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REVIEW PAPER

Structural and functional characterization of tree proteins involved in redox regulation: a new frontier in forest science

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Abstract

• ***Key message*** **This paper describes how the combination of genomics, genetic engineering, and 3D structural characterization has helped clarify the redox regulatory networks in poplar with consequences not only in system biology in plants but also in bacteria and mammalian systems.**

• ***Context*** Tree genomes are increasingly available with a large number of orphan genes coding for proteins, the function of which is still unknown.

• ***Aims and methods*** Modern techniques of genome analysis coupled with recombinant protein technology and massive 3D structural determination of tree proteins should help elucidate the function of many of the proteins encoded by orphan genes. X-ray crystallography and NMR will be the methods of choice for protein structure determination.

• ***Results*** In this review, we provide examples illustrating how the above-mentioned techniques improved our understanding of redox regulatory circuits in poplar, the first forest tree species sequenced. We showed that poplar peroxiredoxins use either thioredoxin or glutaredoxin as electron donors to

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Executive summary This review describes the successful use of technologies to overproduce and purify recombinant enzymes from trees with the aim of solving their 3D structures and identifying their molecular interactions either with other proteins or with potential substrates. Currently only *ca* a dozen of tree genomes have been annotated and released including six forest species, but this will change rapidly with the oncoming release of the chestnut and oak genomes notably. The availability of additional genomes will open the way to identifying the function of a large number of orphan gene products, one of the ways to characterize these functions being to produce and analyze the corresponding protein products.

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reduce hydrogen peroxide. That glutaredoxin could be a reductant was unknown at the time of this discovery even in other biological organisms and was later confirmed notably by the observation that the two genes are fused in some bacteria and by the resolution of the structure of the bacterial hybrid protein. Similarly, genome analysis coupled to in vitro analysis of enzymatic properties led to the discovery that some plant methionine sulfoxide reductases can also use both thioredoxins and glutaredoxins as electron donors. Besides their disulfide reductase activity, it has been demonstrated that some poplar glutaredoxins are also involved in iron-sulfur center biogenesis and assembly. The original 3D structure determination has been made with poplar glutaredoxin C1 and then confirmed in a variety of other biological organisms including human. Our work also showed that in plants, so-called glutathione peroxidases use thioredoxins and not glutathione as electron donors. This is true for all non-selenocysteine-containing glutathione peroxidases. Finally, connections between the thioredoxin and glutaredoxin systems have been elucidated through the study of atypical poplar thioredoxins.

• ***Conclusion*** Altogether, these data illustrate how the combination of genetic engineering and structural biology improves our understanding of biological processes and helps fuel systems biology for trees and other biological species.

Keywords 3D protein structure · Genome sequence · Glutaredoxin · Redox · Thioredoxin · Poplar

1 Introduction

There are currently more than 50 plant genomes sequenced and published (Michael and Jackson 2013; see also: http://en.wikipedia.org/wiki/List_of_sequenced_plant_genomes), and of these, more than ten concern tree species. The majority of those are constituted by fruit trees (peach, plum, pear, sweet orange, clementine, apple, coffee, papaya, and cocoa tree). The first forest tree genome published is *Populus trichocarpa* (Tuskan et al. 2006) followed by *Picea abies* (Nystedt et al. 2013), *Hevea brasiliensis* (Rahman et al. 2013), *Picea glauca* (Birol et al. 2013), *Pinus taeda* (Neale et al. 2014), and *Eucalyptus grandis* (Myburg et al. 2014).

The analysis of the first tree genomes has helped in identifying the total number of genes present in these genomes and also a number of orphan genes that have no counterpart in other biological organisms. A recent estimate of the number of orphan genes in poplar is 44 %, a value much higher than the 14 % estimation in *Arabidopsis* (Guo et al. 2007; Feldmann and Goff 2013). The mechanisms leading to orphan gene emergence have been discussed in a number of biological systems (Wissler et al. 2013). In order to understand the functions and interaction properties of the proteins encoded by these genes (if any), one possibility is to produce the corresponding recombinant proteins. The elucidation of their 3D structures together with molecular docking is one possibility that can be used to reach that understanding. Other techniques as the yeast two-hybrid system screen and co-immunoprecipitation can also help in identifying the potential ligands either metabolites or macromolecules as other proteins, carbohydrates, or lipids. Together with the results of transcriptomic and proteomic experiments, these approaches will help decipher metabolic and regulation networks. We illustrate here the use of genetic engineering and 3D structure determination to describe the redox interaction networks in trees, concentrating on the glutathione/glutaredoxin- (Grx) and thioredoxin- (Trx) dependent pathways in poplar. Most of the enzymes presented are members of the thioredoxin fold class. They share a small thioredoxin domain (consisting of a four-stranded beta sheet surrounded by three alpha helices) to fulfill many molecular functions.

2 Redox regulation background

When biological organisms including trees are submitted to stress either abiotic or biotic, a major response is the establishment of an oxidative burst which has to be controlled in order to avoid excessive accumulation of reactive oxygen species and subsequent subcellular damage. A number of antioxidant systems can be found in trees including the thioredoxin and glutaredoxin systems and structural derivatives such as thiol peroxidases (peroxiredoxins (Prx) and glutathione peroxidases (Gpx)) or methionine sulfoxide reductases (Msr). Notably, the thioredoxin-linked systems control the rate and hence yield of photosynthesis but they are also a good defense line against excess oxidants such as H_2O_2 (Schürmann and Jacquot 2000; Rouhier et al. 2008). The general positioning of the thioredoxin- or glutaredoxin-linked redox networks in plants is shown in Fig. 1. In this review, we provide a number of examples detailing how poplar has served as a model for understanding redox regulation not only in plants but also in other systems including animal and bacterial cells. We will also indicate how poplar has become a model species for establishing the 3D structures of plant proteins, a field still in infancy.

3 Poplar thiol peroxidases: peroxiredoxins and "glutathione" peroxidases

The studies that have been initiated in poplar were initially a follow-up of the previous characterization of a peroxide-detoxifying enzyme called peroxiredoxin or thioredoxin

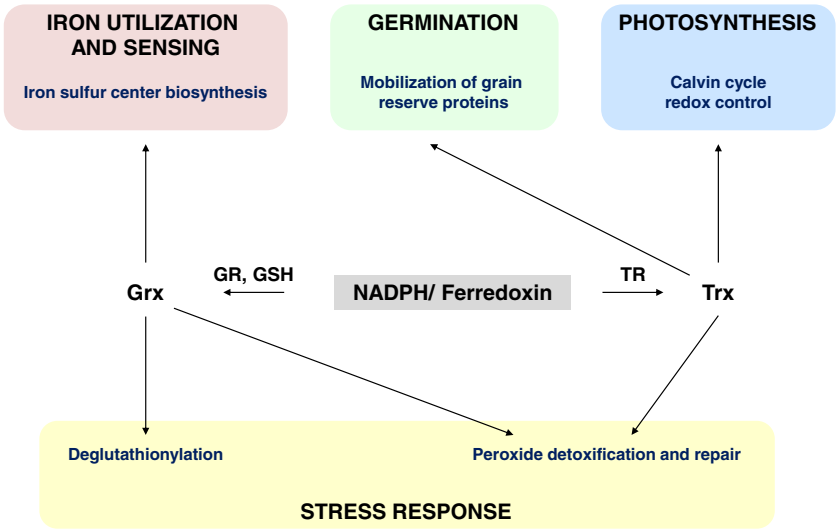


Fig. 1 Positioning and major roles of the glutaredoxin- and thioredoxin-linked systems in plants. This figure emphasizes the central position of the glutaredoxin and thioredoxin systems for the cellular redox control in photosynthetic organisms. Abbreviations used are as follows: *GR* glutathione reductase, *GSH* glutathione, *TR* thioredoxin reductases, *Grx* glutaredoxin, *Trx* Thioredoxin

peroxidase (Verdoucq et al. 1999). Peroxiredoxins are very abundant enzymes which rely on a catalytic cysteine residue to attack various organic peroxides (including hydrogen peroxide) with the release of an alcohol-like molecule and water according to the equation:

$$ROOH \rightarrow ROH + H_2O$$

In the process, the catalytic cysteine residue becomes oxidized into a sulfenic acid which has to be regenerated, the best known regenerating system being the thioredoxin system (NADPH or photoreduced ferredoxin relayed by a thioredoxin reductase and thioredoxin itself). Including glutathione peroxidases, there are five classes of thioredoxin peroxidases in plants based on their amino acid sequence and oligomerization state (Rouhier and Jacquot 2005). Several of them are solely dependent on thioredoxin for their regeneration (Rouhier et al. 2004b; Navrot et al. 2006), but surprisingly our initial data which concerned a specific class called atypical type II peroxiredoxin (later referred to as poplar PrxIIB) indicated that they could be regenerated equally well by thioredoxin and glutaredoxin (Rouhier et al. 2001, 2002a). That glutaredoxins could be an alternate reducing system to atypical type II peroxiredoxins was unknown at that time in other biological organisms. We could in subsequent experiments determine that poplar type II peroxiredoxins are present in several cellular compartments and in particular in the cytosol, mitochondria, and plastids (Gama et al. 2007, 2008). The 3D structure of the cytosolic poplar Prx IIB has been solved both by X-ray crystallography and NMR revealing molecular aspects of its organization, catalytic and regeneration mechanisms (Echalier et al. 2005; Noguera-Mazon et al. 2006). A nice confirmation that glutaredoxin could indeed be a physiological reductant for type II Prx was the detection of hybrid genes in several bacteria. As for thioredoxin reductase-thioredoxin gene fusions observed in some mycobacteria as *Mycobacterium leprae* (Jacquot et al. 2009), Prx and Grx genes can be associated in operons and even fused in some species as *Haemophilus influenzae* and *Neisseria meningitidis* resulting in the generation of a hybrid enzyme (Kim et al. 2003; Rouhier and Jacquot 2003). Based on the sequence of the linker peptide between the two domains (Grx and Prx), we were then able in "fun experiments" to construct functional Prx-Grx and Prx-Trx fusion proteins made with poplar sequences (Rouhier et al. 2006b). We have also studied deeply another variety of peroxiredoxin from poplar called peroxiredoxin Q (Prx Q in plants or BCP in bacteria) (Rouhier et al. 2004b). Prx Q is exclusively chloroplastic and the poplar enzyme is brought back to the reduced form by the thioredoxin system but not via the glutaredoxin system unlike type II Prx. Prx Q expression is modified when poplar leaves are infected by the rust fungus *Melampsora larici-populina*. We could not get a 3D structure of poplar Prx Q but we have been later able to isolate and characterize structurally a hybrid enzyme from *Thermotoga maritima* which contains a Prx Q-like module linked to a nitroreductase domain (Couturier et al. 2013b). Interestingly in *T. maritima*, the hybrid enzyme is reduced by an unusual reduction system composed of thioredoxin reductase and glutaredoxin-like enzymes, indicating that glutaredoxins can be donors in other biological systems than poplar. In non-photosynthetic organisms, another line of defense against excessive peroxide generation is constituted by Gpxs. Those enzymes employ similar catalytic mechanisms and sulfenic acid chemistry as Prxs. There are several classes of Gpxs in mammals

including selenocysteine-containing enzymes which are present only in algae but not in land plants, the Gpxs of which contain cysteines instead of selenocysteines. For the selenoenzymes, the selenenic acid is normally brought back to the thiol state via reduced glutathione. Interestingly, we have observed using the poplar model that plant Gpxs are present in different subcellular compartments but also that they differ from their mammalian selenoenzyme counterparts in using thioredoxin but not glutathione for their regeneration. Whereas it is in principle possible for GSH to reduce sulfenic acids, the presence of a resolving cysteine in plant Gpxs leads to the formation on an intramolecular disulfide which can only be reduced by Trxs. In this respect, cysteine-containing Gpxs can be considered as thioredoxin peroxidases or peroxiredoxins. Indeed, the structural determination of poplar Gpx5 indicated that it has a fold similar to peroxiredoxins, an observation that was not clear from the examination of amino acid sequences (Navrot et al. 2006; Koh et al. 2007). Figure 2 illustrates this structural convergence between type II Prx and so-called plant Gpx (panels a, b). The monomer organization is similar, based on the thioredoxin model plus a helix-beta strand insertion between the second helix and the second β-strand of the Trx fold and a β-hairpin N-terminal extension. Interestingly, plant Gpx can reduce not only hydroperoxides but also peroxynitrites (Selles et al. 2012). In conclusion, this set of experiments has allowed us to determine that unlike their mammalian counterparts, plant Gpxs do not rely on glutathione but rather on the thioredoxin system for their functioning and regeneration. The peroxiredoxin and Gpx "stories" have also provided one of the examples of the interchangeability between the thioredoxin and glutaredoxin pathways with regard to peroxide detoxification.

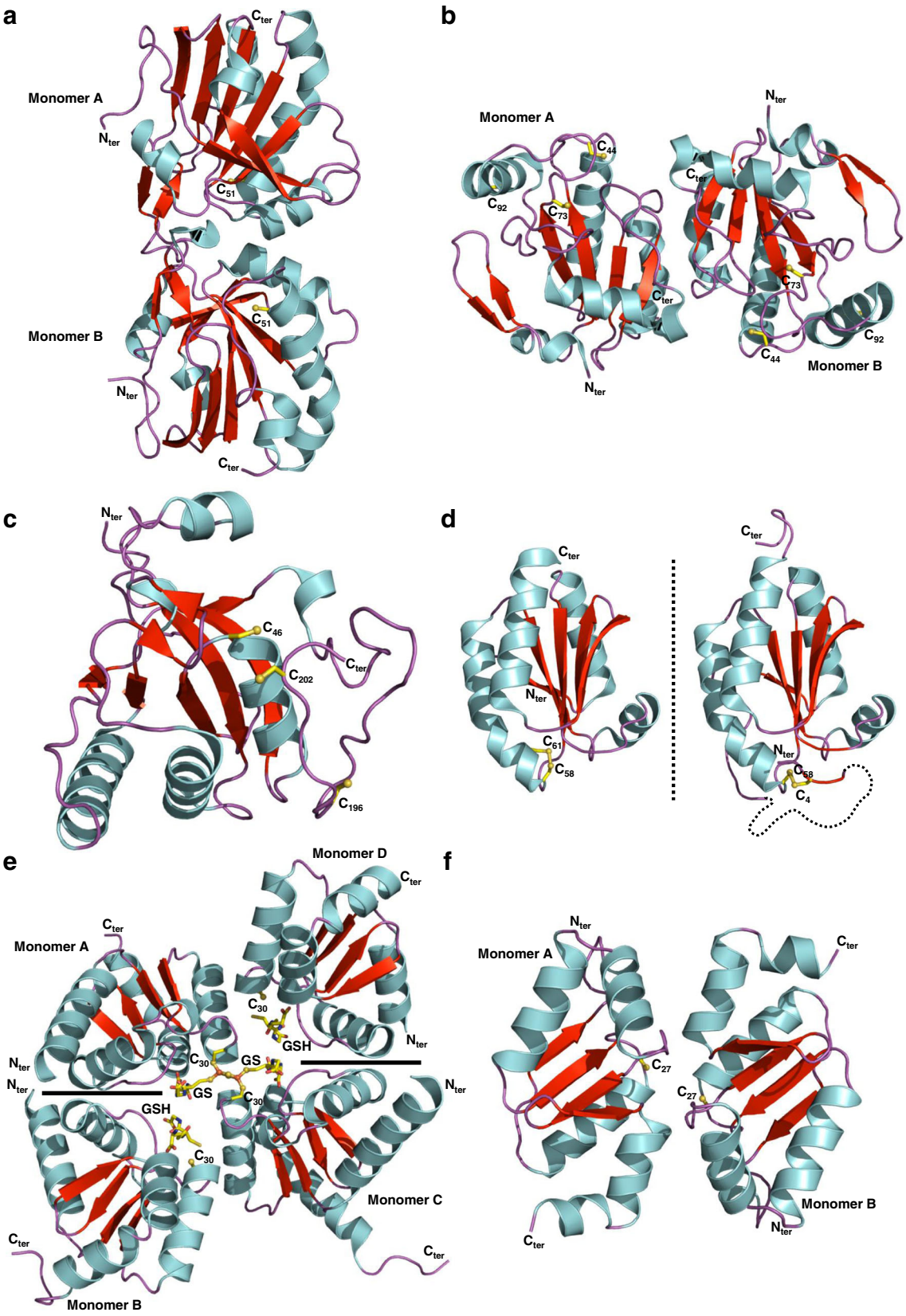
Fig. 2 Selected structures of poplar enzymes. In all these structures, β-strands are shown in *blue*, α-helices are in *red*, and connecting loops are in *purple*. **a** Cartoon representation of the overall fold of the dimeric Prx IIB (PDB number, 1TP9). **b** Cartoon representation of the overall fold of the dimeric Gpx5 in the reduced form (PDB number, 2P5Q). Note that, in the oxidized form, the formation of an intramolecular disulfide bond between Cys44 and Cys92 induces a large conformational rearrangement leading in particular to the complete unwinding of the α1 helix. By comparing both structures, you could note the similar architecture of the monomers and the characteristic double β-strand external to the central pleated β-sheet. **c** Cartoon representation of the overall fold of the plastidial methionine sulfoxide reductase A (PDB number, 2J89). The structure of the monomer in the reduced form is shown here. The positions of the sulfur atoms of the important cysteine residues are indicated by *yellow spheres*. Site-directed mutagenesis experiments coupled to activity assays indicated that the catalytic Cys46 forms a disulfide with Cys202 before its subsequent reduction by Cys196. It is clear that considerable conformational rearrangements are needed to bring the sulfur atoms close enough to form these disulfides. This may explain why we have been unable to get the 3D structure of oxidized forms. **d** Cartoon representation of the overall fold of the monomeric thioredoxin h4 under two different oxidized forms (PDB numbers 3D21, 3D22). The sulfur atoms of the cysteine residues of the disulfide bonds are shown in *yellow spheres*. *Left panel*, disulfide bond between Cys58 and Cys61; *right panel*, disulfide bond between Cys4 and Cys58. Lacking residues (7–15) are shown in an imaginary *gray dashed* loop for a better overview of the right panel structure. **e**, **f** Structural comparison of Grx C1 and Grx C4 dimers. In the Grx C1 structure, there is one homodimer containing a [2Fe-2S] ISC plus two additional monomers symmetrically positioned but bearing no ISC. GSH indicates an external glutathione molecule (shown in *stick*) and GS glutathione molecule covalently linked to the Fe_2S_2 center. Sulfur and iron atoms are represented, respectively, as *yellow* and *red spheres*. Grx C4 makes a homodimer with no ISC. The PDB number of Grx C1 is 2E7P, whereas no coordinates were deposited for Grx C4

4 Poplar methionine sulfoxide reductases

Another example of a repair enzyme using sulfenic acid chemistry is the methionine sulfoxide reductase. Oxidative stress can indeed induce the oxidation of the side chain of methionine residues resulting in methionine sulfoxide generation. Depending on the stereochemistry, two types of Msrs can repair this oxidative modification: MsrA and MsrB. MsrA has been characterized in poplar and its 3D structure deciphered. Unlike Prx whose structure is based on the thioredoxin fold, MsrA exhibits a unique alpha/beta architecture. Figure 2c shows the structure of the subunit of MsrA together with the position of the cysteines involved in catalysis. For catalysis, the two poplar MsrAs characterized rely on a three cysteine mechanism, the order of the cysteine involvement being different from the *Escherichia coli* enzyme (Rouhier et al. 2007a). Initially for poplar MsrA, the most efficient reductant system was found to be the thioredoxin system. However, it was found later that, in *Gracilaria gracilis*, MsrA and Grx are linked in a Grx-Grx-MsrA hybrid enzyme, a situation reminiscent of the earlier identification of Prx-Grx fusions. Additionally, subsequent experiments have shown that Msrs of the two types (A and B) including poplar MsrA are also efficiently regenerated by the glutaredoxin system (Vieira dos Santos et al. 2007; Tarrago et al. 2009; Couturier et al. 2012). Overall, these data provide further evidence concerning the interchangeability of the two reduction systems.

5 Poplar thioredoxins

Additional data connecting the thioredoxin and glutaredoxin systems were provided by the thorough study of poplar


a
Monomer A
Monomer B
b
Monomer A
Monomer B
c
d
e
Monomer A
Monomer B
Monomer C
Monomer D
GSH
GS
f
Monomer A
Monomer B

thioredoxins. We first set out to describe the first structure of an active site variant (CPPC instead of CGPC) in poplar Trxh1 (Coudevylle et al. 2004, 2005). The NMR structural analysis revealed that changing the glycine residue of the active site into a proline has very moderate effect on the structure (Menchise et al. 2001), although based on yeast complementation, CPPC containing thioredoxins are less active in sulfate reduction but more efficient for protection against H_2O_2 than their CGPC counterparts (Bréhelin et al. 2000). Further characterization of poplar thioredoxins included the study of poplar Trxh3 (Gelhaye et al. 2003b) and also showed that the Trxh2 isoform is located in mitochondria where it serves to regulate alternative oxidase and the mitochondrial electron flow (Gelhaye et al. 2004). Incidentally, this was the first clear demonstration of the presence of thioredoxin h in plant mitochondria. A very interesting piece of information arose when studying poplar Trx h4. This protein possesses a classical active site (CGPC) but has the unique characteristic to contain a conserved cysteine near the N-terminus at position 4 (Gelhaye et al. 2003a). Through site-directed mutagenesis and structural determination, we have shown that this was related to an unusual three cysteine mechanism where two successive and different disulfides are formed upon catalysis, one of them involving Cys4. Remarkably, the regeneration system of Trx h4 is the glutaredoxin system and not the NAPDH-thioredoxin reductase system (Koh et al. 2008). Figure 2d shows the structure of poplar Trx h4 with its characteristic pleated β-sheet surrounded by α helices found in all thioredoxins. Unlike in non-photosynthetic organisms, there are in fact a large number of thioredoxin genes in plants and the situation is similar in poplar (Gelhaye et al. 2005; Chibani et al. 2009). One of the newly discovered thioredoxins, Trx z, is located in plastids and reduced by the ferredoxin-thioredoxin reductase (FTR) (Chibani et al. 2011). Several of the thioredoxin genes code for proteins with highly unusual active sites. We showed quite recently that in contrast with their classification based on amino acid sequence similarities, poplar Trx-like2.1 and Trx-lilium2.2 are regenerated in vitro either by the NADPH-thioredoxin reductase system or by glutathione but not the FTR system (Chibani et al. 2012). As a consequence, since these two thioredoxins are plastidial, their likely in vivo reducing system is glutathione, thus resembling glutaredoxins. Consistently, Trx-like2.1 was found to be effective in MsrB1 and PrxIIE regeneration, two known Grx target enzymes. Altogether, these results further exemplify the need to back up phylogenetic studies with "wet lab" biochemistry. A similar need was apparent from the wrong classification of plant "glutathione" peroxidases which turned out to be thioredoxin peroxidases as detailed previously. Concerning our own redox research, these data also reinforce the notion of thioredoxin-glutaredoxin cross talk already previously mentioned. To conclude on the past studies on poplar thioredoxins on a more physiological note, we have very recently obtained evidence that thioredoxin h1 is involved in the gravitropism response being itself localized in the amyloplasts of the endoderm, a localization of high interest for gravitropism signal perception (Azri et al. 2013). Overall, the international recognition of these studies on poplar in a competitive field (including research groups in medicine and other areas of biology) is apparent in a paper by Perez-Jimenez et al. (2009) where two poplar thioredoxin h were selected in a study detailing thioredoxin catalysis at the single molecule and atomic level.

6 Poplar glutaredoxins

Another section of the redox field where we have made significant contributions is the study of glutaredoxins, the initial breakthrough papers having been made with poplar Grxs. As observed for Trxs, Grxs make an extended phylogenetic family in photosynthetic organisms including poplar but conversely the number of *Grx* genes is much reduced in bacteria or mammals (Rouhier et al. 2004a, 2006a; Couturier et al. 2009a, 2013a). Overall, there are three major groups in land plants with active sites in the form of CxxC (class I), CGFS (class II), and CCxC/S (class III). Class III Grxs are present solely in land plants and play a role in flower development (Li et al. 2009). From genomic and phylogenetic analyses, a fourth Grx class is also present in land plants but whether they exhibit glutathione-dependent reductase activity awaits confirmation (Couturier et al. 2009a). Additional classes formed by fusion enzymes are found in lower eukaryotes and prokaryotes. The Grx studies were initiated with poplar Grx C4, a class I member with a "classical" CYPC active site. The corresponding protein was produced in a soluble form by genetic engineering but only after optimization of its DNA sequence and its biochemical properties studied. It did not turn out to be different from other classical Grxs (d'Ambrosio et al. 2003; Rouhier et al. 2002b, 2003). On the other hand, the characterization of poplar Grx C1 (class I, CGYC active site) led to surprising results as the recombinant protein partitioned between an apo monomeric form and

a holo dimeric form. Very interestingly, the holo form was shown to contain an iron sulfur center (ISC) of the Fe_2S_2 type (Feng et al. 2005, 2006; Rouhier et al. 2007b). NMR-linked structural determination of Grx C4 which does not normally assemble such holo structures indicated nevertheless that the protein makes homodimers in solution with the active sites facing one another (Noguera et al. 2005). Figures 2e, f show dimers of Grx C1 and Grx C4, with the ISC present in the former but not the latter. A thorough comparison of the structures of both Grxs revealed that the Pro residue present between the two Cys of the disulfide is probably too bulky to allow the formation of the ISC in Grx C4. In agreement with this observation, the P to G mutation in Grx C4 allows the protein to incorporate an ISC into a homodimer, and the reverse mutation in Grx C1 abolishes that property (Rouhier et al. 2007b). A crucial observation in Grx C1 is that two of the Fe-S ligands are external glutathione molecules, a situation also encountered in a number of non-photosynthetic homologs (Rouhier et al. 2008). Incidentally, these data complexify the bioinformatics prediction of sequences able to bind an ISC. Normally, they should contain four cysteines or a similar number of cysteine and histidine ligand residues, but in the permissive glutaredoxins, only one cysteine per monomer is sufficient (Selles et al. 2009). The structural study of class I poplar Grx S12 and also of *Arabidopsis thaliana* Grx C5 has added more to our understanding concerning the sequence requirements permissive for ISC binding (Couturier et al. 2009b, 2011). The observation that Grxs from class II can bind ISC has shed new light on a completely different research area, the biosynthesis of ISC. Indeed, it was known that in yeast the mitochondrial Grx5 (class II, CGFS active site) is required for Fe-S cluster biogenesis but it was not known why (Rodríguez-Manzaneque et al. 2002). Work performed with CGFS Grxs from poplar or *Arabidopsis* has demonstrated that they also ligate ISC. In addition, they can very efficiently transfer these centers to acceptor proteins as apo ferredoxin (Bandyopadhyay et al. 2008; Rouhier et al. 2010). Overall, these very original contributions made with poplar proteins support an additional role for glutaredoxins in Fe-S protein assembly, separate from the classical reductase activity.

While progress has been remarkable on class I and II members, we know little about the biochemical and structural properties of class III Grxs. Indeed, they are notoriously difficult to work with owing to insolubility problems perhaps linked to the very hydrophobic C-terminus of the protein. Nevertheless, we have made a number of constructs using poplar Grx C1 and Grx C4 as templates in which we have introduced active site signatures (CCMC and CCMS) present in class III Grxs. In agreement with the structural predictions made from earlier work on Grx C1 and C4, the engineered Grxs bind Fe-S centers and possess reductase activity in the monomeric form (Couturier et al. 2010). Whether these data reflect specific physiological functions awaits further characterization.

7 Biochemical and structural studies of redox enzymes in progress

Having isolated to homogeneity a number of poplar thioredoxins and glutaredoxins has allowed us to trap their potential targets. This was performed by genetic engineering and modifying the second cysteine of the active sites into serines which in effect prevents the backup reaction and allows trapping the target disulfide-linked to the redoxin. Using this technology, we have identified *ca* 100 Grx targets suggesting that redox post-translational modifications control a large number of cellular processes. A carbonic anhydrase was further characterized (Rouhier et al. 2005). Likewise, the use of a mutated poplar thioredoxin h led to the identification of more than 50 Trx-linked proteins in the mitochondria (Balmer et al. 2004). The structural and regulatory properties of those potential targets are still largely unknown even though redox regulation in mitochondria was reported for 2-oxoaciddehydrogenase complexes (Bunik et al. 1999).

There are a large number of glutathione-*S*-transferases (GST) genes in photosynthetic organisms and especially in poplar (Lan et al. 2009; Lallement et al. 2014a). These proteins are involved mainly in cellular detoxification. Some of these proteins possess a catalytic cysteine and they are functionally similar to Grxs. Three GSTs of the lambda type (GSTL1 to L3) have been recently characterized until the molecular level. The proteins are monomeric and they bind glutathionylated substrates for deglutathionylation reactions (Lallement et al. 2014b). Overall, this study strengthened the proposal that GSTLs are involved in the management of secondary metabolites via their redox properties. Additional poplar GSTs from different classes are currently being studied.

We have mentioned earlier the existence of thioredoxin-like proteins in poplar (like2- and lilium-types especially), indicating that these proteins functionally behave as glutaredoxins. From this observation, it becomes of high interest to elucidate what in their structure makes them

glutaredoxin-like from a catalytic point of view. As a matter of fact, several of these structures are being solved presently and we should be able to answer this question soon.

Another area where we are currently active concerns the regulatory enzymes of the Benson-Calvin cycle and especially the phosphatases, fructose-1,6-bisphosphatase (FBPase), and sedoheptulose-1,7-bisphosphatase (SBPase). It is known that both enzymes are redox-regulated but the relationship between them is still unclear at the molecular level. Although we know precisely the structure and regulatory properties of pea FBPase, little is known about the structure-function relationship of SBPase (Jacquot et al. 1995; Chiadmi et al. 1999). In a first series of experiments, we have produced the corresponding recombinant proteins from poplar and *Chlamydomonas reinhardtii* but the enzymes turned out not to be sufficiently stable and rather rapidly denatured. We have then turned to another model *Physcomitrella patens* (see companion paper by Müller et al. this issue) from where we could produce stable FBPase and SBPase as recombinant proteins. The biochemical and structural properties of both proteins are currently under investigation. The *Physcomitrella patens* model is also currently being used to create mutants of the ferredoxin-thioredoxin system using site-directed mutagenesis and gene replacement for the catalytic subunit of FTR.

Another area of current excitement is the involvement of class II Grxs in ISC biosynthesis. In three recent papers, the structures of poplar Grx S14 were solved and its Fe-S cluster transfer capacity described (Wang et al. 2011, 2012, 2014). Current emphasis is made on the interactions between class II Grxs and BolA proteins or similar components such as SufE1 protein (Couturier et al. 2014; Roret et al. 2014). A last domain of interest that is being investigated concerns the link between redox and biotic stress. Indeed when poplar leaves are infected by rust there is a very active exchange of information including the release of small secreted proteins (SSP) by both interaction partners. These SSP very often possess unusually high cysteine contents with the possibility to make several disulfides. We are currently studying the properties of some corresponding recombinant proteins up to the 3D molecular level. Interestingly, many of these SSPs would qualify as orphan gene products.

8 Census of tree protein 3D structures

As explained above, the availability of gene models from fully sequenced genomes is extremely helpful and even required for the development of high throughput structural biology with the aim of deciphering tree protein structures. In the pdb database, the structures are listed with a link to their botanical classification and unfortunately the item tree is not present. So making a census of the present tree protein structures is not an easy task. Also the definition of a tree is not so clear; for example, we chose not to define banana (overall nine protein structures available of three different proteins) and coconut (one structure in pdb) as trees as various botanical considerations indicate they should not be labeled as such. Another example is *Vitis vinifera* (overall 20 protein structures nearly all of them describing oxidoreductases of the secondary metabolism) which we chose not to include as a tree although it is an organism which contains lignin but it does not have the stance of a tree. Tables 1 and 2 provide the current data concerning protein structures from forest and non-forest trees, respectively. Using a very empirical approach (looking systematically for most well-known tree species), we have found 141 protein structures belonging to forest species including *H. brasiliensis* and 33 structures to non-forest species (including fruit trees but also plantation trees as coffee). The non-forest tree protein structures describe *ca* 14 different proteins from five different botanical families. The known forest tree protein structures belong to seven genera (Betulaceae, Cesalpinaceae, Euphorbiaceae, Fabaceae, Salicaceae, Taxaceae, and Pinaceae) and they describe *ca* 36 different protein structures. A large number of these structures (18) describe a single pollen allergen of birch, the BET V allergen, and a similar number (17) a hydroxynitryle lyase from *Hevea*. Overall, nearly half of the forest tree structures describe poplar proteins illustrating how genome sequencing influences downstream the structural field (poplar was the first genome tree sequenced and fully annotated). Overall, 19 of the poplar structures describe a single protein plastocyanin either from NMR or crystallographic data in complex with a variety of ligands, one of the structures describing plastocyanin in complex with cytochrome f. In reality, the effort concerning plastocyanin started well before the genome sequence of poplar became available. Our own contribution to the structural field has resulted in 17 structures describing 11 different poplar proteins in various states solved either by NMR or X-ray crystallography. They are marked with a star in Table 1. It now seems clear that poplar is bound to become a model organism for structural biology especially when coupled to molecular biology allowing the production of recombinant proteins. Still it is remarkable that the first plastocyanin structure was obtained with the poplar protein well before the advent of all those molecular techniques (Guss and Freeman 1983). To conclude on those aspects of structural biology, it should be mentioned that poplar is and will most likely become the

Table 1 Known forest tree protein structures

PDB code	Family	Source	Enzyme/protein
1B6F	Betulaceae	*Betula pendula* (birch)	BET V 1 allergen
1BTV	Betulaceae	*Betula pendula* (birch)	BET V 1 allergen
1BV1	Betulaceae	*Betula pendula* (birch)	BET V 1 allergen
1FM4	Betulaceae	*Betula pendula* (birch)	BET V 1 allergen
1LLT	Betulaceae	*Betula pendula* (birch)	BET V 1 allergen
1QMR	Betulaceae	*Betula pendula* (birch)	BET V 1 allergen
4A80	Betulaceae	*Betula pendula* (birch)	BET V 1 allergen
4A81	Betulaceae	*Betula pendula* (birch)	BET V 1 allergen
4A83	Betulaceae	*Betula pendula* (birch)	BET V 1 allergen
4A84	Betulaceae	*Betula pendula* (birch)	BET V 1 allergen
4A85	Betulaceae	*Betula pendula* (birch)	BET V 1 allergen
4A86	Betulaceae	*Betula pendula* (birch)	BET V 1 allergen
4A87	Betulaceae	*Betula pendula* (birch)	BET V 1 allergen
4A88	Betulaceae	*Betula pendula* (birch)	BET V 1 allergen
4A8G	Betulaceae	*Betula pendula* (birch)	BET V 1 allergen
4A8U	Betulaceae	*Betula pendula* (birch)	BET V 1 allergen
4A8V	Betulaceae	*Betula pendula* (birch)	BET V 1 allergen
4B9R	Betulaceae	*Betula pendula* (birch)	BET V 1 allergen
4BK6	Betulaceae	*Betula pendula* (birch)	BET V 1 allergen
4BK7	Betulaceae	*Betula pendula* (birch)	BET V 1 allergen
4BKC	Betulaceae	*Betula pendula* (birch)	BET V 1 allergen
4BKD	Betulaceae	*Betula pendula* (birch)	BET V 1 allergen
4BTZ	Betulaceae	*Betula pendula* (birch)	BET V 1 allergen
1H4B	Betulaceae	*Betula pendula* (birch)	BET V 4 allergen
1CQA	Betulaceae	*Betula pendula* (birch)	Profilin
1R8N	Caesalpinaceae	*Delonix regia*	Kunitz trypsin inhibitor
3QQQ	Cannabaceae	*Trema tomentosa*	Plant hemoglobin
1PXZ	Cupressaceae	Cedar	Allergen
1QYD	Cupressaceae	*Thuya*	Pinoresinol reductase
1P9G	Eucommiaceae	*Eucommia ulmoides*	Antifungal peptide
1P9Z	Eucommiaceae	*Eucommia ulmoides*	Antifungal peptide
4MPI	Euphorbiaceae	*Hevea*	Chitin-binding module of a chitinase-like protein
4HPG	Euphorbiaceae	*Hevea*	Glycosylated glucanase
4IIS	Euphorbiaceae	*Hevea*	Glycosylated glucanase
1HVQ	Euphorbiaceae	*Hevea*	Hevamin A
1LLO	Euphorbiaceae	*Hevea*	Hevamin A
2HVM	Euphorbiaceae	*Hevea*	Hevamin A
1KQY	Euphorbiaceae	*Hevea*	Hevamin mutant
1KQZ	Euphorbiaceae	*Hevea*	Hevamin mutant
1KR0	Euphorbiaceae	*Hevea*	Hevamin mutant
1KR1	Euphorbiaceae	*Hevea*	Hevamin mutant
1HEV	Euphorbiaceae	*Hevea*	Hevein
1QB9	Euphorbiaceae	*Hevea*	Hevein
1T0W	Euphorbiaceae	*Hevea*	Hevein
1WKX	Euphorbiaceae	*Hevea*	Hevein
4YAS	Euphorbiaceae	*Hevea*	Hydroxy nitryle lyase
5YAS	Euphorbiaceae	*Hevea*	Hydroxy nitryle lyase
6YAS	Euphorbiaceae	*Hevea*	Hydroxy nitryle lyase
7YAS	Euphorbiaceae	*Hevea*	Hydroxy nitryle lyase

[a] Indicates the structures deposited by the authors of this paper

Table 1 (continued)

PDB code	Family	Source	Enzyme/protein
1QJ4	Euphorbiaceae	*Hevea*	Hydroxynitryle lyase
1SC9	Euphorbiaceae	*Hevea*	Hydroxynitryle lyase
1SCI	Euphorbiaceae	*Hevea*	Hydroxynitryle lyase
1SCK	Euphorbiaceae	*Hevea*	Hydroxynitryle lyase
1SCQ	Euphorbiaceae	*Hevea*	Hydroxynitryle lyase
1YAS	Euphorbiaceae	*Hevea*	Hydroxynitryle lyase
1YB6	Euphorbiaceae	*Hevea*	Hydroxynitryle lyase
1YB7	Euphorbiaceae	*Hevea*	Hydroxynitryle lyase
2G4L	Euphorbiaceae	*Hevea*	Hydroxynitryle lyase
2YAS	Euphorbiaceae	*Hevea*	Hydroxynitryle lyase
3C6X	Euphorbiaceae	*Hevea*	Hydroxynitryle lyase
3C6Y	Euphorbiaceae	*Hevea*	Hydroxynitryle lyase
3C6Z	Euphorbiaceae	*Hevea*	Hydroxynitryle lyase
3C70	Euphorbiaceae	*Hevea*	Hydroxynitryle lyase
3YAS	Euphorbiaceae	*Hevea*	Hydroxynitryle lyase
1G5U	Euphorbiaceae	*Hevea*	Latex profilin
1FNY	Fabaceae	*Robinia*	Lectin
1FNZ	Fabaceae	*Robinia*	Lectin
2VLC	Laraceae	*Cinnamomum camphora* (camphor)	Cinnamomin (ribosome inactivating protein)
2LVF	Lecythidoideae	*Bertholletia excelsa* (Brazil nut)	Allergen
1J4S	Moraceae	*Artocarpus*	Artocarpin
1J4T	Moraceae	*Artocarpus*	Artocarpin
1J4U	Moraceae	*Artocarpus*	Artocarpin
1VBO	Moraceae	*Artocarpus*	Artocarpin
1VBP	Moraceae	*Artocarpus*	Artocarpin
1JAC	Moraceae	*Artocarpus*	Jacalin
1WS4	Moraceae	*Artocarpus*	Jacalin
1WS5	Moraceae	*Artocarpus*	Jacalin
3P8S	Moraceae	*Artocarpus*	Jacalin
1KU8	Moraceae	*Artocarpus*	Jacalin (lectin)
1KUJ	Moraceae	*Artocarpus*	Jacalin (lectin)
1M26	Moraceae	*Artocarpus*	Jacalin (lectin)
1PXD	Moraceae	*Artocarpus*	Jacalin (lectin)
1UGW	Moraceae	*Artocarpus*	Jacalin (lectin)
1UGX	Moraceae	*Artocarpus*	Jacalin (lectin)
1UGY	Moraceae	*Artocarpus*	Jacalin (lectin)
1UH0	Moraceae	*Artocarpus*	Jacalin (lectin)
1UH1	Moraceae	*Artocarpus*	Jacalin (lectin)
1TOQ	Moraceae	*Artocarpus*	Lectin
1TP8	Moraceae	*Artocarpus*	Lectin
4AK4	Moraceae	*Artocarpus*	Lectin
4AKB	Moraceae	*Artocarpus*	Lectin
4AKC	Moraceae	*Artocarpus*	Lectin
4AKD	Moraceae	*Artocarpus*	Lectin
4CUO	Moraceae	*Ficus bengalensis*	Banyan peroxidase
1JOT	Moraceae	*Maclura pomifera*	Agglutinin
3LLY	Moraceae	*Maclura pomifera*	Agglutinin
3LLZ	Moraceae	*Maclura pomifera*	Agglutinin

Table 1 (continued)

PDB code	Family	Source	Enzyme/protein
3LM1	Moraceae	*Maclura pomifera*	Agglutinin
3S9V	Pinaceae	*Abies*	Abietadiene synthase
3SAE	Pinaceae	*Abies*	Sesquiterpene synthase
3SDQ	Pinaceae	*Abies*	Sesquiterpene synthase
3SDR	Pinaceae	*Abies*	Sesquiterpene synthase
3SDT	Pinaceae	*Abies*	Sesquiterpene synthase
3SDU	Pinaceae	*Abies*	Sesquiterpene synthase
3SDV	Pinaceae	*Abies*	Sesquiterpene synthase
3HBD	Pinaceae	*Picea*	Chitinase
3HBE	Pinaceae	*Picea*	Chitinase
3HBH	Pinaceae	*Picea*	Chitinase
1QYC	Pinaceae	*Pinus*	Phenylcoumaran benzylic ether reductase
1UOU	Pinaceae	*Pinus*	Stilbene synthase
1XES	Pinaceae	*Pinus*	Stilbene synthase
1XET	Pinaceae	*Pinus*	Stilbene synthase
4LEJ	Pinaceae	*Pinus*	Vicilin
4N6T	Salicaceae	*Populus* (poplar)	Adhiron (de novo prot)
4N6U	Salicaceae	*Populus* (poplar)	Adhiron (de novo prot)
3A9U	Salicaceae	*Populus* (poplar)	Coumarate CoA ligase
3A9V	Salicaceae	*Populus* (poplar)	Coumarate CoA ligase
3NI2	Salicaceae	*Populus* (poplar)	Coumarate CoA ligase
1Z7P	Salicaceae	*Populus* (poplar)	Glutaredoxin C1[a]
1Z7R	Salicaceae	*Populus* (poplar)	Glutaredoxin C1[a]
2E7P	Salicaceae	*Populus* (poplar)	Glutaredoxin C1[a]
No deposit	Salicaceae	*Populus* (poplar)	Glutaredoxin C4 NMR crystallography[a]
3FZ9	Salicaceae	*Populus* (poplar)	Glutaredoxin S12[a]
3FZA	Salicaceae	*Populus* (poplar)	Glutaredoxin S12[a]
2LKU	Salicaceae	*Populus* (poplar)	Glutaredoxin S14 apo NMR[a]
2LKU	Salicaceae	*Populus* (poplar)	Glutaredoxin S14 NMR[a]
2P5Q	Salicaceae	*Populus* (poplar)	Glutathione peroxidase Gpx5[a]
2P5R	Salicaceae	*Populus* (poplar)	Glutathione peroxidase Gpx5[a]
4PQH	Salicaceae	*Populus* (poplar)	Glutathione S transferase L1[a]
4PQI	Salicaceae	*Populus* (poplar)	Glutathione S transferase L3[a]
3N0F	Salicaceae	*Populus* (poplar)	Isoprene synthase
3N0G	Salicaceae	*Populus* (poplar)	Isoprene synthase
2 J89	Salicaceae	*Populus* (poplar)	Methionine sulfoxide reductase A[a]
1JXG	Salicaceae	*Populus* (poplar)	Plastocyanin
1PLC	Salicaceae	*Populus* (poplar)	Plastocyanin
1PNC	Salicaceae	*Populus* (poplar)	Plastocyanin
1PND	Salicaceae	*Populus* (poplar)	Plastocyanin
2PCY	Salicaceae	*Populus* (poplar)	Plastocyanin
3PCY	Salicaceae	*Populus* (poplar)	Plastocyanin
4DP0	Salicaceae	*Populus* (poplar)	Plastocyanin
4DP1	Salicaceae	*Populus* (poplar)	Plastocyanin
4DP2	Salicaceae	*Populus* (poplar)	Plastocyanin
4DP4	Salicaceae	*Populus* (poplar)	Plastocyanin
4DP5	Salicaceae	*Populus* (poplar)	Plastocyanin

Table 1 (continued)

PDB code	Family	Source	Enzyme/protein
4DP6	Salicaceae	*Populus* (poplar)	Plastocyanin
4DP7	Salicaceae	*Populus* (poplar)	Plastocyanin
4DP8	Salicaceae	*Populus* (poplar)	Plastocyanin
4DP9	Salicaceae	*Populus* (poplar)	Plastocyanin
4DPA	Salicaceae	*Populus* (poplar)	Plastocyanin
4DPB	Salicaceae	*Populus* (poplar)	Plastocyanin
4DPC	Salicaceae	*Populus* (poplar)	Plastocyanin
4PCY	Salicaceae	*Populus* (poplar)	Plastocyanin
5PCY	Salicaceae	*Populus* (poplar)	Plastocyanin
6PCY	Salicaceae	*Populus* (poplar)	Plastocyanin
1TKW	Salicaceae	*Populus* (poplar)	Plastocyanin cyt f complex
1SI9	Salicaceae	*Populus* (poplar)	Protein SP1 heat stable
1TRO	Salicaceae	*Populus* (poplar)	Protein SP1 heat stable
1YQD	Salicaceae	*Populus* (poplar)	Sinapyl alcohol dehydrogenase
1YQX	Salicaceae	*Populus* (poplar)	Sinapyl alcohol dehydrogenase
1TI3	Salicaceae	*Populus* (poplar)	Thioredoxin h1[a]
3D21	Salicaceae	*Populus* (poplar)	Thioredoxin h4[a]
3D22	Salicaceae	*Populus* (poplar)	Thioredoxin h4[a]
1TP9	Salicaceae	*Populus* (poplar)	Type II peroxiredoxin[a]
1UMZ	Salicaceae	*Populus* (poplar)	Xyloglucan endotransglycolase
1UN1	Salicaceae	*Populus* (poplar)	Xyloglucan endotransglycolase
2YII	Taxaceae	*Taxus*	Phenylalanine amino mutase
4BAA	Taxaceae	*Taxus*	Phenylalanine amino mutase
4BAB	Taxaceae	*Taxus*	Phenylalanine amino mutase
4C5R	Taxaceae	*Taxus*	Phenylalanine amino mutase
4C5S	Taxaceae	*Taxus*	Phenylalanine amino mutase
4C5U	Taxaceae	*Taxus*	Phenylalanine amino mutase
4C6G	Taxaceae	*Taxus*	Phenylalanine amino mutase
4CQ5	Taxaceae	*Taxus*	Phenylalanine amino mutase
3NZ4	Taxaceae	*Taxus*	Taxadiene synthase
3P5P	Taxaceae	*Taxus*	Taxadiene synthase
3P5R	Taxaceae	*Taxus*	Taxadiene synthase

model species for trees but it is of course not the only one for plants in general and remarkable advances have been done in the past with spinach or pea high molecular weight enzymes as RubisCO and LHC (Knight et al. 1990; Standfuss et al. 2005; Barros et al. 2009).

9 Tree system biology: what could be contribution of structural proteomics?

Massive information from genome sequencing needs now to be organized into a systems biology database in order to understand the structure-function relationships of individual proteins (Dietz et al. 2010). Besides, the characterization of protein-protein interactions obviously requires working at the protein level. Still, even the genome organization helps in this respect. We have given above a couple examples explaining how the genes coding for separate proteins working together in poplar can be found linked together in bacteria, leading to the production of hybrid enzymes. A take home message is that one should systematically look for such possible associations in prokaryotic genomes. The results of this systematic genomic data-mining can be sometimes extremely informative. Alternatively, genetic engineering allows the production of recombinant enzymes that can be used

Table 2 Known non-forest tree protein structures

pdf #	Family	Source	Enzyme/protein
3G4F	Malvaceae	Cotton, *Gossypium arboreum*	Cadinene synthase
3G4D	Malvaceae	Cotton, *Gossypium arboreum*	Cadinene synthase
3BRX	Malvaceae	Cotton, *Gossypium hirsutum*	Annexin
1N00	Malvaceae	Cotton, *Gossypium hirsutum*	Annexin
2JON	Oleaceae	Olive	Allergen
1SS3	Oleaceae	Olive	Allergen
1B8G	Rosaceae	Apple	ACC synthase
1M4N	Rosaceae	Apple	ACC synthase
1M7Y	Rosaceae	Apple	ACC synthase
1YNU	Rosaceae	Apple	ACC synthase
3PIU	Rosaceae	Apple	ACC synthase
3ZS3	Rosaceae	Apple	Thaumatin like
1IQQ	Rosaceae	*Pyrus*, pear	Pistil ribonuclease
1E09	Rosaceae	*Prunus*	Pru Av1 cherry allergen
1H2O	Rosaceae	*Prunus*	Pru Av1 cherry allergen
1JU2	Rosaceae	*Prunus*	Hydroxy nitryle lyase
2AHN	Rosaceae	*Prunus*	Pru Av2 allergen
2ALG	Rosaceae	*Prunus*	Pru P (LTP)
2B5S	Rosaceae	*Prunus*	Pru P (LTP)
3EHK	Rosaceae	*Prunus*	Allergenic protein
3FZ3	Rosaceae	*Prunus*	Almond Pru I allergenic
3GDN	Rosaceae	*Prunus*	Hydroxy nitryle lyase
3GDP	Rosaceae	*Prunus*	Hydroxy nitryle lyase
3RED	Rosaceae	*Prunus*	Hydroxy nitryle lyase
2EFJ	Rubiaceae	Coffee	3,7-Dimethylxanthine methyltransferase
2EG5	Rubiaceae	Coffee	Xanthosine methyl transferase
4G22	Rubiaceae	Coffee	Lys HCT
4G0B	Rubiaceae	Coffee	Lys HCT
4G2M	Rubiaceae	Coffee	Lys HCT
2M70	Rutaceae	Citrus	Poly(A)-binding protein 1
3WD7	Rutaceae	Citrus	Polyketide synthase
3WD8	Rutaceae	Citrus	Polyketide synthase
4JJM	Rutaceae	Citrus	Cyclophilin

for biochemical determination and also for protein structural work. Very elegant fishing techniques such as the yeast two-hybrid system or bimolecular fluorescence complementation experiments can be used to fish or validate potential protein ligands for a given protein. Still another avenue for research is the structural determination of tree proteins, though it requires first access to the genes of a given organism. Given the present high-throughput of tree genome sequencing, we postulate that tree protein structural genomics should soon take off. As we have detailed in this paper, the field is still very much in infancy with the structures of only *ca* 70 individual tree proteins that have been solved up to now. We believe that the future of tree protein structure determination is very bright indeed, given its interesting potential for systems biology improvement.

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