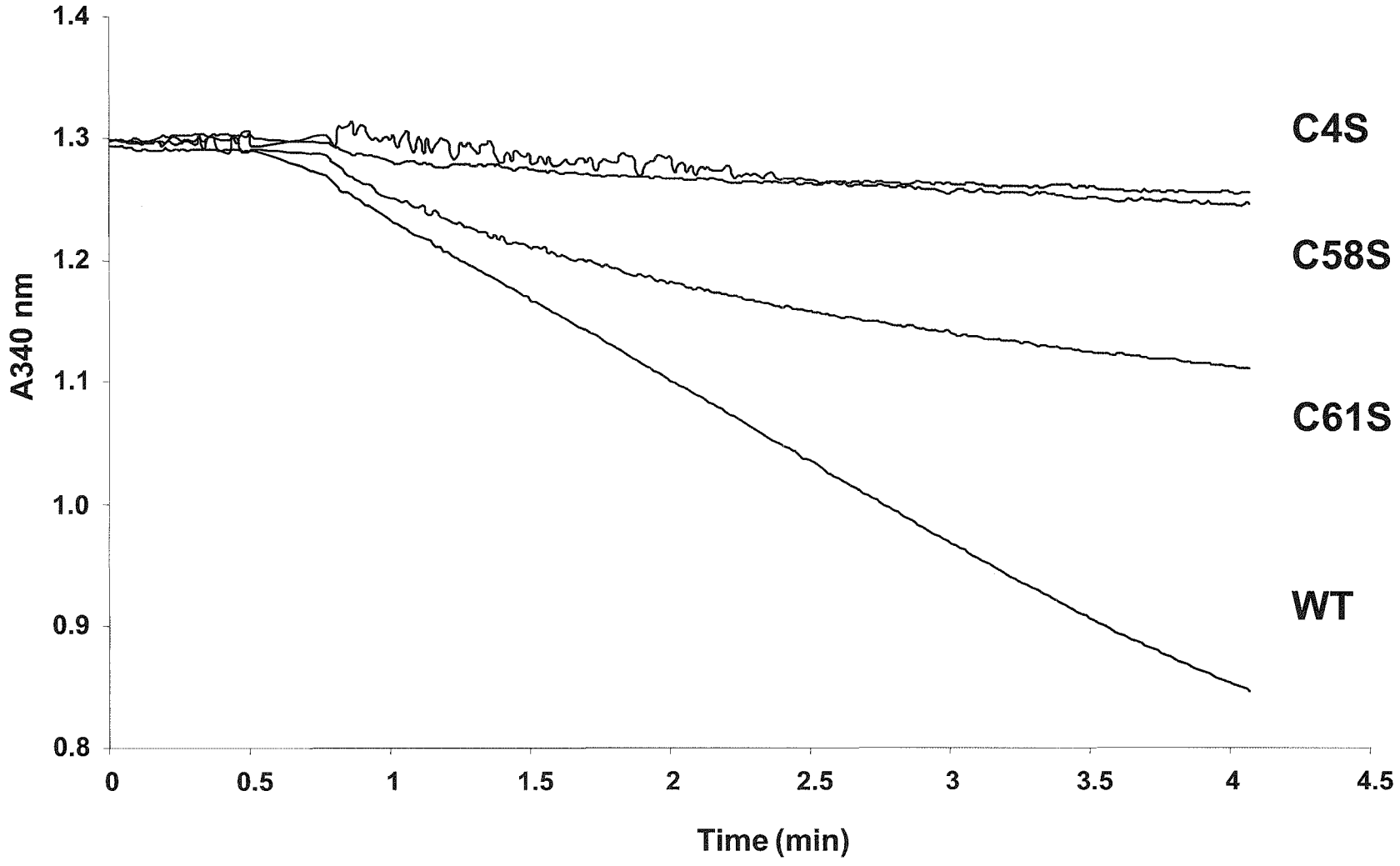
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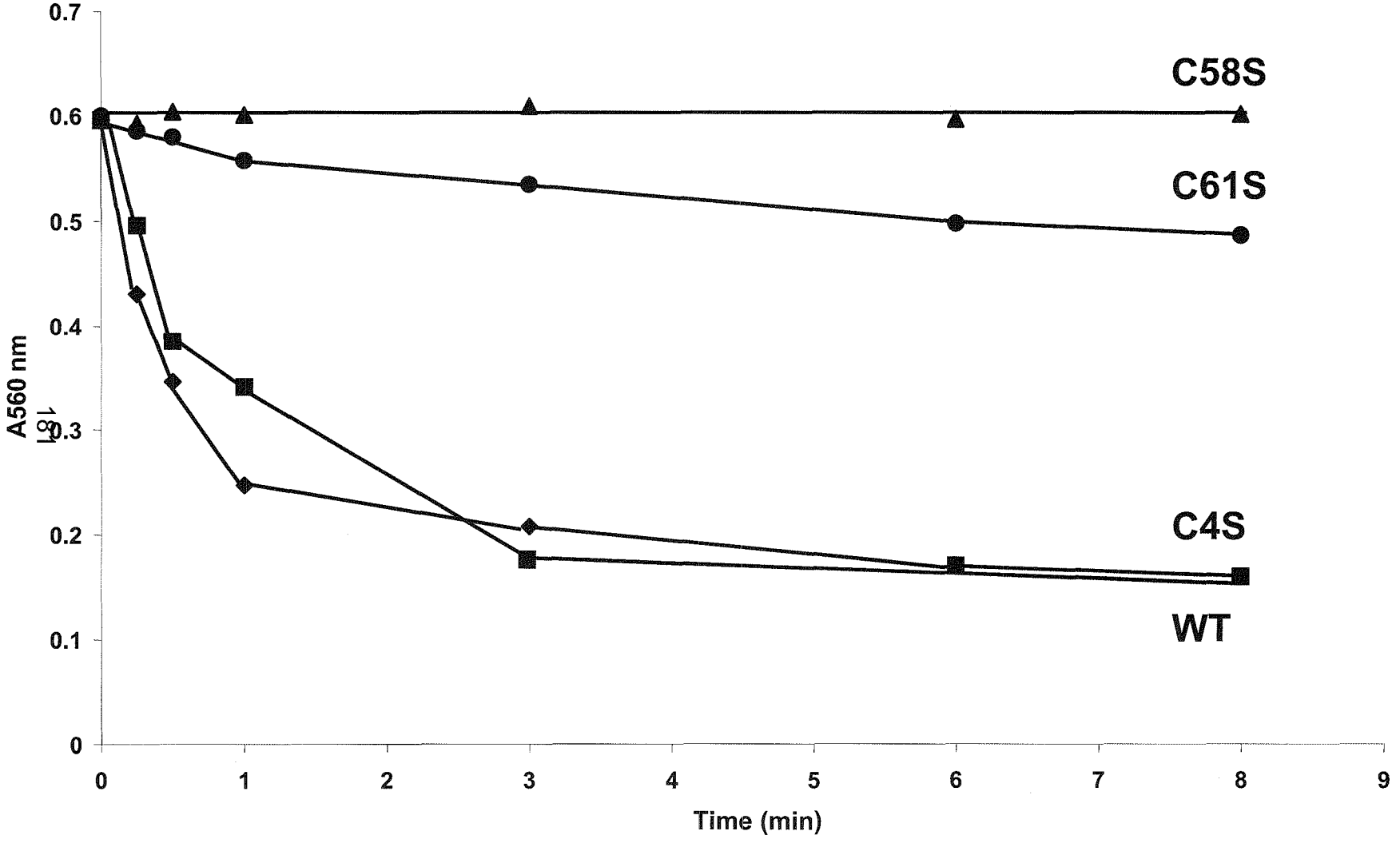
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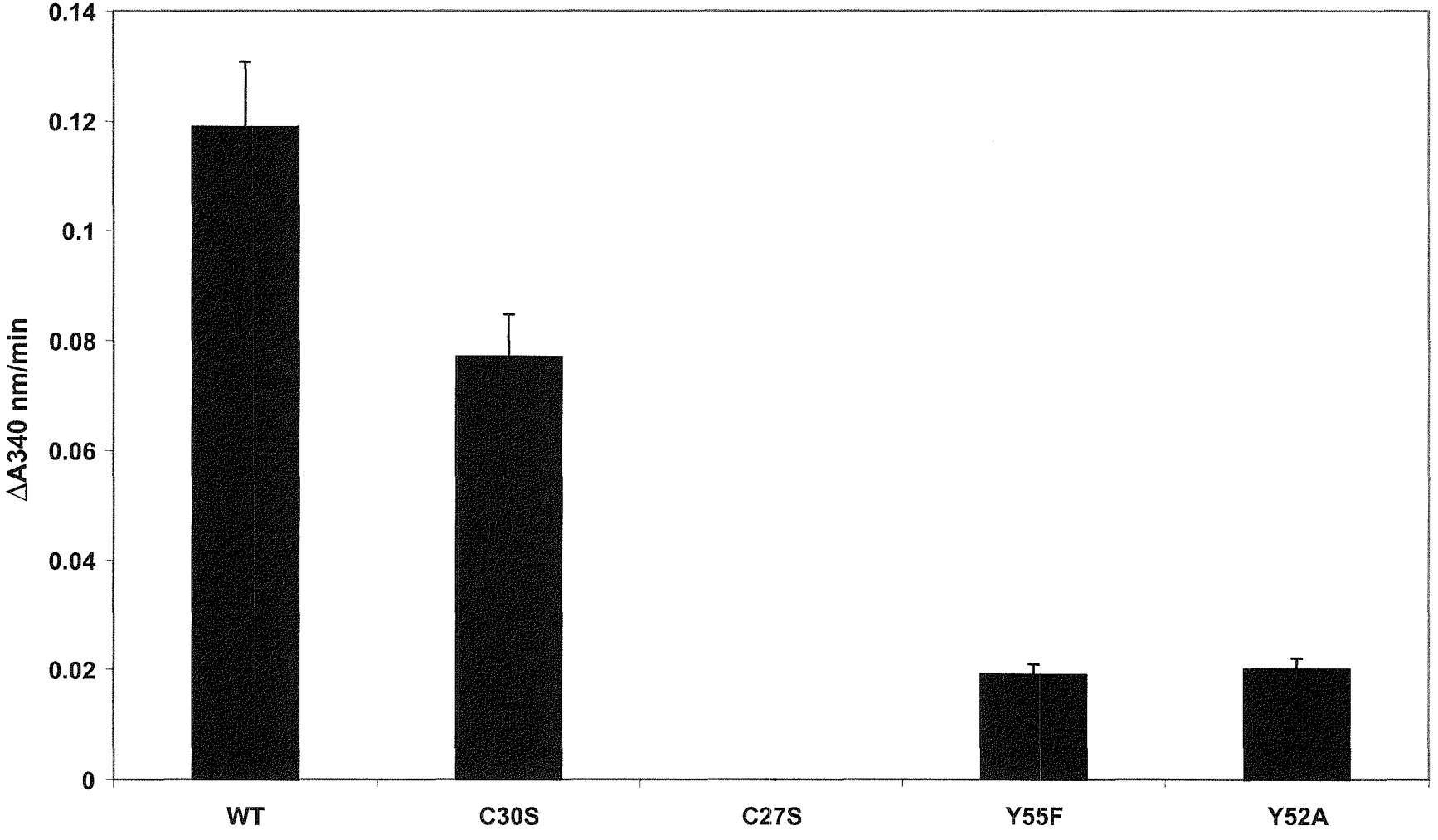
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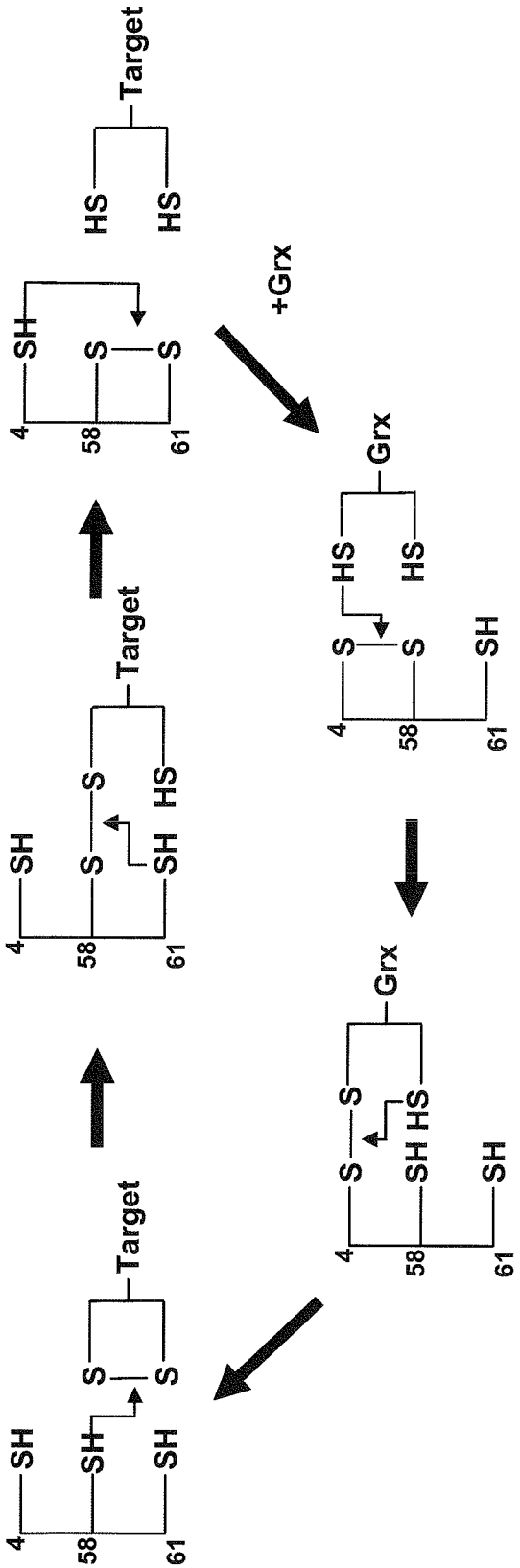

Article VII : Plant glutathione peroxidases are functional peroxiredoxins distributed in several subcellular compartments and regulated during biotic and abiotic stresses

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Nicolas Navrot, Valérie Collin, José Gualberto, Eric Gelhaye, Masakazu Hirasawa, Pascal Rey, David B. Knaff, Emmanuelle Issakidis, Jean-Pierre Jacquot, Nicolas Rouhier

Plant Physiology (2006), 142: 1364-1379

Cet article décrit la caractérisation de la famille des glutathion peroxydases (Gpx) chez le peuplier. Six gènes codant pour des Gpx ont été répertoriés dans le génome. Puisque deux des gènes sont très proches, cinq gènes ont été clonées en vue d'exprimer les protéines dans un système recombinant et des études biochimiques ont été réalisées sur quatre d'entre elles (une protéine s'est révélée insoluble et inactive). Ces protéines sont capables de réduire différents types de peroxydes, avec des efficacités variables selon les isoformes testées et en utilisant le pouvoir réducteur des thiorédoxines. Ainsi, parmi les Trx chloroplastiques, les Gpxs chloroplastiques (Gpx1 et 3) ne sont réduites que par les Trx y. En revanche, aucune activité n'est détectée quand le glutathion et/ou les glutarédoxines sont utilisés comme donneur de pouvoir réducteur. Ces Gpx végétales qui ont une activité de type thiorédoxine peroxydase sont fonctionnellement proches des peroxyrédoxines. La production de mutants cystéiniques de la Gpx3 de peuplier a par ailleurs permis de déterminer l'implication de deux cystéines, en position 107 et 155, dans les mécanismes de réduction des peroxydes et de régénération par les Trx. L'expression des différentes gènes a été analysée par RT-PCR, et l'abondance des protéines par

western blotting. Il apparaît que l'expression des gènes de Gpx de peuplier est variable selon l'organe testé, et que certaines isoformes de Gpx d'*Arabidopsis thaliana* sont induites par un stress, notamment par la présence de métaux lourds. Certaines protéines possèdent des extensions en position N-terminale, ce qui laisse penser que ces protéines sont présentes dans les différents compartiments cellulaires. La localisation de Gpx1 (chloroplaste) et Gpx3 (chloroplaste et mitochondrie) a été confirmée expérimentalement par fusion avec une protéine fluorescente (GFP).

Plant Glutathione Peroxidases Are Functional Peroxiredoxins Distributed in Several Subcellular Compartments and Regulated during Biotic and Abiotic Stresses[1][W]

Nicolas Navrot, Valérie Collin, José Gualberto, Eric Gelhaye, Masakazu Hirasawa, Pascal Rey, David B. Knaff, Emmanuelle Issakidis, Jean-Pierre Jacquot, and Nicolas Rouhier*

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We provide here an exhaustive overview of the glutathione (GSH) peroxidase (Gpx) family of poplar (*Populus trichocarpa*). Although these proteins were initially defined as GSH dependent, in fact they use only reduced thioredoxin (Trx) for their regeneration and do not react with GSH or glutaredoxin, constituting a fifth class of peroxiredoxins. The two chloroplastic Gpxs display a marked selectivity toward their electron donors, being exclusively specific for Trxs of the y type for their reduction. In contrast, poplar Gpxs are much less specific with regard to their electron-accepting substrates, reducing hydrogen peroxide and more complex hydroperoxides equally well. Site-directed mutagenesis indicates that the catalytic mechanism and the Trx-mediated recycling process involve only two (cysteine [Cys]-107 and Cys-155) of the three conserved Cys, which form a disulfide bridge with an oxidation-redox midpoint potential of −295 mV. The reduction/formation of this disulfide is detected both by a shift on sodium dodecyl sulfate-polyacrylamide gel electrophoresis or by measuring the intrinsic tryptophan fluorescence of the protein. The six genes identified coding for Gpxs are expressed in various poplar organs, and two of them are localized in the chloroplast, with one colocalizing in mitochondria, suggesting a broad distribution of Gpxs in plant cells. The abundance of some Gpxs is modified in plants subjected to environmental constraints, generally increasing during fungal infection, water deficit, and metal stress, and decreasing during photooxidative stress, showing that Gpx proteins are involved in the response to both biotic and abiotic stress conditions.

In plant cells, aerobic reactions such as photosynthesis or respiration lead to reactive oxygen species (ROS) production. These ROS, such as superoxide radicals, hydroxyl radicals, or hydrogen peroxide (H_2O_2), can damage biological molecules, including nucleic acids, lipids, and proteins. Rates of ROS generation and cellular ROS levels both increase greatly when plants are subjected to environmental or biotic stresses. Plants have developed several nonenzymatic and enzymatic systems to protect against oxidative damage caused by these ROS. Carotenoids, tocopherols, glutathione (GSH), and ascorbate are the major nonenzymatic antioxidant compounds (Noctor and Foyer, 1998). Enzymatic systems include superoxide dismutases, catalases, ascorbate peroxidases, and peroxiredoxins (Prxs), also named thioredoxin (Trx) peroxidases.

Usually, plant Prxs are classified as belonging to one of four subgroups, called 2-Cys Prx, 1-Cys Prx, type II Prx, and Prx Q. They were initially identified by sequence analysis (i.e. on the number and position of conserved Cys) and on the catalytic mechanism used for peroxide reduction (Rouhier and Jacquot, 2002). All Prxs display a conserved catalytic Cys, located in the N-terminal portion of the protein, which is first

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Nicolas Rouhier (nrouhier@scbiol.uhp-nancy.fr).

[W] The online version of this article contains Web-only data.
www.plantphysiol.org/cgi/doi/10.1104/pp.106.089458

transformed into a sulfenic acid after peroxide reduction. The main difference between the various groups of plant Prxs is the mechanism of regeneration of the reduced enzyme. For the 2-Cys Prx class, the sulfenic acid is reduced via the formation of an intermolecular disulfide bridge using a resolving Cys found on another subunit, whereas for the Prx Q class, the resolving Cys is located on the same subunit and forms an intramolecular disulfide bridge (Rouhier and Jacquot, 2002). In most cases, these disulfides are then reduced via the Trx system. For the two other classes, the sulfenic acid is likely to be reduced without formation of a disulfide bond, as there is no other strictly conserved Cys involved in catalysis. One major difference between these two classes of plant Prxs is that type II Prxs are reduced by the glutaredoxin (Grx) or the Trx system or both (Rouhier et al., 2001; Brehelin et al., 2003; Finkemeier et al., 2005), whereas the 1-Cys Prxs seem to be reduced only by the Trx system (Pedrajas et al., 2000).

Recent studies have shown that some plant and yeast (*Saccharomyces cerevisiae*) GSH peroxidases (Gpx) can reduce peroxides, much more efficiently or sometimes exclusively, by using the Trx system rather than GSH as a reductant (Herbette et al., 2002; Jung et al., 2002; Tanaka et al., 2005). In the unicellular parasite *Plasmodium falciparum*, a Gpx-like protein, closely related to plant Gpxs, has also been shown to be specific for Trx as a reductant (Sztajer et al., 2001). Thus, in a classification based on biochemical feature rather than on phylogenetic linkage, the plant Gpxs constitute a fifth group of Prxs (Rouhier and Jacquot, 2005).

Most of the Gpxs found in animal cells are well-characterized selenium-containing enzymes. Because of the high reactivity of the active site seleno-Cys, enzymes of this family are among the most efficient antioxidant systems in animal cells (Maiorino et al., 1990). One of these selenium-containing enzymes has also been found in a unicellular photosynthetic organism, the green alga *Chlamydomonas reinhardtii* (Fu et al., 2002; Novoselov et al., 2002). In higher plants, the Gpx-like proteins identified so far possess a Cys instead of a seleno-Cys at their active site. Some studies showing that plant Gpxs are far less reactive than their animal counterparts have attributed the lower activity of plant Gpxs to the lower reactivity of Cys compared to seleno-Cys (Eshdat et al., 1997). However, replacement of the active-site Cys with seleno-Cys did not significantly increase the GSH-dependent peroxidase activity of a citrus Gpx (Hazebrouck et al., 2000). Thus, at least in some cases, other differences between animal and plant enzymes exist and produce different biochemical properties. Avery et al. (2004) have shown some gaps in the amino acid sequences of yeast Gpxs compared to mammalian Gpxs, raising the possibility of some important biochemical differences between these two types of Gpxs. It should also be noted that yeast and plant Gpxs may play a role distinct from that of their animal counterparts and could be part of an alternative pathway that scavenges peroxides, especially phospholipid hydroperoxides (Eshdat et al., 1997).

The level of Gpx mRNA from various organisms is affected by stress conditions (Criqui et al., 1992; Holland et al., 1994; Sugimoto and Sakamoto, 1997; Depege et al., 1998; Roeckel-Drevet et al., 1998; Li et al., 2000; Agrawal et al., 2002; Rodriguez Milla et al., 2003; Kang et al., 2004). Other studies have followed changes in Gpx activity in stress conditions (Aravind and Prasad, 2005; Kuzniak and Sklodowska, 2005; Ali et al., 2006; Gajewska and Sklodowska, 2006). In these studies, the Gpx expression or activity is generally up-regulated in response to stress. However, there are exceptions to this pattern. For example, while the expression of two Gpx isoforms increases in barley (*Hordeum vulgare*) under osmotic or methyl viologen-induced stress, a third Gpx isoform (HVGPH3) is down-regulated under these conditions (Churin et al., 1999). These proteins may thus have different functions in plant cells, with one or more isoforms functioning in a signal transduction pathway, while other isoforms serve as enzymes involved in catalyzing modification of potentially harmful stress-related chemical agents. A signal-transducing role for a Gpx has already been demonstrated in yeast, which possesses three plant-type Gpxs, where Gpx3 functions as a redox sensor and is involved in gene activation by regulating the AP1 transcription factor (Delaunay et al., 2002).

The annotation of the first release of the poplar (*Populus trichocarpa*) genome indicates the presence of six complete *Gpx* genes. To understand why so many Trx-dependent peroxidases are present in plant cell compartments, we first focused our attention on the specificity of Gpx proteins toward different electron donors and substrates. All of them use Trx but not GSH as a donor, but most importantly, a specific interaction was found between chloroplastic Gpxs and y-type Trxs. No specificity was observed with regard to substrates. Moreover, we have demonstrated that only two of the three conserved Cys present in plant Gpxs participate in the catalytic cycle and in Trx-mediated regeneration. The subcellular localization of two Gpx isoforms was characterized by green fluorescent protein (GFP) fusions in two distinct subcellular compartments, and the gene expression and protein abundance were analyzed in various plant tissues and stress conditions. The amount of some Gpxs is modified upon pathogen infection and in response to water deficit and photooxidative and metal stresses, revealing their participation in stress responses.

RESULTS

Sequence and Genome Analysis

Five different isoforms of Gpx, termed PtrcGpx 1 to 5 (for poplar Gpx) were initially identified in the different poplar expressed sequence tag (EST) databases available. With the recent release of the first version of the poplar genome sequence by the Department of

Energy (DOE) Joint Genome Institute (JGI), a sixth isoform, very similar to PtrcGpx3, was identified, and these two isoforms were named PtrcGpx3.1 and PtrcGpx3.2. The only obvious difference between the two sequences is the presence of an N-terminal extension in PtrcGpx3.2, otherwise they are 90% identical and display a 99% functional homology in their predicted mature form. The protein sequences of all poplar Gpxs identified are shown in Figure 1 and compared with Gpxs from yeast and human. The percentage of strict identity ranges from 62% to 90% among all poplar Gpxs and from 20% to 48% versus yeast and mammalian Gpxs. The lengths of the proteins vary from 168 to 238 amino acids, depending on the presence of N-terminal extensions in some isoforms.

From this amino acid sequence comparison, it appears that poplar Gpxs are more closely related to yeast Gpxs than to human Gpxs. Indeed, three Cys (residues generally assumed to be essential for Gpx catalysis) are not only strictly conserved in all poplar Gpxs but also in all higher plant and yeast sequences (Fig. 1; data not shown). This is in clear contrast to animal Gpxs, which display either two conserved Cys residues or in some isoforms a Cys and a seleno-Cys. The difference in the number of conserved Cys could result in different reactivities for plant and yeast Gpxs compared to mammalian enzymes. The closest human homolog to plant and yeast Gpxs is Gpx4, also known as phospholipid hydroperoxide Gpx. Some residues are highly conserved in all these sequences, especially in the neighborhood of the two first conserved Cys (the respective consensus sequences are VASx[C/U]G and FPCNQF, with U being the symbol for seleno-Cys). The three residues, Gly, Gln, and Trp denoted with asterisks in Figure 1, which are involved in the catalytic mechanism of the mammalian seleno-Gpxs (Prabhakar et al., 2005), are also conserved in plant and yeast sequences. However, no information is available about the role of these residues in plant or yeast Gpxs.

Another interesting feature, arising from the poplar genome release, is that the *gpx* gene structure is very conserved in poplar. All the genes contain six exons of almost the same length, whereas the size of the intron sequences varies greatly. The major differences are found in the first exon, because it includes putative targeting sequences for some isoforms (Supplemental Table S1). Another difference is the slightly smaller size of exon 6 for *PtrcGpx1* (30 nucleotides instead of 33 in the other *Gpx* sequences, resulting in a PtrcGpx1 sequence shortened by one amino acid in the C terminus). This organization is similar in Arabidopsis (*Arabidopsis thaliana*), where the eight genes also contain six exons. In Arabidopsis, the last exon is also shorter for some genes, in particular for the homologs of *PtrcGpx1* (Rodriguez Milla et al., 2003; data not shown).

Some characteristics of poplar Gpxs are summarized in Table I. The consensus subcellular predictions obtained with different software (Predotar, TargetP, Mitoprot, and Psort) are as follows: PtrcGpx1 carries a putative plastidic transit peptide; PtrcGpx3.2, a mitochondrial or plastidic one; and PtrcGpx2 and PtrcGpx5, a peptide presumably directing the proteins into the secretory pathways, whereas PtrcGpx4 and PtrcGpx3.1 should be localized in the cytosol.

We then built a phylogenetic tree using most of the plant Gpx sequences present in the databases with an emphasis on the eight and five Gpxs identified in Arabidopsis and *Oryza sativa* genomes, respectively (Fig. 2). Although the tree was constructed using sequences devoid of N-terminal extensions, five subgroups of Gpx, which are likely to correspond to the predicted intracellular protein localization, can be defined. This means that there are probably some amino acid signatures specific for each subgroup. It also appears that some organisms possess two members of the same group. For example, Arabidopsis has two putative chloroplastic and two cytosolic Gpxs, explaining the higher gene content in this plant. In contrast, the subgroup including the predicted secreted proteins is not found in *O. sativa*.

Biochemical Characterization

We have cloned five of the six Gpx sequences, minus their N-terminal extensions, in the pET-3 d plasmid for subsequent expression in *Escherichia coli*. Because of the high similarity between PtrcGpx3.1 and PtrcGpx3.2, we have only expressed the mature form of PtrcGpx3.2, which was the most abundant in terms of ESTs and also the first identified. The sizes of the mature forms that we have expressed range from 164 to 171 amino acids. All the proteins, except PtrcGpx2, were soluble. After purification to homogeneity, the yields ranged from 5 mg for PtrcGpx2 (after resolubilization from inclusion bodies) to about 100 mg of protein/L culture for the other isoforms.

Electron Donor Specificity

As it was demonstrated earlier that Trx was a better electron donor than GSH for some Gpx-like proteins, we have tested different electron donor systems, i.e. the Trx and Grx systems or GSH alone. We have first measured the Gpx activity using a spectrophotometric test coupled to NADPH oxidation.

Except for PtrcGpx2, which was completely inactive, all the proteins (PtrcGpx1, PtrcGpx3.2, PtrcGpx4, and PtrcGpx5) catalyzed H_2O_2 reduction at similar rates using poplar Trx h1 as the electron donor, NADPH, and recombinant Arabidopsis type B NADPH-thioredoxin reductase (data not shown; Table II). In contrast, no activity was found when using either GSH alone or in combination with different poplar Grxs belonging to the same subgroup, either dithiol Grx with a classical CxxC active site (Grx C1, C2, C3, and C4) or a monothiol chloroplastic Grx (Grx S12), which possesses a CxxS active site (see Rouhier et al., 2006 for the nomenclature; data not shown).

Because all recombinant proteins, with the exception of PtrcGpx2, were equally active in transferring

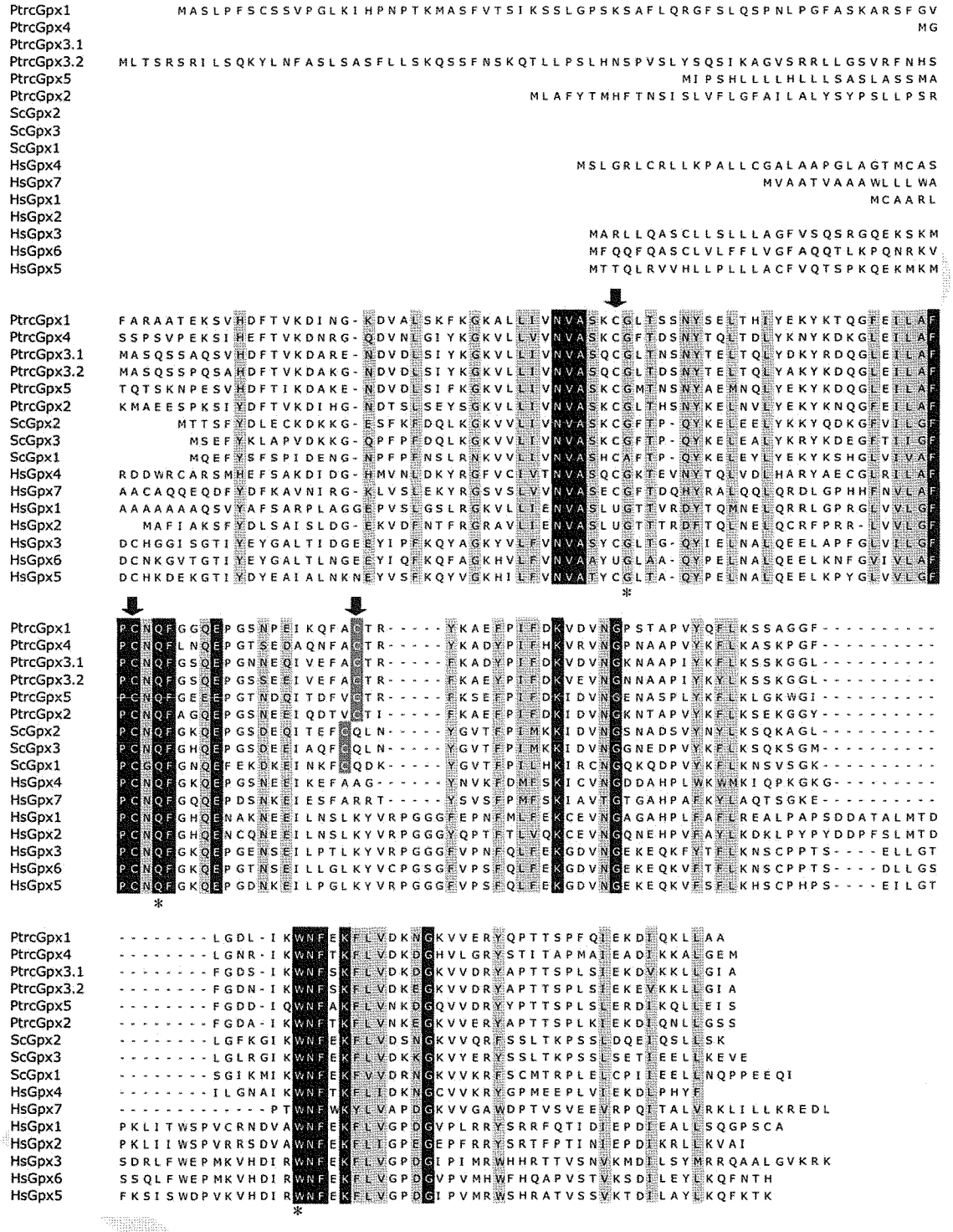

Figure 1. Amino acid sequence alignment of Gpxs from poplar, yeast, and human. Poplar Gpx gene models from DOE JGI *Populus trichocarpa* v1.0 database (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html): PtrcGpx1, grail3.0013048601; PtrcGpx2, estExt_Genewise1_v1.C_LG_VII0868; PtrcGpx3.1, estExt_Genewise1_v1.C_LG_I8960; PtrcGpx3.2, fgenesh4_kg.C_LG_III000037; PtrcGpx4, estExt_fgenesh4_kg.C_LG_XIV0026; PtrcGpx5, eugene3.00010913. Accession numbers of yeast and human Gpx: ScGpx1, P36014; ScGpx2, P38143; ScGpx3, NP012303; HsGpx1, P07203; HsGpx2, AAH05277; HsGpx3, P22352; HsGpx4, NP_002076; HsGpx5, NP_001500; HsGpx6, NP_874360. Strictly conserved amino acids are on black background, functionally conserved amino acids are on gray background. Arrows show the three conserved Cys in positions 107, 136, and 155 (PtrcGpx1 numbering). Asterisks show three residues involved in the catalytic mechanism in human selenoenzyme. U indicates seleno-Cys in human enzymes.

Table I. *Characteristics of poplar Gpxs*

The molecular masses of the recombinant mature proteins are indicated. The masses of the full-length proteins including signal peptide are in parentheses. Poplar gene models were found in the DOE JGI poplar v1.0 database. Arabidopsis homologs are named according to Rodriguez Milla et al. (2004), except for AtGpx8, which was not described in this paper. *O. sativa* homologs were found using the Institute for Genomic Research *O. sativa* genome annotation database (http://www.tigr.org/tdb/e2k1/osa1/index.shtml). The localization predictions are the consensus from results obtained with Predotar (http://genoplante-info.infobiogen.fr/predotar/predotar.html), TargetP (http://www.cbs.dtu.dk/services/TargetP/), Mitoprot (http://ihg.gsf.de/ihg/mitoprot.html), and WoLF Psort (http://wolfpsort.seq.cbrc.jp/) software. Ptrc, Poplar.

Protein	Gene Model	Length (Amino Acids)	Molecular Mass	EST No.	Predicted Localization	Predicted TP Length	Arabidopsis Homolog	*O. sativa* Homolog
			D					
PtrcGpx1	grail3.0013048601	164 (232)	18,250 (25,359)	48	Chloroplastic	71	AtGPX1 (At2g25080), AtGPX7 (At4g31870)	LOC_Os06g08670
PtrcGpx2	estExt_Genewise1_ v1.C_LG_VII0868	170 (203)	18,842 (22,850)	10	Secreted	38	AtGPX2 (At2g31570), AtGPX3 (At2g43350)	LOC_Os11g18170
PtrcGpx3.1	estExt_Genewise1_ v1.C_LG_I8960	168	18,691	23	Cytosolic	–	AtGPX6 (At4g11600)	LOC_Os04g46960 LOC_Os02g44500
PtrcGpx3.2	fgenesh4_kg.C_LG_ III000037	168 (238)	18,550 (26,306)	85	Mitochondrial chloroplastic	70	AtGPX6 (At4g11600)	LOC_Os04g46960 LOC_Os02g44500
PtrcGpx4	estExt_fgenesh4_ kg.C_LG_XIV0026	171	18,950	17	Cytosolic	–	AtGPX4 (At2g48150), AtGPX5 (At3g63080)	LOC_Os03g24380
PtrcGpx5	eugene3.00010913	169 (203)	19,250 (21,498)	8	Secreted	31	AtGPX8 (At1g63460)	–

electrons from reduced Trx to H_2O_2, only one of them, PtrcGpx3.2, was selected for more detailed study.

As PtrcGpx3.1, the cytosolic isoform, is very similar in sequence to PtrcGpx3.2, we tested in vitro various cytosolic Trxs of the h type in an attempt to find a potential physiological electron donor for this protein, using NADPH oxidation coupled to the NTR-catalyzed reduction of Trx to monitor activity. All the poplar Trx h tested (Trx h1, h3, and h5) are efficient electron donors for PtrcGpx3.2 in vitro, with apparent K_m values being around 10 μM (Table II). It was previously shown that Trx h2, although it colocalizes with PtrcGpx3.2 in mitochondria, is a very poor electron donor (Gelhaye et al., 2004). We next tried to identify a preferred, and hopefully physiological, electron donor for the two chloroplastic Gpxs, PtrcGpx1 and PtrcGpx3.2. A colorimetric method was used for these assays as an alternative to the assay based on monitoring NADPH oxidation, because chloroplastic Trxs are reduced by a light-dependent system involving ferredoxin and ferredoxin Trx reductase. As chloroplastic Trxs are not reduced by NTR, the reducing capacity of Arabidopsis chloroplastic Trxs was tested in the presence of 500 μM dithiothreitol (DTT), a reagent that will reduce these Trxs but does not support the peroxidase activity of Gpxs during the relatively short time used for the assay. Of all the chloroplastic Trxs tested, only the two Arabidopsis y-type Trxs (Trx y1 and Trx y2) were able to reduce Gpx to a significant extent. Similar results were obtained for PtrcGpx1 and PtrcGpx3.2, with the reduced y-type Trxs leading to an 80% decrease of the H_2O_2 content with both Gpxs (Fig. 3; data not shown). The other isoforms tested did not support Gpx activity. The cytosolic poplar Trx h1, present at the same concentration as the chloroplastic Trxs, was approximately as effective as the y-type Trxs in supporting H_2O_2 reduction catalyzed by either PtrcGpx1 or PtrcGpx3.2. Despite the similar reactivity of the cytoplasmic Trx h1, the fact that the two y-type Trxs present in the poplar genome colocalize with PtrcGpx1 and PtrcGpx3.2 in chloroplasts suggests that the two Trx y may well constitute in vivo reductants for these two Gpxs.

Peroxide Specificity

The substrate specificity of PtrcGpx3.2 was tested under steady-state conditions using Trx h1 as reductant and different types of peroxides, ranging from H_2O_2 to more complex molecules, such as tert-butyl hydroperoxide (tBOOH) or cumene hydroperoxide (COOH), as electron acceptors. The K_m values for the different substrates and the catalytic efficiencies of the enzymes are summarized in Table II. Although slightly different (K_m ranging from 239 μM to 1.41 mM), the K_m values for the three substrates are all of the same order of magnitude in the millimolar range. In terms of catalytic efficiency (k_{cat}/K_m), PtrcGpx3.2 is slightly more efficient in reducing COOH (5.3×10^4 M^{-1} s^{-1}) compared to H_2O_2 (20×10^3 M^{-1} s^{-1}) and to tBOOH (6.4×10^3 M^{-1} s^{-1}).

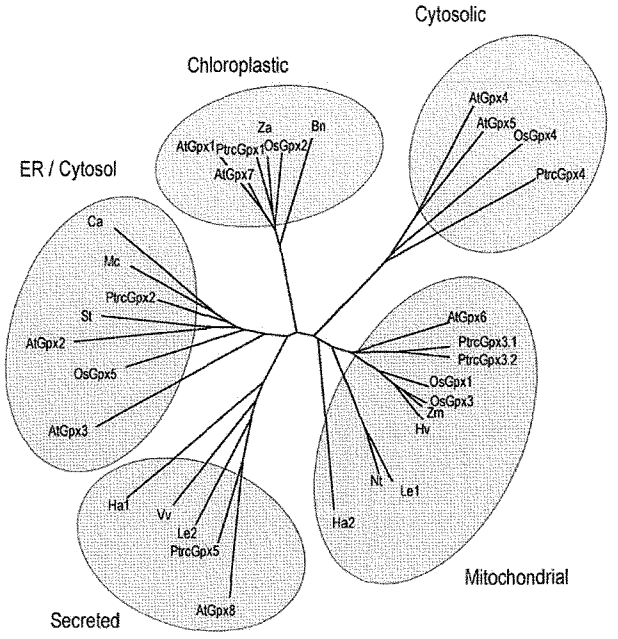

Figure 2. Phylogenetic tree of plant Gpxs. The gene models used for poplar Gpxs are indicated in Figure 1. The other accession numbers (full-length cDNAs or ESTs) are as follows. Arabidopsis: AtGpx1, At2g25080; AtGpx2, At2g31570; AtGpx3, At2g43350; AtGpx4, At2g48150; AtGpx5, At3g63080; AtGpx6, At4g11600; AtGpx7, At4g31870; AtGpx8, At1g63460. *O. sativa*: OsGpx1, AY100689; OsGpx2, CB666847; OsGpx3, CF306402; OsGpx4, CF281628; OsGpx5, AAX96834. *Helianthus annuus*: Ha1, CAA74775; Ha2, CAA75009. *L. esculentum*: Le1, BI209257; Le2, AI898013. *Brassica napus*: AF411209. Ca, *Cicer arietinum*, CAD31839; Hv, *H. vulgare*, BJ448530; Mc, *Momordica charantia*, AF346906; Nt, *N. tabacum*, CK286614; St, *Solanum tuberosum*, BM404021; Vv, *Vitis vinifera*, CB978870; Za, *Zantedeschia aethiopica*, AAC78466; Zm, *Zea mays*, BI319059. Only the predicted mature sequences were used for tree building.

We then examined the stoichiometry of the reaction catalyzed by Gpxs, by mixing known concentrations of reduced enzyme with various known concentrations of tBOOH in the absence of reductants. After completion of the reaction, the remaining peroxide content was measured using the ferrous oxidation of xylenol orange (FOX) colorimetric method. All the enzymes tested (PtrcGpx1, 3.2, and 5) can reduce 1 mol substrate per mole enzyme, indicating that Gpxs use only one or two Cys but certainly not three.

It is known that, for enzymes that use sulfenic acid chemistry for their catalysis, the stoichiometry depends on the number of Cys involved. When one or two Cys are involved, the stoichiometry of the reaction is 1 mol of enzyme oxidized per mol of peroxide reduced (Boschi Muller et al., 2000). In one case, the first peroxidatic Cys attacks the substrate and is converted to a sulfenic acid, with the reaction stopping at this point. More commonly, a second resolving Cys attacks the sulfenic acid, leading to the formation of a disulfide bridge. When a third Cys is involved in the catalytic mechanism, as it is the case for some Met sulfoxide reductases, the stoichiometry of the reaction is 2 (Boschi Muller et al., 2005). In this case, the third Cys (or the second resolving Cys) reduces the first disulfide bridge formed between the peroxidatic Cys and the first resolving Cys, leading to a new intramolecular bridge. Then, the peroxidatic Cys would be free and able to attack another molecule of peroxide, leading to a consumption of 2 mol peroxide per mole of reduced enzyme.

Catalytic Mechanism

To understand the mechanism used by PtrcGpxs for peroxide reduction and for the Trx-dependent regeneration, site-directed mutagenesis was used to generate four variants of PtrcGpx3.2 (PtrcGpx3.2 C107S, C136S, C155, or C136/155S) in which Cys residues were replaced by Sers. The activity of the mutated proteins was tested in the presence of the Trx system. As expected, the Cys 107S mutant is totally inactive. While PtrcGpx3.2 C155S or the double mutant PtrcGpx3.2 C136/155S are able to reduce peroxides in single-turnover experiments (i.e. prereduced samples of these two variants can reduce an equal amount of peroxide), they are completely inactive with the Trx-reducing system, regardless of the peroxide substrate used. This indicates that Trx is probably not able to directly reduce the sulfenic acid formed on Cys-107. In contrast, PtrcGpx3.2 C136S displays almost identical enzymatic properties to PtrcGpx3.2 (Table II). The only difference is a small modification of the K_m value for peroxides that results in a decreased catalytic efficiency. These results indicate unequivocally that Cys-107 and Cys-155 are required for Trx regeneration, whereas Cys-136, albeit strictly conserved and found in a very conserved motif, does not have a catalytic role.

Table II. *Steady-state kinetic parameters of PtrcGpx3.2 and PtrcGpx3.2 C136S*

Values for the different peroxides were obtained using Trx h1 as electron donor. A total of 200 nM of enzyme was used in the tests. PtrcGpx C107S, C155S, and C136/155S mutants are inactive with the Trx system.

		K_m	k_{cat}	k_{cat}/K_m
		mM	s^{-1}	$(M^{-1}\ s^{-1}) \times 10^3$
H_2O_2	PtrcGpx3.2	0.545	11	20
	PtrcGpx3.2 C136S	1.388	13	12
tBOOH	PtrcGpx3.2	1.41	9.08	6.4
	PtrcGpx3.2 C136S	4.66	12	2.6
COOH	PtrcGpx3.2	0.239	13	53
	PtrcGpx3.2 C136S	0.454	16	35
		K_m		
		μM		
tBOOH	Trx h1	12		
	Trx h3	13		
	Trx h5	10.3		

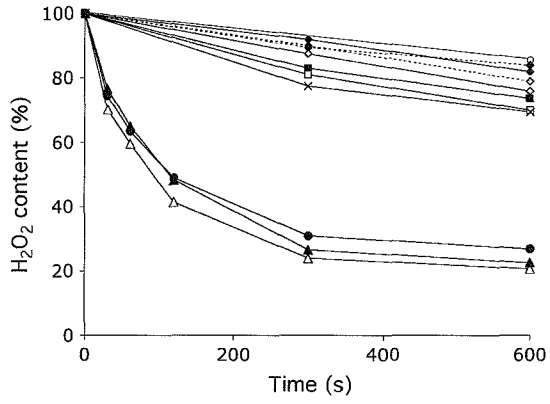

Figure 3. Time-course-dependent H_2O_2 reduction by PtrcGpx1 in the presence of chloroplastic Trxs. Activity of PtrcGpx1 was tested using different chloroplastic Arabidopsis Trxs by following H_2O_2 consumption in function of time. Reactions contain 2 μM PtrcGpx1, 500 μM DTT, 400 μM H_2O_2, and 10 μM of the different Arabidopsis Trx. From top to bottom: control without addition of Trx (○), Trx m3 (◆, dotted line), Trx m1 (◆), Trx m4 (◇, dotted line), Trx m2 (◇),Trx f1 (■), Trx f2 (□), Trx x (x), poplar Trx h1(●), Trx y2 (▲), and Trx y1 (△).

Redox and Oligomerization States

We examined the behavior of PtrcGpx3.2 and its various Cys/Ser variants during denaturing polyacrylamide gel electrophoresis (SDS-PAGE) under different conditions. Under reducing conditions (30 mM DTT), all the proteins migrated at their expected molecular masses, around 19 kD (Fig. 4A, lanes 2, 4, 6, 8, and 10). Under oxidizing conditions (30 mM H_2O_2), the migration of PtrcGpx3.2, as well as of its C107S and C136S variants, is shifted to a lower apparent molecular mass (around 13 kD), whereas the migration of C155S and C136S/155S mutated proteins was not altered (Fig. 4A, lanes 1, 3, 5, 7, and 9). The change in the migration profile under oxidizing conditions for all the proteins in which Cys-155 has not been mutated suggests that an intramolecular disulfide bond involving Cys-155 can be formed. The disulfide, formed between Cys-107 and Cys-155 in the case of the C136S mutant and between Cys-136 and Cys-155 in the case of the C107S mutant, apparently modifies the overall structure of the proteins sufficiently to alter the migration behavior during electrophoresis. It should also be pointed out that a covalent dimer, involving Cys-107 of two subunits, can be observed for the double mutant under nonreducing conditions but not under reducing conditions, as has been previously observed for mutated proteins in which only the catalytic Cys remains (Rouhier et al., 2004). These results were confirmed by following changes in intrinsic Trp fluorescence emission of the proteins in reduced or oxidized conditions (Fig. 4B). Only one Trp found in position 196 in the WNF motif and conserved among organisms is present in PtrcGpx3.2. The behavior of PtrcGpx3.2 is shown in Figure 4B as an example, but all Gpxs behave similarly. While the protein is in the oxidized form after purification (the number of detectable thiol groups is generally close to zero), adding 250 μM DTT changed the emission fluorescence spectrum of the protein, with a decrease of approximately 3-fold in the fluorescence intensity and a shift of the maximum of fluorescence emission from 350 to 316 nm. The addition of 250 μM peroxide in the cuvette completely restored the initial spectrum, suggesting that changes of Trp environment and thus of fluorescence emission are linked to changes in oxidation state. Among the cysteinic-mutated proteins, only PtrcGpx3.2 C136S showed similar differences in the fluorescence properties of the oxidized and reduced forms. All other mutated proteins (PtrcGpx3.2 C107S, PtrcGpx3.2 C155S, and PtrcGpx3.2 C136S/155S) did not share this property (data not shown). As observed under SDS-PAGE, where it abolished the shift between oxidized and reduced

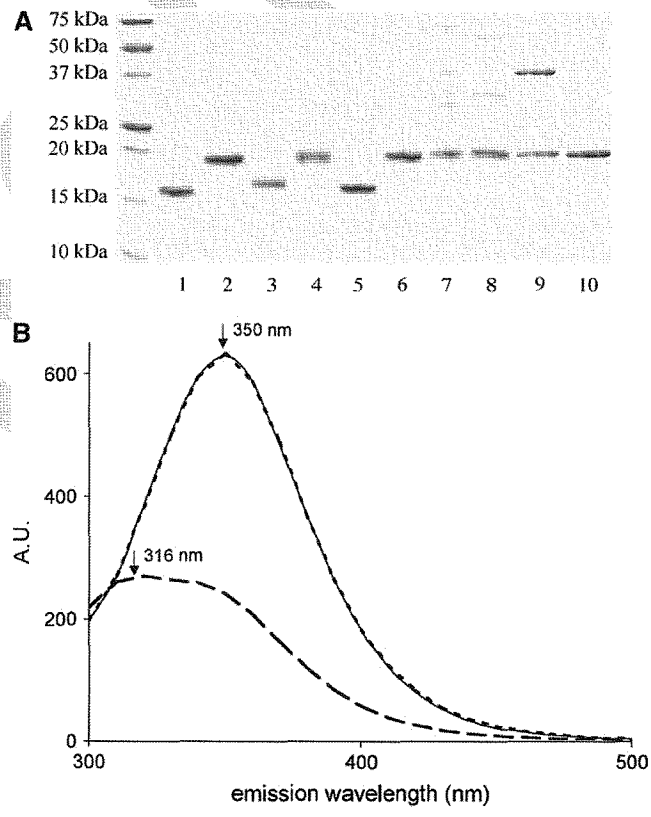

Figure 4. Effects of redox conditions on PtrcGpx3.2. A, The proteins were either oxidized with 30 mM H_2O_2 (lanes 1, 3, 5, 7, and 9) or reduced with 30 mM DTT (lanes 2, 4, 6, 8, and 10) and separated on 14% SDS-PAGE. Lanes 1 and 2, Gpx3.2; lanes 3 and 4, Gpx3.2 C107S; lanes 5 and 6, Gpx3.2 C136S; lanes 7 and 8, Gpx3.2 C155S; and lanes 9 and 10, Gpx3.2 C136S/155S. A total of 1 μg of protein was loaded per lane. B, Fluorescence emission spectrum of PtrcGpx3.2 in different redox conditions. Excitation was set at 290 nm. All spectra were recorded for 4 μM PtrcGpx3.2 concentration in 30 mM Tris, pH 8, 1 mM EDTA at room temperature. Untreated sample spectrum is figured in continuous line, spectrum for sample treated for 10 min with 250 μM DTT is figured in discontinuous line, and sample treated with DTT then incubated for 1 min with 250 μM H_2O_2 is figured in dotted line. Maximum emission peaks are indicated.

states, the mutation of Cys-155 prevents modification of Trp fluorescence and thus of the formation of the disulfide bond. No change of fluorescence was observed for PtrcGpx3.2 C107S, while a shift, attributed to the formation of a Cys-136-Cys-155 disulfide bond, was visible on SDS-PAGE. Altogether, these results indicate that Trp fluorescence is modified only when the peroxidatic Cys (Cys-107) and the resolving Cys (Cys-155) are involved in the formation of a disulfide bond.

Gel-filtration experiments performed with reduced or untreated PtrcGpx3.2 showed that it consistently elutes with an apparent molecular mass of 38 kD, corresponding to a homodimer (data not shown). As the protein migrates as a monomer (around 19 kD) under both reducing and nonreducing conditions during denaturing SDS-PAGE, the dimer observed during gel filtration cannot involve an intermolecular disulfide and must be stabilized by noncovalent forces (e.g. electrostatic or hydrophobic interactions).

Redox titrations of PtrcGpx3.2 using redox equilibration buffers composed either of defined mixtures of oxidized and reduced DTT or of defined mixtures of GSH plus oxidized GSH (GSSG) are most readily interpreted in terms of a single disulfide/dithiol redox couple per monomer, with a midpoint redox potential (E_m) value of 295 ± 10 mV at pH 7.0 (Fig. 5). Redox titrations (data not shown) over a more positive range than that shown in Figure 5 (i.e. E_h values ranging from −250 mV to −80 mV), in which defined mixtures of GSH plus GSSG were used as redox equilibration buffers, demonstrated that no additional more positive dithiol/disulfide couple is present in the protein. As Cys-136 is not involved in catalysis and the protein is not present as a disulfide-linked covalent dimer, one can conclude that the value observed corresponds to an intramolecular disulfide bridge linking Cys-107 to Cys-155.

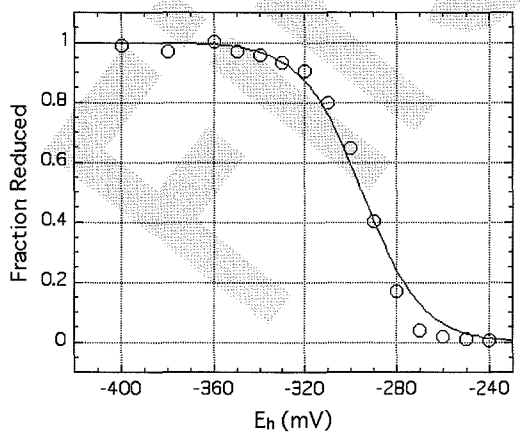

Figure 5. Redox titration of PtrcGpx3.2. The titration was carried out as described in "Materials and Methods" using a total DTT concentration of 2 mM in the redox buffer and with a redox equilibration time of 2 h.

Expression of Prx in Plant Organs and in Stress Conditions

We first performed an in silico analysis based on ESTs found in the DOE JGI database and found 191 ESTs coding for poplar Gpxs (Table I). The different isoforms are not equally represented among these ESTs; PtrcGpx3.2 seems to be the most frequently expressed gene, with 85 corresponding ESTs (i.e. approximately 45%). We also found 48 ESTs for PtrcGpx1, 10 for PtrcGpx2, 23 for PtrcGpx3.1, 17 for PtrcGpx4, and eight for PtrcGpx5. These results were combined with a semiquantitative reverse transcription (RT)-PCR approach. As these genes belong to the Prx family, we have studied the transcript expression pattern of all Gpxs together with the nine Prxs existing in poplar in seven different tissues (roots, young leaves, expanded leaves, fruits, stems, petioles, and stamen). For most genes, we have been able to find specific hybridizing areas. However, we were sometimes unable to distinguish some closely related sequences (i.e. Prx Q1 and Q2 or 2-Cys Prx A and B). In these two cases, we used nonspecific primers able to amplify both transcripts (Prx Q1 and Q2 or 2-Cys Prx A and B), but we were also able to define one primer to specifically amplify one gene (Prx Q1 and 2-Cys Prx A). From the difference between these two amplifications, the expression of the second gene can be deduced. For example, as the 2-Cys Prx A is only expressed in expanded leaves, we can estimate from the 2-Cys Prx A and B expression profile that 2-Cys Prx B is expressed in fruits, stems, petioles, and stamens and possibly in expanded leaves. All the genes exhibit different expression patterns, but there is at least one gene expressed in each organ tested (Fig. 6A). These data, coupled with the in silico analysis, are indicative of a broad distribution and expression of Prxs in plants, at least at the transcript level. Nevertheless, the multiplicity of Prx genes can be explained by some specificity in the expression pattern. For example, out a total of 17 genes, only three (coding for PtrcGpx2, PtrcGpx 3.1, and PtrcPrx IIE) are expressed at detectable levels in roots under the growth conditions used. One gene (PtrcPrx IIE) encodes a plastidic protein, another (PtrcGpx3.1) appears to encode a cytosolic Gpx, and the third (PtrcGpx2) appears to encode a secreted Gpx. In contrast, all the genes are expressed in expanded leaves. Given the specificity of the two chloroplastic Gpxs (PtrcGpx1 or PtrcGpx3.2) for a Trx of the y type as an electron donor, it was also of interest to examine the expression patterns of these two poplar Trxs. Both Trx y1 and y2 were found to be expressed in all tissues tested, and thus their role cannot simply be restricted to serving as specific reductants for plastidial Gpxs.

To get a deeper view of the expression of *Gpx* genes, we used both microarray data available using Genevestigator and the results presented by Rodriguez Milla et al. (2003) to follow the expression pattern of the different Arabidopsis Gpx genes in the whole plant (Fig. 6B; Zimmermann et al., 2004). Based on the

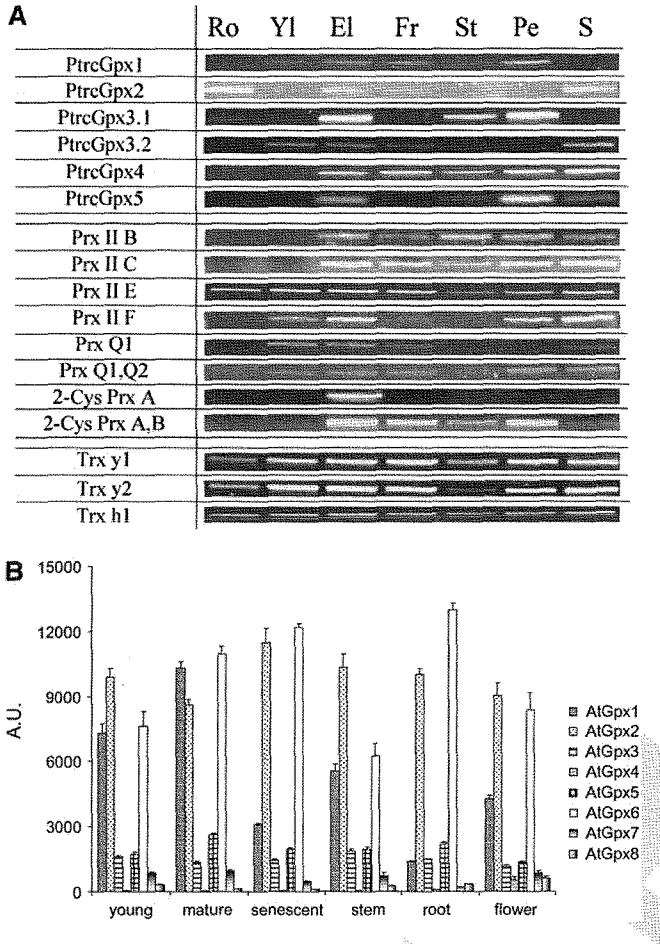

Figure 6. Prx transcript expression profiles. A, Expression of Gpx, Prx, and Trx y transcripts. RT-PCR reactions were performed with RNA extracted from various poplar organs (Ro, Root; Yl, young leaf; El, expanded leaf; Fr, fruit; St, stem; Pe, petiole; and S, stamen), loaded on 0.8% agarose gel, and stained with ethidium bromide. B, AtGpx gene expression pattern in the different plant organs, as compiled from microarray data available using the GeneAtlas function of Genevestigator (Zimmermann et al., 2004). Accession numbers: AtGpx1, At2g25080; AtGpx2, At2g31570; AtGpx3, At2g43350; AtGpx4, At2g48150; AtGpx5, At3g63080; AtGpx6, At4g11600; AtGpx7, At4g31870; and AtGpx8, At1g63460.

results of Rodriguez Milla et al. (2003), AtGpxs1 to 7 are expressed in all tissues analyzed, except AtGpx2, which does not seem to be expressed in dry seeds. AtGpx4 and 7 are the less expressed genes, as they were only detected by RT-PCR and few or no ESTs are found for these genes (Rodriguez Milla et al., 2003). Using Genevestigator, we observed that AtGpx1, AtGpx2, and AtGpx6, respectively homologous to PtrcGpx1, PtrcGpx2, and PtrcGpx3.2, were highly expressed in all organs or tissues analyzed (i.e. in young, expanded, and senescent leaves, as well as in stems, roots, and flowers), while AtGpx3, AtGpx5, AtGpx7, and AtGpx8, and especially AtGpx4, generally show a low expression level in these organs. Nevertheless, as expected for a chloroplastic protein, AtGpx1 is preferentially expressed in green tissues (young and expanded leaves, stems, and flowers). The main difference between the two sources concerns the expression of AtGpx6, which was found to be low in all organs except dry seeds by northern blot, while microarray data indicates a strong expression in all organs.

Using an antibody raised against purified recombinant PtrcGpx3.2, we first checked its specificity against the recombinant proteins. It reacts with all five recombinant proteins (which incidentally all migrate at slightly different positions on SDS-PAGE) but only faintly with PtrcGpx1 and 5 and more strongly with PtrcGpx2, 3.2, and 4 (Fig. 7A). This antibody is thus specific for Gpx as a whole, including the sixth protein, PtrcGpx3.1, which is very similar to PtrcGpx3.2 but does not discriminate between the isoforms. We could, however, differentiate at least two isoforms with two slightly different molecular masses in poplar protein extracts. The higher molecular mass band seems specific to roots, and because the signals obtained with the extracts from all the other organs tested have a similar size, they could thus correspond to a single isoform that we cannot identify for the reasons explained above (Fig. 7B).

In Arabidopsis (Fig. 7C), two bands of different sizes arising from two or more different isoforms have been detected in almost all organs, except roots. The protein(s) of smaller molecular mass is most easily detected in leaves and flowers, while in contrast the protein(s) of higher molecular mass is present in stems and roots and to a lesser extent in leaves and flowers. Using proteins extracted from purified leaf chloroplasts, only the lower band was detected (data not shown). Given the fact that AtGpx7 is weakly expressed at the transcriptional level, only the other two predicted chloroplastic proteins (i.e. AtGpx1 and AtGpx6) are likely to be detectable. Thus, we conclude that the lower band consists predominantly of AtGpx1 and/or AtGpx6. The upper band is likely to arise from the presence of AtGpx2, as it is not a chloroplastic protein and because AtGpx3, AtGpx4, AtGpx5, and AtGpx8 also seem to be weakly expressed at the transcriptional level. We next

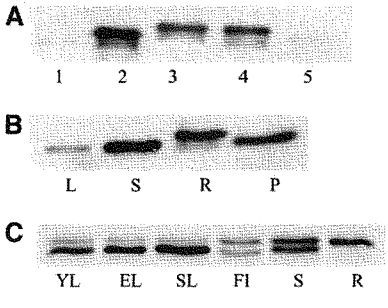

Figure 7. Gpx expression in poplar and Arabidopsis organs. A, Specificity of PtrcGpx3.2 antibodies. A total of 30 ng of each recombinant poplar Gpx (PtrcGpx1 to 5) was used. B, Distribution of Gpx expression in different poplar organs. L, Leaf; S, stem; R, root; and P, petiole (30 μg of total proteins loaded). C, Distribution of Gpx expression in different Arabidopsis organs. YL, Young leaf; EL, expanded leaf; SL, senescent leaf; Fl, flower; S, stem; and R, root (30 μg of total proteins loaded).

investigated the abundance of these proteins in stress situations (Fig. 8). Gpx abundance as a function of time was followed after infection of poplar by the agent of poplar rust, the fungus *Melampsora larici-populina*, and using two different isolates, a virulent one that induces a compatible reaction and an avirulent one that induces an incompatible reaction (Fig. 8A). As is the case for untreated leaf extracts, a single band is visible. During the incompatible reaction, the expression of Gpx increased greatly 2 h after infection and even more after 48 h. The pattern of expression during the compatible reaction is strikingly different, with a lower protein level observed at both 2 h after infection and then again at 24 h after infection but with levels very similar to those seen prior to infection found at all other times. Because this expression pattern is surprising, we have analyzed the expression of Trx h1, which is constitutively expressed and was never found to vary with stress, as a control (N. Rouhier, unpublished data). As expected, the Trx h1 abundance is not dependent on the pathogen infection, regardless of which fungal isolate was used.

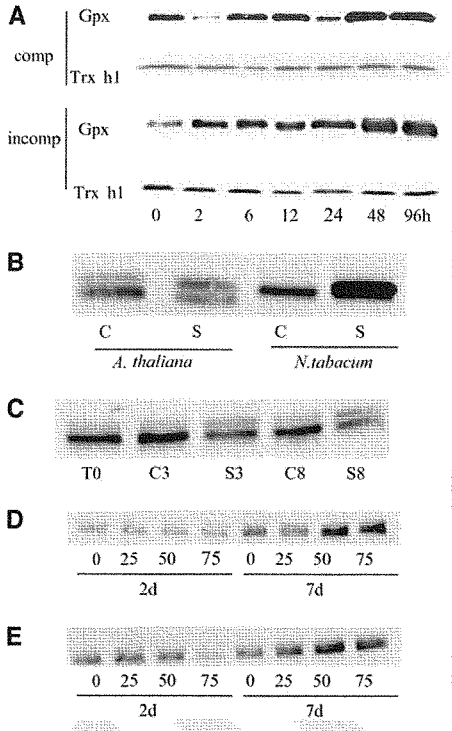

Figure 8. Gpx protein expression in poplar and Arabidopsis subjected to biotic or abiotic stresses. A, Abundance of poplar Gpxs during infection by the rust fungus *M. larici-populina* between 0 and 96 h after a compatible or an incompatible reaction. The variation of Trx h1 amount was used as a control. B, Abundance of Gpxs in young leaves of Arabidopsis or tobacco plants exposed to a water deficit of 6 d (S) compared to control plants (C). C, Abundance of Gpxs in expanded leaves of Arabidopsis plants exposed to photooxidative stress (low temperature [8°C] combined to high light [1,400 μE m^{-2} s^{-1}; S]) for 3 or 8 d compared to control plants (C). D, Abundance of Gpxs in expanded leaves of Arabidopsis plants subjected to various concentrations of cadmium (0, 25, 50, or 75 μM added to hydroponic plants) for 2 or 7 d. E, Abundance of Gpxs in expanded leaves of Arabidopsis plants subjected to various concentrations of copper (0, 25, 75, or 150 μM added to hydroponic plants) for 2 or 7 d. Each western blot (30 μg of protein were loaded per lanes) was done at least in duplicates with two independent treatments.

In addition, we followed Gpx levels in young leaves from Arabidopsis or tobacco (*Nicotiana tabacum*) plants submitted to various abiotic stresses. After 6 d of water deficit, the intensity of the lower band decreased, while that of the upper band increased (Fig. 8B). This was even more pronounced in young tobacco leaves subjected to the same conditions with an increased intensity of the Gpx signal, which could correspond to the presence of one or more Gpx isoforms (Fig. 8B). Similar profiles were observed using expanded leaves (data not shown). In plants subjected to photooxidative stress (Fig. 8C), the amount of the two proteins varied in the opposite way; the intensity of the upper band increased slightly under stress conditions in expanded leaves, whereas the intensity of the lower band decreased after 3 or 8 d of treatment.

In plants exposed to metal stresses induced by application of various concentrations of cadmium or copper for 2 and 7 d, only the amount of the lower M_r protein(s) detected is modified. It decreased at 150 μM copper after 2 d (Fig. 8E) but increased strongly after 7 d at cadmium concentrations of 50 and 75 μM (Fig. 8D) and at copper concentrations of 25, 75, and 150 μM (Fig. 8E).

Subcellular Localization of PtrcGpx1 and PtrcGpx3.2

PtrcGpx2 and PtrcGpx5 are predicted to be secreted but were not studied here, as we were unable to amplify the 5′ end coding for their N-terminal extensions from either cDNA libraries or genomic DNA. Although Arabidopsis and *O. sativa* homologs possess this extension, it is not yet clear whether these extensions really belong to the cDNA or are annotation or assembly errors. PtrcGpx4 and PtrcGpx3.1 contain neither an N-terminal extension nor known C-terminal signals, suggesting that the proteins are cytosolic. To determine the subcellular localization of PtrcGpx1 and PtrcGpx3.2, the 5′ portions of the open reading frame encoding only the N-terminal parts were fused to the sequence coding for a GFP. The recombinant pCK GFP S65C plasmids were then used to transiently transform tobacco cells by bombardment. In the guard cells shown here, it appears that Gpx1 is specifically targeted to chloroplasts and that Gpx3.2 is directed both to chloroplasts and mitochondria (Fig. 9). Similar results were obtained in mesophyll cells (data not shown).

DISCUSSION

As more and more genomes are sequenced and annotated, it is now clear that multigenic families are very common in organisms. Here, we describe the

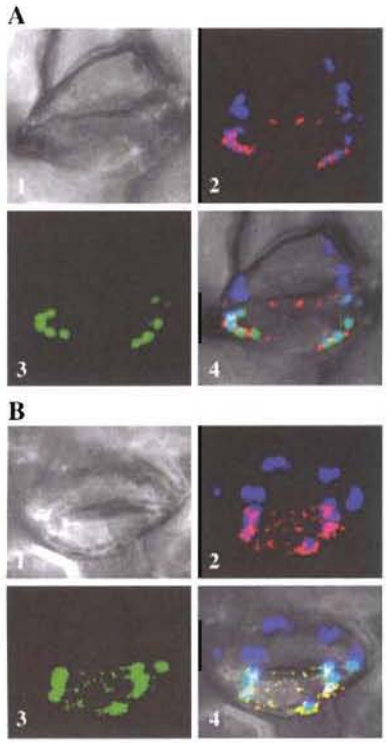

Figure 9. Subcellular localization of PtrcGpx1 (A) and PtrcGpx3.2 (B). N-terminal extensions of the two Gpxs were fused to the GFP and used to bombard tobacco cells. The pictures presented here are guard cells. 1, Visible light; 2, autofluorescence of chlorophyll (blue) and mitochondrial marker (red); 3, fluorescence of the construction; and 4, merged images.

existence of six Gpx isoforms in the model tree poplar. By comparison, eight and five Gpx isoforms are present in the genome of Arabidopsis and *O. sativa*, respectively. The number of *Gpx* genes thus varies among species, probably because of gene duplication events. A continuing challenge is to understand why so many genes or proteins with similar functions are needed in plants. To answer this question, both the abundance and localization of the proteins and the substrate specificities of these enzymes were studied. As a result of our study, it is now clear that the poplar enzymes originally named Gpxs are reduced by Trxs rather than by GSH and belong to the Prx family. This large family thus includes 15 different Prxs in poplar.

Substrate and Electron Donor Specificities

The poplar Gpxs do not exhibit any difference in the reduction of H_2O_2 compared to complex hydroperoxides (COOH and tBOOH), as the catalytic efficiencies (k_{cat}/K_m) vary only over a 20-fold range (i.e. from 2.6 to 53 $\times$ 10^3 M^{-1} s^{-1}). The reduction of complex peroxides is consistent with a role for Gpxs (and for Prxs in general) that complements other systems, like catalases or ascorbate peroxidases, which specifically reduce H_2O_2. The catalytic efficiencies of poplar Gpxs lie in the range previously described for other Prxs. Rouhier et al. (2004) determined kinetics parameters for poplar chloroplastic Prx Q, with k_{cat} around 1 s^{-1} and k_{cat}/K_m of 10^3 to 10^4 M^{-1} s^{-1}, depending on the substrate considered. Horling et al. (2002) reported an activity of Prx IIC that is in the same range. Previous studies (Herbette et al., 2002; Jung et al., 2002; Tanaka et al., 2005) also indicate that plant or yeast Gpxs may display kinetic parameters similar to those of the other classes of the broad Prx family. The catalytic efficiencies reported to date for Gpxs and Prxs are far lower than those of catalases or ascorbate peroxidases, but they are likely to reduce a broader range of substrates than these two enzymes. Gpxs may also provide the graduated response necessary for the metabolic fine tuning in case of oxidative stress, as has been demonstrated in the interaction with transcription factors (Delaunay et al., 2002). All these data are consistent with a possible role for Gpxs and Prxs in ROS detoxication in plant cells and, as has been demonstrated in other organisms, in redox signaling (Delaunay et al., 2002; Rhee et al., 2005; Vivancos et al., 2005).

In so far as the electron donor specificity of poplar Gpxs is concerned, these enzymes seem to behave like previously characterized plant Gpx in that they can be reduced by Trxs, as earlier demonstrated for some plant Gpx (Herbette et al., 2002; Jung et al., 2002) but not at all by GSH. We have also demonstrated that Grxs, which can provide a pathway for disulfide reduction alternative to the Trx pathway, are unable to reduce poplar Gpxs. However, because only five Grxs belonging to the same subgroup were tested, the possibility exists that one or more other members of the large Grx family might serve as an effective electron donor to Gpx. The oxidation-reduction titrations carried out in this study (see Fig. 5) provide a thermodynamic basis for this donor specificity. The intramolecular Cys-107/Cys-155 disulfide present in the oxidized PtrcGpx3.2 was shown to have an E_m value at pH 7.0 of -295 ± 10 mV. While reduction of this PtrcGpx3.2 disulfide by Trxs, which have E_m values ranging from -275 mV to -330 mV (Brehelin et al., 2003; Collin et al., 2003), would be expected to be thermodynamically

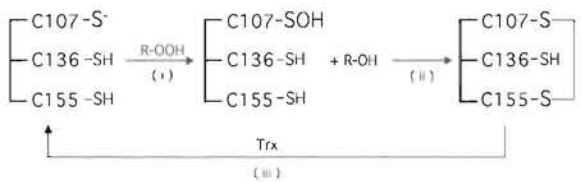

Figure 10. Proposed catalytic and Trx-dependent recycling mechanisms for poplar Gpx: (i) nucleophilic attack of Cys-107 on peroxide (ROOH) leading to the formation of a sulfenic acid and the concomitant release of an alcohol; (ii) formation of an intramolecular disulfide bridge between Cys-107 and Cys-155; and (iii) reduction of the intramolecular disulfide bridge by Trx leading to a reduced enzyme and an oxidized Trx.

favorable, the more positive E_m values of GSH (E_m = −245 mV at pH 7.0) and plant Grxs (E_m values around −180 mV; N. Rouhier, unpublished data) would make reduction of the PtrcGpx3.2 disulfide thermodynamically unfavorable.

The question of which Trx is the actual physiological electron donor for different Gpxs remains unsettled. Taking the Trx h family as an example, we were unable to establish a preference between Trx h1, h3, and h5 in in vitro assays. It was previously demonstrated that neither a poplar Trx h2 nor an Arabidopsis Trx o were able to serve as reductants to PtrcGpx3.2 (Gelhaye et al., 2004), and thus the identity of the physiological reductant for this mitochondrial Gpx remains unclear.

Considering the putative secreted isoforms, namely PtrcGpx2 and PtrcGpx5, we found that Trx h5 is able to reduce PtrcGpx5 in a catalytic manner (data not shown). Juarez-Diaz et al. (2005) recently showed that a homolog of poplar Trx h5 was secreted by *Nicotiana alata* cells. Assuming that PtrcGpx5 is actually secreted, these two proteins could be part of an extracellular redox signaling system. Pignocchi and Foyer (2003) suggested that changes in the apoplastic redox state could be sensed by a system involving dithiol-disulfide exchanges. Secreted Gpxs may be candidates as apoplastic redox sensors.

There are at least 11 chloroplastic Trxs in Arabidopsis (NtrC, CDSP32, Trx x, Trx y1 and y2, Trx f1 and f2, Trx m1, m2, m3, and m4) and even more in poplar (E. Gelhaye, unpublished data). Although we did not test the efficiency of the bimodular Trxs (NtrC and CDSP32), it appears that only the two Trx y are able to efficiently support the activity of PtrcGpx1 and PtrcGpx3.2. These chloroplastic Trxs all have very similar E_m, and so the specificities observed must arise from properties of the Trxs other than their E_m values (e.g. the distributions of surface charges and of surface hydrophobic patches). In a previous study, it was found in Arabidopsis that only Trx x is able to reduce 2-Cys Prx, and Trx y is the most efficient electron donor to Prx Q (Collin et al., 2004). It is then likely that the major function of x-type and y-type chloroplastic Trxs is the reduction of chloroplastic Prxs (this family includes 2-Cys Prxs, Prx Q, Prx IIE, and Gpxs). The f-type Trxs are specifically needed in the regulation of chloroplastic metabolism (in particular, through the activation of FBPase and of the CF_1 ATP synthase), but they can also reduce 2-Cys Prxs and Prx Q. The m-type Trxs may play a dual role, regulating metabolism through control of NADP-dependent malate dehydrogenase and also serving as possible reductants for 2-Cys Prx and Prx Q (Collin et al., 2003). Overall, this would constitute a system with partial redundancy that could be linked to each subcellular compartment redox state.

Using the digital northern function of Genevestigator online database (https://www.genevestigator.ethz.ch; Zimmermann et al., 2004), we noticed that the mRNA profiles of the Arabidopsis ortholog of PtrcGpx1 (AtGpx1, accession no. At2g25080) are more closely related to that of AtTrx y1 (accession no. At1g76760) than to that of AtTrx y2 (accession no. At1g43560). This may indicate which of the two y-type Trxs is likely to be the favored electron donor to this enzyme in vivo. As there are also two isoforms of Trx y in poplar, the situation might be similar. RT-PCR experiments showed that the two genes (Trx y1 and y2) are expressed in all the organs tested, and thus at the moment one cannot identify specific and distinct roles for the two isoforms.

Catalytic Mechanism and Trx-Mediated Recycling Process

When starting this work, one question was to determine whether one, two, or three Cys were involved in the reactions catalyzed by plant Gpxs. Although yeast Gpx2 had been shown to possess a Trx-dependent activity, involving only the two Cys equivalent to Cys-107 and Cys-155 in poplar (Tanaka et al., 2005). Jung et al. (2002) had previously suggested a catalytic mechanism involving three conserved Cys residues for a Chinese cabbage (*Brassica napus*) Gpx. This proposal was based on mass spectrometry data that showed that a disulfide bridge could be formed between the Cys corresponding to poplar Cys-136 and Cys-155 (Jung et al., 2002) and on their observation that the mutated protein equivalent to PtrcGpx C136S retained only 30% of the activity displayed by the wild-type enzyme. Here, we show that, although three Cys are absolutely conserved in all Gpxs from higher plants, only two are involved both in catalysis and in the Trx-dependent regeneration. Indeed, three observations support this conclusion: (1) the stoichiometry of the reaction catalyzed by the wild-type enzyme is consistently 1:1 (in terms of peroxide reduced to enzyme oxidized) and never 2:1; (2) the mutated PtrcGpx3.2 C136S is as active as PtrcGpx3.2; and (3) we have detected only one disulfide bridge when titrating PtrcGpx3.2. Another characteristic of these enzymes is a shift during migration under SDS-PAGE similar to that previously reported by Tanaka et al. (2005) for yeast Gpx2, as the redox state of the protein is altered. Both the redox-dependent shift in SDS-PAGE migration properties and the redox-dependent changes in the Trp fluorescence parameters of the proteins clearly indicate that Cys-155 is required for the formation of an intramolecular disulfide with Cys-107. One additional piece of evidence that Gpx belongs to the Prx family is its formation of noncovalent dimers. Indeed, the only plant Prx for which a structure is known is a homodimer with a conserved interface found in other nonplant Prx structures (Echalier et al., 2005). A structural study designed to determine the structural determinants responsible for substrate binding, for dimerization, and to elucidate a possible role for the highly conserved Cys-136 is currently under way.

Unlike the case for many other eukaryotic Prx characterized so far, we did not observe the formation of overoxidized forms of Gpx. Indeed, the stoichiometry of the reaction catalyzed by Gpxs is consistently 1, the

activity measured in the presence of the Trx system is linear with time, and H_2O_2-treated proteins do not undergo the mass increment expected for sulfinic or sulfonic formation (data not shown). We thus propose a three-step reaction mechanism for plant Gpxs similar to the one used by Prx Q, with two Cys forming an intramolecular disulfide bridge in the oxidized state (Fig. 10). These steps are: (1) a nucleophilic attack of the catalytic Cys (Cys-107) on the peroxide with the release of an alcohol and the concomitant formation of a sulfenic acid; (2) an attack of the sulfenic acid by Cys-155 and formation of an intramolecular disulfide bridge between Cys-107 and Cys-155; and (3) a reduction of the disulfide bridge by Trx.

Expression and Localization of Gpx in Plants

We analyzed the expression of all the Prxs in poplar organs. All the members of this family are expressed at the transcriptional level in at least one of the organs tested, with a specific expression pattern for each of them. Gpxs are likely to be present in all organs and in nearly all cell compartments, suggesting a redundancy with the Prxs identified previously. Interestingly, many isoforms are expressed in flowers or in fruits, but their precise physiological function is yet unknown. Nevertheless, to explain the need for proteins with identical functions, we can imagine that some genes could be expressed in specific conditions under stress, for example, or could be temporally regulated.

When poplar leaves are subjected to a biotic stress (infection by the rust fungus *M. larici-populina*), the abundance of these proteins is modified. This is in line with previous observations that the levels of Prx Q, Prx IIC, and Prx IIF but not of 2-Cys Prx are modified in this situation (Rouhier et al., 2004; Gama et al., 2007). Abiotic stress conditions (water deficit, photooxidative, and metal stresses) also induce modifications of the Gpx levels. The amount of isoform(s) of lower molecular masses is especially modified, generally decreasing in water deficit and photooxidative stress but strongly increasing in the presence of toxic metals such as cadmium or copper. In the case of photooxidative stress, it was previously shown that among members of the Prx family, only the amount of Prx Q increased in this situation, whereas the level of Prx IIE and 2-Cys Prx remained unchanged (Havaux et al., 2005). In our case, the amount of chloroplastic Gpx(s) decreased, suggesting there is a fine control and a functional specialization among members of the same family. It was previously demonstrated at the transcript levels that AtGpx2, AtGpx5, and AtGpx6 were strongly and rapidly increased in Arabidopsis plants treated with 200 μM of $FeSO_4$ or $CuSO_4$ (Rodriguez Milla et al., 2003). Other studies provided evidence for an increased Gpx activity in plants treated with copper (Ali et al., 2006) or nickel (Gajewska and Sklodowska, 2006) but decreased activity in the presence of cadmium (Aravind et al., 2005). The conclusion, based on our results and those of others, that Gpxs are Trx-dependent enzymes suggests that the GSH-dependent peroxidase activity found in these studies corresponds to the peroxidase activity of GSH *S*-transferases. Taken as a whole, the results presented above provide evidence at the protein level that supports the conclusions based on previous observations in mRNA studies that Gpx are involved in stress responses and especially in response to metals. For example, it was previously demonstrated that overexpression of a Gpx from *C. reinhardtii* both in the cytosol and in the chloroplast and overexpression of a tomato (*Lycopersicon esculentum*) Gpx in yeast and plant increased tolerance to oxidative stress (Chen et al., 2004; Yoshimura et al., 2004).

Another possible explanation for the redundancy of Trx peroxidases as a whole could be linked to their subcellular distribution. Using GFP fusion, we demonstrated that two Gpxs (PtrcGpx1 and 3.2) are localized in chloroplast, one of these (PtrcGpx3.2) being also targeted to mitochondria. This dual targeting is not unusual, having already been observed for some other antioxidant proteins (GSH reductase [GR], monodehydroascorbate reductase, and ascorbate peroxidase; Chew et al., 2003). The plastidic localization of PtrcGpx1 was expected, as a pea (*Pisum sativum*) Gpx homolog was previously shown to be imported into chloroplasts (Mullineaux et al., 1998). Only one other plant Gpx was previously localized experimentally. This protein, from tomato, called GPXle-1 and homologous to PtrcGpx3.1, was found in the cytosol near the plasma membrane and to a lesser extent in the cell wall of some leaf cells (Herbette et al., 2004).

Adding the results of this investigation to the previously available data on distribution of Prxs, eight poplar Prxs are now known to be expressed in plastids (Prx IIE, 2-Cys Prx A and B, Prx Q1 and 2, and PtrcGpx1 and PtrcGpx3.2), two in mitochondria (Prx IIF and PtrcGpx3.2), three in the cytosol (Prx IIB, Prx IIC, and PtrcGpx3.1), one in the nucleus (Prx 1-Cys), and two predicted to be directed to the secretory pathways (PtrcGpx2 and PtrcGpx5), assuming that these proteins indeed contain an N-terminal extension. This situation is slightly different from Arabidopsis, in which there is only one Prx Q but an additional cytosolic Prx II and eight Gpxs instead of the six found in poplar.

MATERIALS AND METHODS

Materials

H_2O_2, tBOOH, COOH, NADPH, GSH, and GR from yeast (*Saccharomyces cerevisiae*) were purchased from Sigma.

Expression of Poplar Gpxs in Various Organs

RT-PCR

Semiquantitative RT-PCR experiments were used to estimate the distribution of Gpx, Prx, and y-type Trx transcripts in various poplar (*Populus trichocarpa*) organs. Total RNA isolation was performed with the RNeasy Plant Mini kit (Qiagen) from approximately 100 mg of various frozen tissues obtained from 4-month-old poplar plants (young and expanded leaves, roots,

stems, and petioles) or from older poplar trees (fruits and stamen). To remove contaminating DNA, the samples were treated with DNAseI (Qiagen). Except for the stem (for which we used 300 ng), a total of 1 μg of total RNA was used to generate cDNAs by RT using the Omniscript RT kit (Qiagen). These products were used as templates for subsequent PCR reactions using the primers described in Supplemental Table S2. The program used was as follows: 94°C for 3 min and 35 cycles of 94°C for 30 s, 54°C for 45 s, and 72°C for 1 min 45 s. The primers used were sometimes identical to those used for the cloning experiments (Gpx1, 2, and 4; 1-Cys Prx). When two open reading frames were very similar (e.g. Gpx3.1 or 3.2; Prx IIC, D, and E; Prx Q1 and Q2, Prx 2-Cys A and B, Trx y1 and 2), we used one or two new primers hybridizing in the 3′ or 5′ part of mRNAs, sometimes in combination with one of the cloning primers. When no selective amplification was possible for one isoform out of a pair of very close sequences, primers that can hybridize the two forms were used. In the case of Gpx5, the amplification was done with two specific internal primers. The Trx h1 gene from poplar, which is expressed in all tissues tested, was amplified simultaneously and used as a control. The PCR fragments were then loaded on a 0.8% agarose gel.

Western Blotting

Infection of poplar leaves with either avirulent or virulent fungal isolates of the rust fungus *Melampsora larici-populina* was carried out as described in Rouhier et al. (2004). Arabidopsis (*Arabidopsis thaliana*) plants subjected to water deficit and photooxidative or metal stresses were treated as described in Vieira dos Santos et al. (2005) and Gama et al. (2007). A total of 30 μg of proteins was loaded per lane on 15% denaturing SDS-PAGE then transferred onto polyvinylidene difluoride membranes (Millipore) when using poplar protein extracts or onto nitrocellulose membranes (Pall) for Arabidopsis extracts. Antibodies raised against PtrcGpx3.2 were diluted 1,500×. Detection of proteins recognized by antibodies on polyvinylidene difluoride membranes was done by autoradiography and using the Odyssey Infrared Imager from LICOR in the case of nitrocellulose membranes.

Cloning and Site-Directed Mutagenesis

After identification in the databases (see "Results"), the nucleotide sequences of PtrcGpx2, 3.2, and 4 were then amplified by PCR using the primers described in Supplemental Table S2 from a *Populus tremula* × *trichocarpa* cv Beaupre poplar root cDNA library (Kohler et al., 2003), and PtrcGpx1 and PtrcGpx5 were amplified directly from isolated *Escherichia coli* colonies containing the cDNA of interest (gift from Dr. Gunnar Wingsle, University of Umeå, Sweden). Each of the three Cys of the mature PtrcGpx3.2, at positions 107, 136, and 155, respectively, were replaced individually by Ser using site-directed mutagenesis following a procedure described in Jacquot et al. (1997). The double mutant PtrcGpx3.2 C136S, C155S, which only contains the first Cys, was also generated and cloned. After digestion with the *Nco*I and *Bam*HI restriction enzymes, PCR fragments were inserted into the pET-3 d expression plasmid.

Production and Purification of Recombinant Gpxs

The recombinant plasmids obtained were used to transform the BL21(DE3) pSBET strain of *E. coli* (Schenk et al., 1995). The bacteria were then grown to a final volume of approximately 3.2 L at 37°C, and protein production was induced during exponential phase by adding 100 μM isopropyl-β-D-thiogalactoside. For PtrcGpx3.2, the amount of soluble protein increased when bacteria were grown overnight at 30°C without induction by isopropyl-β-D-thiogalactoside. The bacteria were harvested by centrifugation at 5,000 rpm for 20 min and then resuspended in TE buffer (Tris-HCl 30 mM, pH 8.0, EDTA 1 mM). Cells were broken by sonication, and all Gpxs were found in the soluble fraction after high speed centrifugation, except for PtrcGpx2, which was present mostly in inclusion bodies.

The insoluble portion of PtrcGpx2 was first denatured by resuspending the pellet in TE buffer containing 8 M urea. The soluble proteins obtained after centrifugation at 16,000 rpm for 30 min were then renatured slowly by two successive dialyses of 6 h, first against TE buffer containing 0.5 M urea and then against TE buffer alone. A centrifugation step (30 min at 16,000 rpm) was performed after each dialysis to remove denatured proteins. For the soluble isoforms, ammonium sulfate fractionation was used as the first step in the purification procedure. For all of these soluble Gpxs, most of the protein precipitated between 40% and 80% saturation.

The next purification step, used for all Gpxs, was gel filtration carried out on ACA44 (Biosepra), equilibrated in TE buffer containing 200 mM NaCl. After removing NaCl by ultrafiltration, the Gpx fractions were loaded onto a DEAE-Sephacel ion-exchange column (Amersham). A gradient ranging from 0 to 400 mM NaCl was used for the elution of PtrcGpx2, 4, and 5, whereas PtrcGpx1 and 3.2 passed through the column. After dialysis and concentration, the proteins were analyzed for purity by SDS-PAGE and stored frozen at −20°C in TE buffer.

Biochemical Characterization of Poplar Gpx

NADPH-Coupled Spectrophotometric Method

These experiments were performed at 30°C and were based on monitoring the decrease in absorbency at 340 nm arising from NADPH oxidation. All Gpxs were tested using this method and showed similar catalytic parameters. The catalytic properties of PtrcGpx3.2 and its cysteinic mutants (PtrcGpx3.2 C107S, C136S, C155S, and C136/155S; 200 nM) were investigated in more detail and were tested using either a reduced Trx-generating system (3 μM Arabidopsis recombinant NTRb and various concentrations of Trx h1, h3, and h5 from poplar), or the Grx system (0.5 units GR, 1 mM GSH, and 100 μM Grx C4), or GSH alone (0.5 units GR, 2 mM GSH) as possible electron donors. These assays were carried out in a total volume of 500 μL in 30 mM Tris-HCl, pH 8, buffer, 1 mM EDTA, and 200 μM NADPH. The catalytic parameters for one substrate were measured by varying its concentration at saturating concentrations of the other substrate (typically between 1 and 100 μM for Trx, and between 40 μM and 12 mM for the peroxides [H_2O_2, tBOOH, or COOH]).

FOX Colorimetric Method

The reduction of peroxides by PtrcGpx1 and PtrcGpx3.2, the two chloroplastic isoforms of poplar, was also followed using the FOX colorimetric method (Wolff, 1994). All the monomodular chloroplastic Trxs from Arabidopsis were assayed as possible electron donors. A 50-μL reaction mixture contained 2 μM of recombinant PtrcGpx1 or 3.2, 500 μM DTT, and 10 μM of Trx f1, f2, m1, m2, m3, m4, x, y1, or y2 recombinant proteins from Arabidopsis, purified as described in Collin et al. (2003). The reaction was started by adding the peroxide (either H_2O_2, tBOOH, or COOH) to a final concentration of 400 μM. At different times, 5 μL of the reaction mixture was added to 495 μL of the colorimetric reagent and the absorbency read at 560 nm after 30 min incubation in the dark.

Stoichiometry of the Reaction

The stoichiometry of the reaction was also measured using the FOX method with a known concentration of reduced enzyme (typically 100 μM or 200 μM) in the absence of any other reductants and in the presence of 400 or 500 μM peroxides. The residual amount of peroxide remaining after reduction by a known amount of protein was titrated after completion of the reaction (30 min). The enzyme was reduced with a large excess of DTT (10 mM), which was removed, prior to the assay, by two successive dialyses, each time against 1 L of TE buffer.

Oligomerization and Redox State of Gpx

The redox state of PtrcGpx3.2 and of the mutated proteins was analyzed by incubating the proteins with either 30 mM DTT or 30 mM H_2O_2 for 30 min before separating them on 14% acrylamide SDS-PAGE gels. It was also analyzed by following intrinsic Trp emission fluorescence using a Cary Eclipse fluorimeter (Varian). Measurements were recorded at room temperature in a 1- × 1-cm cuvette. The excitation wavelength was set to 290 nm (5 nm bandpass). Emitted light was detected at 90° between 300 and 500 nm. Spectra of a 1-mL solution containing 4 μM of various PtrcGpxs were recorded before adding successively 250 μM DTT and then 250 μM H_2O_2.

The native oligomerization state of PtrcGpx3.2 was determined both in oxidized (i.e. as isolated) or reduced (30-min incubation with 20 mM DTT) conditions using an ACA 44 gel-filtration column (5 × 75 cm) and proteins of known molecular masses as standards (bovine serum albumin, ovalbumin, and chymotrypsinogen; Sigma).

Intracellular Localization via GFP Fusion

The nucleotide sequences corresponding to the predicted N-terminal extension of PtrcGpx1 and PtrcGpx3.2 were cloned into the *Nco*I and *Bam*HI sites of pCK-GFP S65C using the primers detailed in Supplemental Table S2 as

PtrcGpx1 FOR GFP, PtrcGpx1 REV GFP, PtrcGpx3.2 FOR GFP, and PtrcGpx3.2 REV GFP. These primers were designed to amplify the sequences corresponding to the predicted signal peptides of Gpx1 (from MASLPF to TEKSVH) and Gpx3.2 [from M(A) LTSRS to SQSSPQ; see Fig. 1]. The PCR fragments were fused to GFP at the *Bam*HI site, resulting in chimeric proteins where the N-terminal extensions of the two Gpxs are present at the N terminus of enhanced GFP, under the control of a double 35S promoter (Menand et al., 1998). *Nicotiana benthamiana* cells were then transfected by bombardment of leaves with tungsten particles coated with plasmid DNA, and images were obtained with a Zeiss LSM510 confocal microscope.

E_m Determination

Oxidation-reduction titrations, using the fluorescence of the adduct formed between the protein and mBBr to monitor the thiol content of the protein, were carried out at ambient temperature as described previously (Krimm et al., 1998; Hirasawa et al., 1999). The reaction mixtures contained 100 μg of protein in 100 mM MOPS buffer, pH 7.0, containing defined mixtures of oxidized and reduced DTT to set the ambient potential (E_h). Identical titrations were obtained at total DTT concentrations of 1, 2, and 4 mM. The titration behavior was also independent of redox equilibration time over the range from 1.5 to 3 h. Titrations at more positive (E_h) values were carried out in a similar fashion, except that GSH replaced DTT in the redox equilibration buffer total GSH plus GSSG concentration of 2 mM. The ambient potential was set by varying the ratio of GSSG to GSH as described previously (Setterdahl et al., 2000). Redox equilibration times of either 2 or 3 h were used.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers CF936448 (Gpx1), DT518382 (Gpx2), DT516214 (Gpx3.2), DT487747 (Gpx4), and BU863119 (Gpx5).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Structure of the poplar Gpx genes.

Supplemental Table S2. Primers used in this study.

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DISCUSSION

L'origine et la formation des ROS dans les cellules végétales sont maintenant assez bien connues: les principaux sites de production de ROS sont détaillés et sont pour la plupart localisés dans les chaînes de transport d'électrons chloroplastiques ou mitochondriales. Des systèmes complexes régulent la concentration en ROS dans chaque compartiment, afin que les ROS ne perturbent pas le fonctionnement cellulaire, et que la cellule puisse utiliser ces molécules dans les voies de signalisation intra- et intercellulaires. Un inventaire des principales enzymes de détoxication cellulaire recensées dans le génome du peuplier est présenté dans la figure 12.

De nombreuses protéines, impliquées ou non dans ces cascades de transduction ou dans le contrôle de la teneur en ROS, sont régulées au niveau post-traductionnel par des échanges dithiol-disulfures, faisant intervenir les Trx et les Grx. L'inventaire de ces deux familles de protéines et de leurs protéines cibles potentielles a d'ailleurs fait l'objet de nombreuses recherches ces quinze dernières années (Jacquot et al 2002, Gelhaye et al 2004, Balmer et al 2004, Lemaire 2004, Rouhier et al 2004b, Meyer et al 2005). La réalité des interactions entre les Trx ou les Grx et leurs partenaires reste bien souvent à démontrer, mais la caractérisation biochimique des protéines recombinantes (Verdoucq et al 1999), de même que les techniques de co-immunoprécipitation (Rey et al 2005), ou de complémentation double-hybride (Vignols et al 2005) fournissent des moyens de les confirmer. Ces deux dernières techniques donnent un autre point de vue sur les interactions, et pourraient être

précieuses là où la biochimie seule ne permet pas d'affirmer quelles sont, parmi les nombreuses isoformes de Trx ou Grx végétales, celles qui sont réellement actives dans les cellules. Parmi les cibles potentielles potentielles des Trx et Grx, on trouve des facteurs de transcription, des kinases, des phosphatases ou des protéines impliquées dans les mécanismes de résistance aux pathogènes, régulées par des échanges dithiol-disulfures ou par des phénomènes de glutathionylation/déglutathionylation (Bower et al 1996, Cabrillac et al 2001, Despres et al 2003, Rivas et al 2004, Dixon et al 2005, Nekrasov et al 2006, Sokolov et al 2006).

Parmi les cibles des Trx et Grx impliquées dans la régulation de la teneur en ROS, la famille des Prx a fait l'objet de nombreuses études chez les plantes mais aussi chez les autres eucaryotes et chez les bactéries. Les Prx et Gpx sont omniprésentes dans la cellule, leur fonction principale étant d'éliminer les peroxydes de différentes natures, par des réactions d'oxydoréduction impliquant des cystéines.

I Les peroxyrédoxines

Depuis plusieurs années, la caractérisation du rôle des Prx dans ce domaine est maintenant bien avancée, avec leur large répartition au sein de la cellule, leur forte concentration intracellulaire, leur relativement faible efficacité catalytique si on la compare à d'autres systèmes enzymatiques, et leur propension à être inactivées par leur substrat. De plus, elles sont capables de réduire une large gamme de substrats, qui comprend des peroxydes mais aussi des espèces réactives dérivées de l'azote, comme les peroxynitrites (Wong et al 2002, Sakamoto et al 2003). Toutes ces propriétés confèrent aux Prx un statut qui n'est pas simplement celui d'une enzyme

antioxydante, mais aussi un système de perception et de transduction spécifique du signal généré par les ROS en général et par H_2O_2 en particulier. Le séquençage de génomes de plantes a permis d'effectuer un recensement exhaustif de cette famille de protéines. Chez le peuplier, il existe 9 Prx qui se divisent 4 Prx de type II, 2 Prx 2-Cys, 2 Prx Q et 1 Prx 1-Cys. Dans le génome du riz, on trouve 8 Prx: 4 Prx de type II, 1 Prx à 2-cys et 2 Prx 1-Cys (Gama et al, sous presse). Chez *Arabidopsis thaliana*, il existe 9 Prx exprimées, qui se répartissent ainsi : 5 Prx de type II, 2 Prx à 2 cystéines, 1 Prx Q et une Prx à une cystéine (Rouhier et Jacquot 2005). Elles sont présentes dans les différents compartiments cellulaires : les Prx 2-Cys A et B, Prx Q et Prx IIE sont chloroplastiques, les Prx IIB, C, D sont cytosoliques, la Prx IIF est mitochondriale et la Prx 1-Cys nucléaire. Toutes ces Prx possèdent une activité de réduction des peroxydes très similaire, mais diffèrent au niveau des mécanismes de régénération de l'enzyme, et au niveau de leur structure quaternaire. Enfin, si l'élimination des ROS est vraisemblablement une fonction commune, certaines pourraient avoir une fonction plus spécialisée, comme la protection des photosystèmes pour la Prx Q, ou comme la signalisation par H_2O_2 dans le chloroplaste pour les Prx 2-Cys qui seraient les seules Prx dont l' « hyperoxydation » est réversible (Konig et al 2003, Dietz et al 2006, Lamkemeyer et al 2006).

II Les glutathion peroxydases

Les Gpx constituent chez les animaux un système très efficace d'élimination des ROS grâce à la présence d'une sélénocystéine très réactive, chez la plupart des isoformes. Chez les végétaux, non seulement les protéines homologues de ces enzymes ne dépendent pas du GSH mais des Trx pour leur fonctionnement, mais

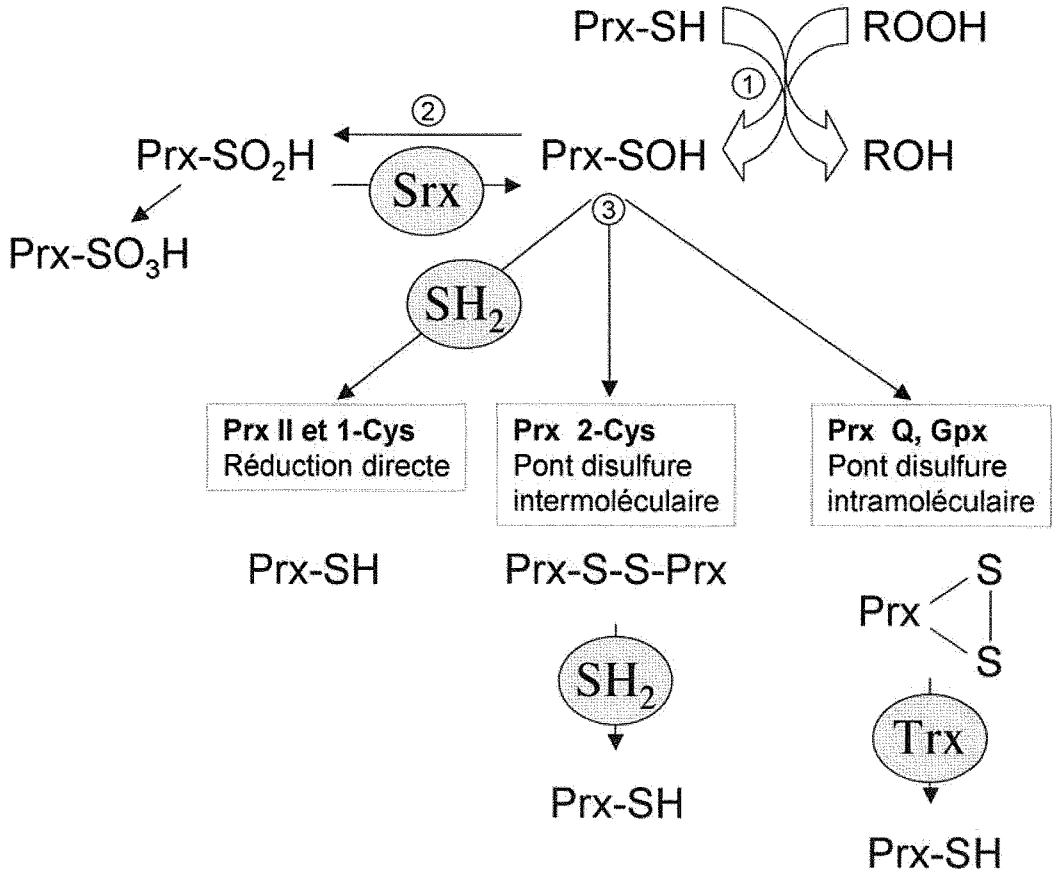

Figure 13. Différents mécanismes réactionnels des Prx. 1 : Le péroxyde ROOH réagit avec la cystéine catalytique de la Prx, pour former un acide sulfénique et relarguer une molécule d'alcool. 2 : L'acide sulfénique des Prx 2-Cys peut réagir avec une seconde molécule de péroxyde et former un acide sulfinique, réductible par la Srx. 3 : Selon la sous-famille considérée, l'acide sulfénique va être converti : en thiol par l'intermédiaire d'un thiol cellulaire dans le cas des Prx de type II et 1-Cys; en pont disulfure intermoléculaire, réduit par la suite par un thiol cellulaire dans le cas des Prx 2-Cys; en pont disulfure intramoléculaire réduit par la suite par une Trx, dans le cas des Prx Q et Gpx.

elles sont aussi dépourvues de sélénocystéine. Elles possèdent une cystéine classique dans leur site actif. Ces particularités suggèrent que les Gpx végétales pourraient être inclues dans la famille des peroxydases thiol- (ou thiorédoxine-) dépendantes. Cependant, leur structure primaire est différente, de même que le nombre et la position des cystéines conservées impliquées dans le mécanisme catalytique. Celui des Gpxs implique deux des trois cystéines conservées, et est strictement dépendant des Trx pour la régénération de l'enzyme (Figure 13). Au niveau structural, Prx et Gpx possèdent un motif de type Trx-fold, avec leur cystéine catalytique située à l'extrémité N-terminale d'une hélice alpha. De plus, les Gpx végétales sont organisées en homodimères, liés par des liaisons électrostatiques (travail réalisé en collaboration avec le laboratoire de cristallographie), ce qui est aussi le cas d'une Prx II de peuplier (Echalier et al 2005). Cette situation est par exemple très différente de celle des Prx 2-Cys, qui sont organisées sous forme de monomères, dimères covalents ou de décamères selon les conditions (Wood et al 2002).

En considérant ces différentes propriétés, on peut néanmoins envisager des rôles assez proches pour les Prx et les Gpx végétales. Les capacités catalytiques des Gpx de peuplier, établies au cours de ce travail, sont comparables à celles établies sur d'autres Gpx et Prx de plantes (Herbette et al 2002, Horling et al 2002, Jung et al 2002, Rouhier et al 2004a, Tanaka et al 2005, Yang et al 2005) et sont compatibles avec un rôle de détoxication *in vivo.* Les Gpx sont présentes dans la plupart des organes de la plante (ce travail et Rodriguez-Milla et al 2003) et dans la plupart des compartiments intracellulaires. Récemment, une isoforme mitochondriale a été caractérisée chez *Raphanus sativus* (Yang et al 2006) Des isoformes mitochondriale et chloroplastique ont ainsi été mises en évidence chez le peuplier, et

des peptides transit putatifs existent aussi dans les séquences de Gpx d'autres plantes.

Une différence importante entre Prx et Gpx semble être l'absence d'inactivation de l'enzyme par H_2O_2. Georgiou et Masip (2003), dans leur théorie du « floodgate », proposent que les Prx jouent un rôle de détoxication des ROS quand ils sont présents à faible concentration. Si cette concentration augmente, en cas de stress oxydant, les Prx deviennent hyperoxydées et transitoirement inactivées, entraînant la diffusion de ROS dans la cellule et l'activation de mécanismes de réponse au stress oxydant. Cette théorie ne serait donc pas directement applicable aux Gpx de plantes, même si on peut imaginer que certaines isoformes puissent avoir un rôle comparable à celui de la Gpx3 de levure qui active un facteur de transcription (Yap1) impliqué dans la réponse au stress oxydant (Delaunay et al 2002). Leur rôle pourrait donc être plus spécifiquement lié à l'élimination des peroxydes. L'expression d'une Gpx de plante chez un mutant déficient de levure augmente la tolérance de celui-ci aux peroxydes de phospholipides (Yang et al 2004). De même, l'expression d'un gène de Gpx de plante inhibe la mort cellulaire programmée chez la levure et la tomate (Chen et al 2005). Les Gpx sont de plus surexprimées dans de nombreuses conditions de stress (Criqui et al 1992, Holland et al 1993, Sugimoto and Sakamoto 1997, Depege et al 1998, Roeckel-Drevet et al 1998, Li et al 2000, Agrawal et al 2002, Rodriguez Milla et al 2003, Kang et al 2004, ce travail). Cette activité de protection de la cellule, notamment au niveau des membranes, a déjà été démontré extensivement pour l'isoforme humaine, nommée PHGpx ou Gpx4, la plus proche des Gpx végétales (Sattler et al 1994). Cette PHGpx possède aussi d'autres rôles chez les mammifères: elle est impliquée entre autres dans la voie des cytokines (Brigelius-Flohé et al 1997), et joue un rôle structural dans

les capsules des mitochondries de spermatozoïdes (Ursini et al 1999). Cependant, ces rôles sont peu transposables aux cellules végétales, surtout parce que la PHGpx de mammifères est impliquée dans des fonctions métaboliques absentes chez les plantes. (pour revue sur les Gpx de mammifères, voir Brigélius-Flohé 1999, Drevet 2000).

La Gpx3 de levure, qui sert d'activateur de Yap1, est également impliquée dans la régulation d'une autre enzyme, une méthionine sulfoxyde réductase (Kho et al 2006). De manière inattendue, un pont disulfure intermoléculaire se forme entre les cystéines de recyclage des deux protéines et non pas entre les cystéines catalytiques. Les auteurs proposent que l'interaction entre les deux protéines soit un moyen de réguler l'activité des enzymes impliquées dans la détoxication des ROS et dans la réparation des protéines. En effet, l'interaction est rompue en situation de stress, permettant aux deux enzymes de fonctionner. Il serait donc intéressant de savoir si les Gpx végétales peuvent aussi être impliquées dans des régulations de ce type.

III Les fonctions physiologiques des peroxydases thiol-dépendantes : redondance ou spécificité ?

III.A Activités des Prx et des Gpx

Chez notre modèle d'étude, le peuplier, l'existence de six isoformes de Gpx couplées aux 9 Prx existantes, peut paraître difficilement compatible avec un rôle spécifique de chacune d'entre elles au sein de la cellule. Les études biochimiques

n'ont pas toujours permis de faire apparaître des spécificités entre les différentes isoformes. En effet, les Gpx et Prx ont souvent des efficacités catalytiques similaires vis-à-vis des différents peroxydes. Toutefois, il existe des spécificités qui peuvent être notées. Ainsi, les Prx Q réduisent peu les substrats plus complexes qu'H_2O_2 tels que les peroxydes complexes ou les lipides peroxydés (Lamkemeier et al 2006). Les Prx 2-Cys semblent capables de réduire toutes sortes de peroxydes, y compris les peroxydes de lipides, avec une plus faible efficacité cependant (Konig et al 2003). On retrouve la même variabilité dans les études concernant la gamme de substrats que les Gpx sont capables de réduire. Certaines réduisent toutes sortes de peroxydes testés (Jung et al 2002, Tanaka et al 2005, Rho et al 2006, ce travail), tandis que d'autres ne semblent pas pouvoir réduire le peroxyde d'hydrogène (Herbette et al 2002). Les Gpx végétales ont aussi une activité qui peut dépendre de différents réducteurs. Certaines études montrent que cette activité est dépendante du GSH ou des Trx (Jung et al 2002). Une Gpx de tournesol réduit quant à elle l'H_2O_2 en utilisant une Trx mais elle n'en est pas capable en présence de GSH (Herbette et al 2002). Enfin, dans notre étude, les Gpx caractérisées sont strictement dépendantes des Trx pour leur activité de détoxication. La grande variété de réducteurs (Trx, Grx), testés au cours de ce travail permet de conclure que dans le cas du peuplier, l'activité des Gpx est probablement strictement dépendante des Trx, et non du GSH et/ou des Grx. Ceci contraste avec les connaissances de la PHGpx humaine, homologue aux Gpx végétales, qui est capable de réduire tout type de peroxydes, depuis H_2O_2 jusqu'aux peroxydes de phospholipides complexes, en utilisant le GSH (Maiorino et al 1991). Cependant, cette enzyme est monomérique, alors que les Gpx de plantes sont dimériques, ce qui peut faciliter ses interactions avec des molécules complexes, et possède une sélénocystéine dans son site actif. Les Gpx végétales semblent donc

définitivement plus proches des Prx que des Gpx déjà connues dans les autres règnes.

Au niveau du chloroplaste, les Gpx montrent une spécificité pour les Trx y. Cette spécificité se retrouve aussi chez les Prx chloroplastiques: les Prx Q utilisent aussi préférentiellement les Trx de type y, alors que les Prx 2-Cys d'*Arabidopsis thaliana* sont réduites plus efficacement par les Trx x (Collin et al 2003; 2004, Rouhier et al 2004a).

Finalement, si l'on considère que les Gpx végétales sont strictement dépendantes des Trx pour leur fonctionnement, la question de la présence d'une activité peroxydase dépendante du glutathion dans les extraits végétaux reste posée. Il semble que cette activité ne proviennent pas des Gpx proprement dites, mais plutôt de certaines glutathion S-transférases (GST), enzymes impliquées dans l'adduction de GSH à certaines molécules, mais qui possèdent aussi une activité Gpx (Bartling et al 1993, Cummins et al 1999). La surexpression d'une GST de coton entraîne par ailleurs une forte augmentation de l'activité Gpx dans des feuilles de tabac, et leur résistance au stress oxydant (Yu et al 2003). Il existe de plus un grand nombre d'isoformes de GST, ainsi que des familles spécifiques aux plantes (Wagner et al 2002), qui sont encore très peu caractérisées.

III.B Localisation, expression des Prx et Gpx

Alors que ce travail de thèse montre qu'une isoforme au moins est strictement chloroplastique (Gpx1), tandis qu'une autre est co-localisée dans la mitochondrie et le chloroplaste (Gpx3.2), la localisation des Gpx à l'intérieur de chaque compartiment

			Fleur	Etamine	Pollen	Graine	Tige	Feuilles	Xylème	Racine
Cyto	Prx IIA	At1g65990	-	-	-	-	-	-	-	-
	Prx IIB	At1g65980	++	++	++	+++	++	++	++	+++
	Prx IIC	At1g65970	++	+++	+++	+	-	-	-	++
	Prx IID	At1g60740	++	+++	+++	+	-	-	-	++
	Gpx2	At2g31570	++	++	-	++	++	++	-	++
	Gpx3	At2g43350	+	+	-	+	+	+	+	+
	Gpx4	At2g48150	-	+	+	-	-	-	-	-
	Gpx5	At3g63080	+	+	+	+	+	+	+	+
Chloro	Prx IIE	At3g52960	+	+	-	++	+	++	-	+
	Prx 2-cys A	At3g11630	++	+	-	++	+++	+++	+	+
	Prx 2-cys B	At5g06290	+	-	-	+	++	++	-	+
	Prx Q	At3g26060	++	+	-	+	++	++	-	-
	Gpx1	At2g25080	+	+	-	+	++	++	+	+
	Gpx6	At4g11600	++	+++	+	+++	++	++	++	++
	Gpx7	At4g31870	-	-	-	-	-	+	-	-
Mito	Prx IIF	At3g06050	+	+	+	+	+	+	+	+
	Gpx6	At4g11600	++	+++	+	+++	++	++	++	++
Secr	Gpx8	At1g63460	+	+	-	-	+	+	+	+
Noyau	Prx 1-cys	At1g48130	-	-	-	+++	-	-	-	-

-	<1000
+	1000-5000
++	5000-15000
+++	>15000

Figure 14. Expression des différents gènes de Prx et Gpx d'*Arabidopsis thaliana*. Les données sont tirées du logiciel GeneAtlas, sur le site Genevestigator (www.genevestigator.ethz.ch). Les valeurs numériques des signaux ont été converties pour faciliter une lecture comparative.

cellulaire n'est pas connu, mais pourrait être déterminante pour comprendre quelles sont les rôles de ces enzymes. Il a ainsi déjà été montré que la Prx Q et les Prx 2-Cys d'*A. thaliana* sont majoritairement situées au niveau des thylacoïdes, et sont impliquées dans la protection des photosystèmes (Lamkemeyer et al 2006, Konig et al 2002).

En ce qui concerne la localisation des Gpx, les Gpx2 et 5 de peuplier possèderaient un peptide de transit qui les dirigeraient vers les voies de sécrétion. Elles seraient donc les seules peroxydases thiol-dépendantes dans ce cas, puisqu'aucune Prx ne semble présenter ce type de localisation. Concernant, les réducteurs potentiels pour ces enzymes, des Trx et Grx (Grx C2, C4, Trx h5 de peuplier) possèdent des séquences d'adressage de ce type et sont donc des candidats d'intérêt. Il a d'ailleurs été montré que la Trx h5 de tabac est secrétée *in vitro* (Juarez-Diaz et al 2006). Chez le peuplier, Gpx4 et Gpx3.1 sont, *a priori*, des formes redondantes du cytosol, qui s'ajouteraient aux Prx IIB et IIC identifiées. L'expression différentielle des gènes codant pour ces protéines pourrait expliquer cette apparente redondance.

En effet, l'analyse des profils d'expression des gènes de Prx *d'Arabidopsis thaliana* par microarrays, en utilisant le logiciel Genevestigator (Zimmermann et al 2004) montre des variations importantes de l'expression suivant l'organe considéré (Figure 14). On observe que certaines isoformes sont exprimées très spécifiquement, la Gpx4 dans les étamines et le pollen, la Gpx7 dans les feuilles, ou encore la Prx 1-cys dans les graines. D'autres profils sont moins tranchés, tandis que certaines isoformes sont omniprésentes dans toute la plante, comme les Prx IIB et IIF ou les Gpx5 et 6. Cette dernière est homologue à la Gpx3.2 de peuplier, qui est la plus représentée en termes d'EST. On peut aussi remarquer que globalement,

l'expression des gènes de Prx semble plus importante que celle des Gpx, ce qui est en accord avec les résultats obtenus par RT-PCR chez le peuplier (voir Article VIII). On obtient donc un profil global assez hétérogène pour cette famille, qui peut encore être affiné en fonction du compartiment cellulaire considéré, ou encore grâce aux résultats obtenus en conditions de stress. Ainsi, dans les graines par exemple, la Gpx6 est susceptible d'être la principale Prx présente dans la mitochondrie. A partir des données de microarrays pour *Arabidopsis thaliana* (site Genevestigator: http://www.genevestigator.ethz.ch), on peut observer que la transcription de la Prx IID est celle qui est la plus régulée en conditions de stress. En particulier, l'infection par des pathogènes de type *Agrobacterium*, *Botrytis* ou certains *Pseudomonas* induit une augmentation de la transcription d'un facteur compris entre 5 et 70, alors qu'aucune augmentation importante n'est visible pour les autres isoformes. La plus forte augmentation est observée pour les genres *P. infestans* (64 fois) et *P. syringae* (66 fois). L'implication de cette Prx dans la réponse à l'infection par ce pathogène est donc probable. Cette hypothèse est appuyée par les résultats obtenus en présence d'un éliciteur de *Pseudomonas*, la syringoline, qui induit une augmentation du taux de transcrit de 96 fois. En ce qui concernent les isoformes de Gpx, le gène de Gpx4 est surexprimé lui aussi en cas d'infection par *Agrobacterium tumefaciens* (11 fois), tandis que celui de la Gpx2 est induit en condition de sénescence (9 fois). Mittler et al (2004) ont aussi montré que la transcription des gènes de Gpx était influencée par des conditions de stress froid. Les Gpx semblent par ailleurs être surexprimées en conditions de stress en présence de métaux lourds, ce qui n'est pas le cas pour les autres Prx (Pascal Rey, communication personnelle). Il paraît donc possible de postuler qu'il existe bien une fonction spécifique pour chacune de ces enzymes.

Les Gpx possèdent un mécanisme commun à toutes les isoformes, contrairement à ce qui est observé chez les Prx. De même, contrairement aux variations dans le nombre de cystéines conservées et impliquées dans la catalyse, qui sont observées pour les Prx, les Gpx de plantes possèdent toutes trois cystéines, dont deux sont nécessaires à l'activité des enzymes. Ces enzymes ont aussi un donneur d'électrons unique, les Trx, ce qui contraste avec les capacités des Prx à utiliser le GSH, les Grx ou les Trx pour leur recyclage. La famille des Gpx est aussi plus restreinte, avec 8 isoformes chez l'arabette, mais seulement 6 chez le peuplier. Il est donc posssible d'envisager que ces Gpx aient d'autres fonctions dans la cellule, en particulier dans une position plus « en amont » dans la réponse de la cellule au stress oxydant. Pour tester cette hypothèse, l'obtention de mutants sous-exprimant, ou n'exprimant pas un ou plusieurs gènes de Gpx a déjà été utilisée chez d'autres organismes, et des études similaires chez les plantes commencent à être publiées.

Chez les mammifères, la mutation homozygote du gène PHGpx, par exemple, est létale (Imai et al 2003). Ces protéines protègent les cellules contre le stress oxydant, et ont aussi d'autres rôles, notamment structuraux dans la capsule mitochondriale des spermatozoïdes (Maiorino et al 2005). Chez la levure, la délétion des gènes de Gpx n'est pas létale. Cependant, la suppression du gène Gpx3 entraîne une augmentation de la sensibilité des levures au stress oxydant (Inoue et al 1999). Chez les plantes, on sait déjà que la surexpression d'une Gpx de *Chlamydomonas reinhardtii* dans des plants de tabac augmente la tolérance au stress et l'activité de réduction des peroxydes complexes (Yoshimura et al 2004). Récemment, il a été démontré qu'un mutant d'insertion pour la Gpx3 d'*Arabidopsis thaliana* est plus sensible au stress oxydant, et que sa réponse à un stress hydrique est perturbée (Miao et al 2006).

Ainsi, si les rôles spécifiques des Gpx au sein des systèmes redox chez les plantes sont aussi encore sujets à hypothèses, ces enzymes sont probablement essentielles dans le réseau de transduction du signal et de défense antioxydante des cellules végétales. Bien que nous ayons pu éclaircir les mécanismes enzymatiques utilisés par ces enzymes ainsi que leurs localisations tissulaire et intracellulaire, la plupart des fonctions physiologiques potentielles : peroxydase, senseur de stress, transducteur direct ou indirect de signaux, activateur d'enzymes, ainsi que leur importance relative *in vivo* restent encore à évaluer chez les plantes.

PERSPECTIVES

Le peuplier est la principale plante modèle représentant les végétaux ligneux. Son génome assez restreint et ses capacités à être transformé par *Agrobacterium tumefaciens* compensent les difficultés expérimentales liées à ce matériel telles que sa lenteur de croissance (comparée à celle des herbacées). Les études accumulées permettent aussi de disposer de la séquence du génome d'une espèce (*Populus trichocarpa*) et de nombreuses séquences EST. Des puces à ADN permettant des analyses transcriptomiques à grande échelle sur le génome entier sont déjà à disposition. L'étude au niveau protéique reste limitée par l'existence de familles multigéniques ce qui ajoute un degré de complexité à tous les systèmes enzymatiques déjà connus, et en particulier aux systèmes de détoxication redox. Ceci est exacerbé chez les végétaux, puisque le nombre de gènes composant ces différentes familles est généralement beaucoup plus important.

Les travaux réalisés au cours de ma thèse ont ainsi permis de caractériser une famille de protéines thiol-dépendantes, appartenant au groupe des Prx. Cette caractérisation doit encore être approfondie notamment par l'identification de protéines (enzymes, facteurs de transcription) partenaires des Gpx, ROS mis à part, comme cela a été montré dans d'autres organismes. La caractérisation *in vitro* est une première étape, de même que des techniques *in vivo*, telles que la co-immunoprécipitation, qui s'imposent de plus en plus comme des techniques incontournables pour prouver les interactions entre protéines. L'implication des Gpx dans la réponse au stress, causé par les métaux lourds en particulier, reste également à approfondir, avec la surexpression de Gpx *in planta* ou en utilisant des mutants d'*Arabidopsis thaliana* par exemple. La recherche de phénotypes particuliers

chez des mutants déficients en une ou plusieurs isoformes de Gpx pourrait ainsi permettre d'éclaircir la fonction physiologique de ces protéines. La fonction de senseur du stress oxydant et de transduction du signal par les Gpx, déjà mises en évidence chez la levure, sont aussi des pistes de recherche intéressantes, notamment par la recherche de protéines partenaires des Gpx qui seraient oxydées par celles-ci en conditions de stress. Par des approches de protéomique classique (interactions sur gel ou sur colonne), puis des expériences de complémentation double-hybride, la mise en évidence de ces protéines semblent être envisageable chez les végétaux. Enfin la localisation des Gpx au sein des compartiments cellulaires pourrait permettre de définir les rapports entre Gpx et membranes, ou encore d'étudier la localisation exacte et le rôle des Gpx potentiellement secrétées.

Les études structurales qui sont en cours vont apporter des informations supplémentaires sur le site actif et le mécanisme catalytique de ces enzymes, sur les acides aminés impliqués dans la formation du dimère et dans l'interaction avec les Trx. En particulier, il sera intéressant de comprendre pourquoi seules certaines Trx interagissent avec les Gpx. L'étude de protéines mutées pour un ou plusieurs acides aminés est envisagée, et pourrait éclaircir et confirmer les informations déjà obtenues.

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DOCTORAT DE L'UNIVERSITE HENRI POINCARE, NANCY 1

en BIOLOGIE FORESTIERE

VU, APPROUVÉ ET PERMIS D'IMPRIMER N° 1823

Nancy, le 5/11/06

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RESUME

Dans les cellules végétales, la concentration en espèces oxygénées réactives (ROS), est contrôlée par différents systèmes. Les thiorédoxines (Trx) et les glutarédoxines (Grx) jouent un rôle crucial en fournissant leur pouvoir réducteur aux péroxyrédoxines (Prx), ou peroxydases thiol-dépendantes, qui assurent l'élimination des différents composés oxygénés (ou nitrés) dans les cellules. Le travail présenté dans ce manuscrit décrit essentiellement la famille multigénique des glutathion peroxydases (Gpx) chez une espèce modèle, le peuplier. Bien qu'appartenant aux peroxydases thiol-dépendantes, la structure primaire des Gpx est différente de celle des Prx déjà caractérisées. L'expression de cette famille de gènes a été évaluée et comparée aux autres Prx dans les organes de peuplier. L'abondance de certaines Gpx est modifiée au cours de certains stress, notamment en présence de métaux comme le cadmium. La localisation de deux isoformes de Gpx dans le chloroplaste (Gpx1, Gpx3) ou la mitochondrie (Gpx3) a été expérimentalement démontrée par des fusions GFP. Les études enzymatiques réalisées sur la Gpx3 recombinante et sur des mutants cystéiniques de cette protéine a permis de mettre en évidence : (i) un mécanisme catalytique différent de celui des Gpx de mammifères, faisant intervenir seulement deux des trois cystéines conservées, qui forment (ii) un pont disulfure intramoléculaire de potentiel rédox –295 mV, réduit spécifiquement par certaines Trx *in vitro*. Ces enzymes sont en outre actives en solution sous la forme d'homodimères non-covalents.

Mots-clés : glutathion peroxydases, thiorédoxines, péroxyrédoxines, peuplier, stress oxydant.

ABSTRACT

In plant cells, reactive oxygen species (ROS) concentration is controlled by different systems. Thioredoxins (Trx) and glutaredoxins (Grx) play a crucial role in providing reducing power to peroxiredoxins (Prx), also called thiol-dependent peroxidases, which scavenge oxygen- or nitrogen-derived reactive compounds. The work presented in this manuscript describes mainly the glutathione peroxidases (Gpx) family in a model species, the poplar tree. Although Gpx are thiol-dependent peroxidases, their primary sequence differs from the Prx sequences characterized to date. The gene expression of this family was assessed in different plant organs and it was compared to those of other Prx. The abundance of different Gpx is also modified under stress conditions, notably in the presence of heavy-metals like cadmium. Localization of two isoforms in the chloroplast (Gpx1, Gpx3) and in mitochondria (Gpx3) was demonstrated using GFP fusion proteins. Enzymatic studies performed on wild-type and cysteine-mutated Gpx3 recombinant proteins showed: (i) a catalytic mechanism different from the mammalian Gpx, involving two of the three conserved cysteines, which form (ii) an intramolecular disulfide bridge (redox potential: -290 mV) that will then specifically be reduced by certain Trx *in vitro*. Studies also showed that these enzymes are active as non-covalent homodimers in solution.