Biochemical Processes and Macromolecular Structures

**Running head:** Atypical thioredoxins from poplar

**Corresponding author:** Rouhier Nicolas

UMR 1136 Lorraine University INRA
Interactions Arbres-Microorganismes. IFR 110 EFABA
Faculté des Sciences BP 239
54506 Vandœuvre-lès-Nancy Cedex, France
Tel (+33) 3 83 68 42 25
Fax (+33) 3 83 68 42 92
Email: nrouhier@scbiol.uhp-nancy.fr
Atypical thioredoxins in poplar: the glutathione-dependent thioredoxin-like 2.1 supports the activity of target enzymes possessing a single redox active cysteine

Kamel Chibani\textsuperscript{a,b}, Lionel Tarrago\textsuperscript{a}, José Manuel Gualberto\textsuperscript{c}, Gunnar Wingsle\textsuperscript{b}, Pascal Rey\textsuperscript{d,e,f}, Jean-Pierre Jacquot\textsuperscript{a}, Nicolas Rouhier\textsuperscript{a,*}

\textsuperscript{a} UMR 1136 Lorraine University-INRA, Interactions Arbres-Microorganismes. IFR 110 EFABA, Faculté des Sciences BP 239, 54506 Vandœuvre-lès-Nancy Cedex, France.
\textsuperscript{b} Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, Umeå Plant Science Centre, 90183 Umeå, Sweden.
\textsuperscript{c} Institut de Biologie Moléculaire des Plantes, CNRS-UPR2357, 67084 Strasbourg, France.
\textsuperscript{d} CEA, DSV, IBEB, Lab Ecophysiol Molecule Plantes, Saint-Paul-lez-Durance, F-13108, France
\textsuperscript{e} CNRS, UMR 7265 Biol Veget & Microbiol Environ, Saint-Paul-lez-Durance, F-13108, France
\textsuperscript{f} Aix-Marseille Université, Saint-Paul-lez-Durance, F-13108, France

*Corresponding author: nrouhier@scbiol.uhp-nancy.fr
Tel: +33 3 83 68 42 25; Fax: +33 3 83 68 42 92


Keywords: chloroplast, glutathione, poplar, redox potential, thioredoxin.
**Footnotes**

This work was supported by funding from the INRA-FORMAS program to KC, GW and NR. NR and JPJ acknowledge grants from Institut Universitaire de France.

Present address of Lionel Tarrago:

Center for Redox Medicine
Division of Genetics, Department of Medicine
Brigham and Women’s Hospital and Harvard Medical School
New Research Building, Room 435
77 Ave. Louis Pasteur
Boston, Massachusetts 02115, USA

* Corresponding author; e-mail: nrouhier@scbiol.uhp-nancy.fr.
ABSTRACT

Plant thioredoxins (Trx) constitute a complex family of thiol-oxidoreductases generally sharing a WCGPC active site sequence. Some recently identified plant Trxs (Clot, Trx-like1 and 2, Trx-lilium1, 2, and 3) display atypical active site sequences with altered residues between the two conserved cysteines. The transcript expression patterns, subcellular localizations and biochemical properties of some representative poplar isoforms were investigated. Measurements of transcript levels for the 10 members in poplar organs indicate that most genes are constitutively expressed. Using transient expression of GFP fusions, Clot and Trx-like1 were found to be mainly cytosolic, whereas Trx-like2.1 was located in plastids. All soluble recombinant proteins, except Clot, exhibited insulin reductase activity although with variable efficiencies. Whereas Trx-like2.1 and Trx-lilium2.2 were efficiently regenerated both by NADPH-thioredoxin reductase and glutathione, none of the proteins were reduced by the ferredoxin-thioredoxin reductase. Only Trx-like2.1 supports the activity of plastidial thiol-peroxidases and methionine sulfoxide reductases employing one single cysteine residue for catalysis and using a glutathione recycling system. The second active site cysteine of Trx-like2.1 is dispensable for this reaction, indicating that the protein possesses a glutaredoxin-like activity. Interestingly, the Trx-like2.1 active site replacement, from WCRKC to WCGPC, suppresses its capacity to use glutathione as a reductant but is sufficient to allow the regeneration of target proteins employing two cysteines for catalysis, indicating that the nature of the residues composing the active site sequence is crucial for substrate selectivity/recognition. The present study provides another example of the crosstalk existing between the GSH/Grx and Trx-dependent pathways.
INTRODUCTION

In proteins, cysteines are prone to oxidation, leading for example to the formation of sulfenic acids or nitrosothiols upon reaction with reactive oxygen or nitrogen species. Overoxidation can be prevented through the formation of disulfide bridges either internal or between two distinct peptides or with the cysteine of glutathione, a process named glutathionylation (Zaffagnini et al., 2012). All these oxidized forms are reversible and reduced back to cysteines by thioredoxins (Trxs) or glutaredoxins (Grxs), two types of thiol oxidoreductases displaying redox active cysteines in a CxxC or CxxS active site motif (Rouhier et al., 2008; Zaffagnini et al., 2012). These dithiol-disulfide exchange reactions are thus essential for many processes, regulating or regenerating the activity of many proteins. In most living organisms, Trxs are thought to be the major disulfide reductases, whereas Grxs preferentially catalyze deglutathionylation reactions. The reduction of disulfide bonds is a two-step process where the first cysteine of the active site, named catalytic cysteine, performs a nucleophilic attack on the target disulfide forming a transient heterodisulfide and the second cysteine, also referred to as resolving cysteine, then reduces this intermolecular disulfide (Collet and Messens, 2010). A few years ago, Trxs were defined as small proteins of 10 to 14 kDa with a specific WCGPC active site signature. However, the recent sequencing of many genomes, in particular from plant species, helped identifying many variations in the size of the proteins, in their domain organisation and/or in their active site sequence (Chibani et al., 2009). The conventional Trxs have generally a low midpoint redox potential compared to other redoxins, comprised between -270 and -330 mV at pH 7.0 and an N-terminal cysteine with a pKa around 7 (Collin et al., 2003; Ren et al., 2009; Collet and Messens, 2010). Site-directed mutagenesis established that the active site proline is a key residue determining the reducing property of Trx and that the glycine maintains the conformation of the active site (Holmgren, 1985; Eklund et al., 1991; Roos et al., 2007). Besides, it was shown that the tryptophan preceding the catalytic cysteine contributes to Trx stability. Indeed, the altered redox potential and cysteine pKa value observed for a W28A variant of a Staphylococcus aureus Trx was attributed to partial protein unfolding (Roos et al., 2010). This tryptophan is also part of the contact area involved in substrates recognition (Menchise et al., 2000). A few other residues have also been demonstrated to be important both for the reactivity and redox properties of Trxs. In E. coli Trx1, Asp27 and Lys57 contribute to the lowering of the cysteine thiol group pKa
(Dyson et al., 1997). In addition, the residue (usually an isoleucine) positioned just before the conserved cis-Pro found in all members of the Trx superfamily downstream of the active site and facing it in 3D structures, is also very important since its mutation strongly modifies the redox potential, the cysteine pKa and substrate recognition (Ren et al., 2009).

Photosynthetic organisms contain a large number of Trx isoforms (~40), as compared to non-photosynthetic organisms such as E. coli, S. cerevisiae or humans. The classical Trxs with WC[G/P]PC active sites, formerly studied, are located in several sub-cellular compartments including the cytosol (h-type Trxs, tetraricopeptide domain-containing thioredoxins (TDX)), the nucleus (h-type Trxs and nucleoredoxin), mitochondria (h- and o-type Trxs) and plastids (m-, f, x-, y- and z-type Trx, NTRc) (Meyer et al., 2005; Chibani et al., 2009; Meng et al., 2010). The subcellular localizations of several Trxs, especially of members of the nucleoredoxin and Trx-h classes, are only based on predictions and have not yet been experimentally confirmed. The plastids constitute the subcellular compartment possessing the highest number of Trxs. Trxs m and f are primarily involved in the light-dependent regulation of key carbon metabolism enzymes (Lemaire et al., 2007). Trxs x, y, NTRc and CDSP32 (chloroplast drought-induced protein of 32 kDa) are likely involved in stress response serving as reductants for antioxidant enzymes such as thiol-peroxidases (Tpxs) and methionine sulfoxide reductases (MSRs) (Vieira Dos Santos and Rey, 2006). Trx z is implicated in plastid development (Arsova et al., 2010). Most plastidial Trxs with a single domain are likely maintained reduced by light through the ferredoxin (Fdx)/ferredoxin-Trx reductase (FTR) system, whereas Trxs h and o, usually found in the cytosol and in mitochondria, are reduced by NADPH via NADPH-thioredoxin reductase (NTR) (Schürmann and Jacquot, 2000; Laloï et al., 2001; Gelhaye et al., 2004; Balmer et al., 2006; Reichheld et al., 2007; Schürmann and Buchanan, 2008; Chibani et al., 2011). The peculiar plastidial NTRc, formed by a fusion between a NTR and a Trx module, is dependent on NADPH (Perez-Ruiz et al., 2006). It is also worth mentioning that a plant Trx h isoform is specifically reduced by a glutathione reductase/glutathione/glutaredoxin (GR/GSH/Grx) system instead of the classical thioredoxin reductase pathway (Gelhaye et al., 2004; Koh et al., 2008).

More recently, new classes of atypical Trxs named Clot, Trx-like and Trx-lilium have been identified (Meyer et al., 2005). Higher plants generally contain 1 Clot isoform, 2 to 4 Trxs-like and 4 to 5 isoforms of Trxs-lilium (Chibani et al., 2009). These proteins are specific to photosynthetic eukaryotes as these classes are also present in some algae but not in
cyanobacteria. Although sharing a clear homology with other Trxs, they possess very different active site sequences, Clot exhibits a WCPDC active site, Trxs-like proteins have WCRVC or WCRKC active sites whereas Trxs-lilium most often possess GCGGC, SCGSC and WCASC active sites. The Trxs-lilium and the so-called Trx-like2 (WCRKC active site) from *A. thaliana* are localized in chloroplasts, most likely in the stromal fraction (Cain et al., 2009; Dangoor et al., 2009). However, only little biochemical information is available on these atypical Trxs. Some Trxs-lilium, though having a less negative redox potential compared to other plastidial Trxs, could constitute good reductants of 2-Cys PrxA but are poor activators of NADP-malate dehydrogenase (NADP-MDH) (Dangoor et al., 2009).

In this study, we have investigated the transcript expression patterns of the poplar genes encoding Clot, Trxs-like and -lilium in various plant organs and we have experimentally determined the sub-cellular localization of the isoforms, for which it has not been previously performed. On the other hand, selected members, as well as protein variants constructed by site-directed mutagenesis, were produced as recombinant proteins and purified. Using a combination of biochemical approaches and activity assays, the specificity of these Trxs vs different known target proteins (NADP-MDH, Tpxs (either glutathione peroxidases (Gpx) or peroxiredoxins (Prx)) and MSR) or vs the possible reducing systems (NADPH/NTR, NADPH/NTRc, NADPH/FNR/Fdx/FTR or NADPH/GR/GSH) was tested.
RESULTS

Sequence characteristics and sub-cellular localization of Clot, Trx-like1 and Trx-like2.1

Previous phylogenetic studies on photosynthetic and non-photosynthetic organisms revealed the presence of a large number of Trxs with undefined roles. For instance, the Populus trichocarpa genome contains 45 thioredoxin-related sequences and 4 thioredoxin reductases (Chibani et al., 2009). Beside classical Trxs, poplar contains 1, 4 and 5 isoforms of Clot, Trx-like and Trx-lilium, respectively. Clot proteins (WCPDC active site) constitute a single phylogenetic subgroup, whereas Trx-like proteins are distributed in two distinct subgroups called Trx-like1 (WCRVC active site) and Trx-like2 (WCRKC active site). Trx-lilium proteins are split in three subgroups named Trx-lilium1, Trx-lilium2 and Trx-lilium3 (usually GCGGC, WCASC and SCGSC active sites respectively) (Supplementary Fig.1) (Chibani et al., 2009).

While the sub-cellular localization has been addressed previously for most Arabidopsis orthologs, some doubt subsisted for a few isoforms (Cain et al., 2009; Dangoor et al., 2009). The analysis of protein targeting by different prediction programs suggested a cytosolic localization for poplar Clot, Trx-like1 and also for Trx-like2.1 despite the presence of an N-terminal extension in Trx-like2.1. Their sub-cellular localization was assessed by fusing the whole protein to the N-terminus of green fluorescent protein (GFP). As expected from the lack of an N-terminal transit peptide in their sequence, Clot and Trx-like1 were found in the cytosol (Fig. 1A and B). In addition, fluorescence was also observed in the nucleus. Whether this signal was due to the transient overexpression or whether it has a physiological significance will have to be further investigated. On the other hand, Trx-like2.1 was clearly targeted to the chloroplast (Fig. 1C).

Expression analysis of atypical Trx genes in different poplar organs

In order to gain information about the transcript expression patterns, RT-PCR experiments have been performed using RNA extracted from different poplar organs (roots, stems, young leaves, mature leaves, petioles, stamens, female catkins and fruits) (Fig. 2). With the exception of Trx-like1 and Trx-lilium1.2, which were not expressed or below the detection level in roots and stamens and in stems and stamens respectively, transcripts have been detected in all organs for all other Trxs tested. The transcripts of Clot, Trx-lilium1.1, Trx-lilium2.1, Trx-lilium2.2 and Trx-lilium3 were present in all tissues, indicating that they are constitutively
expressed (Fig. 2). On the other hand, some transcript variations were observed for members of the Trx-like subgroup. For example, Trx-like2.1 and Trx-like2.3 transcripts were only barely detected in fruits and petioles, Trx-like2.1 being also weakly expressed in roots and young leaves. Trx-like2.2 was also less expressed in roots, petioles and stems. The fact that Trx-like2.1 was more expressed in photosynthetic organs rather than in non-photosynthetic organs is consistent with its plastidial localization.

**Reductase activity of atypical thioredoxins**

To characterize their biochemical properties, the mature forms (i.e. devoid of N-terminal targeting sequences when present) of all these proteins were expressed in *E. coli* and purified to homogeneity by conventional purification techniques, and in an untagged form unless otherwise indicated. All the recombinant proteins were produced at high level in the soluble fraction, except Trx-like1. For the latter, all attempts to solubilize the protein either by adding an N-terminal His-tag, by changing culture conditions or by co-expressing chaperones were unsuccessful (data not shown). In addition, to explore the importance of the active site for protein reactivity, some amino acids constituting the active site of two representatives, Trx-like2.1 and Trx-lilium3, have been mutated. For Trx-like2.1, the WCRKC active site has been changed into WCRKS (C45S variant) or into WCGPC (R43G/K44P variant). For Trx-lilium3, the SCGSC active site was mutated into WCGSC (S84W variant), SCGPC (S87P variant) and WCGPC (S84W/S87P variant). The reductase activity was first tested by measuring *in vitro* their ability to reduce insulin disulfide bridges using DTT as a reductant in comparison to poplar Trxh1 used as a positive control (Fig. 3) (Behm and Jacquot, 2000). Although less efficient than Trxh1, Trx-like2.1 was able to reduce insulin. As expected, the activity drops down with the monocysteinic mutant Trx-like2.1 C45S indicating that the resolving cysteine is important for disulfide reduction. The remaining activity might be attributed to a reaction involving the successive nucleophilic attack of two Trx molecules. Accordingly, protein dimers were observed in non-reducing SDS-gels (data not shown). Interestingly, the Trx-like2.1 R43G/K44P variant (WCGPC active site) has an efficiency very comparable to Trxh1, showing that the dipeptide motif separating the two catalytic cysteines is very important for protein reactivity and/or substrate recognition. Among Trx-lilium isoforms, Trx-lilium2.2 was more efficient than Trx-lilium1.2 and Trx-lilium3. Since the major difference is the presence of a tryptophan in the active
site of Trx-lilium2.2, which is absent in the two other isoforms, we tested Trx-lilium3 variants with WCGSC, SCGPC and WCGPC active sites. None of these mutated proteins became more efficient in insulin reduction. In marked contrast with Trx-like2.1, changing the SCGSC active site into WCGPC did not improve the capacity of this protein to reduce insulin. Clot was not active at all in this classical activity assay, raising the question of its reductase activity.

**Regeneration of oxidized Trxs**

We further characterized the redox properties of these proteins by examining their possible regeneration pathways. We first tested their ability to reduce DTNB, an artificial substrate containing a disulfide, in the presence of a NADPH/NTR system that allows to investigate the capacity of Trxs to be regenerated by NTR. Only Trx-like2.1 and to a lesser extent Trx-lilium2.2 efficiently reduced DTNB with catalytic efficiencies \( \frac{k_{cat}}{K_{Trx}} \) of 35 and 3.5 \( \times \) \( 10^3 \) M\(^{-1}\) s\(^{-1}\) respectively, comparable, in the case of Trx-like2.1, to the one obtained with Trx h1 (39 \( \times \) \( 10^3 \) M\(^{-1}\) s\(^{-1}\)) (Fig. 4). The catalytic efficiency of the reaction catalyzed by the R43G/K44P variant was also similar (around 35 \( \times \) \( 10^3 \) M\(^{-1}\) s\(^{-1}\)). Surprisingly, the Trx-like2.1 C45S variant conserved the capacity to reduce DTNB, although the catalytic efficiency was decreased by a factor 2 (16 vs 35 \( \times \) \( 10^3 \) M\(^{-1}\) s\(^{-1}\)). The fact that Trx-lilium1.2 and Trx-lilium3 mutants were able to reduce insulin but not DTNB indicates that they are not reduced by NTR.

Since Trx-like2.1 is plastidial, we have tested the physiological FTR or NTRc reducing systems for its regeneration. The reduction by FTR was measured using a recently developed procedure employing a four component electron transfer system, NADPH/FNR/Fdx/FTR (Chibani et al., 2011). Following incubation of oxidized Trx with this reducing system, the redox state was assessed after alkylation of free thiol groups with 2 kDa m-PEG maleimide and subsequent separation in non-reducing SDS-gels. Contrary to Trx-z systematically used as a control, and although they contain an intramolecular disulfide, none of these atypical Trxs were reduced by the Fdx/FTR system as illustrated for Trx-like2.1 (Fig. 5 and data not shown). The reduction of Trx-like2.1 by NTRc was then investigated by taking advantage that Trx-like2.1, unlike NTRc, directly reduces Prx IIE (see below). Although NTRc was functional (as tested in the DTNB reduction assay, not shown), it was not able to support Trx-like2.1 activity, indicating that, in the case of the full-length NTRc, neither its NTR domain nor its Trx domain was able to reduce Trx-like2.1 (Supplementary Fig.2).
The fact that some Trxs exhibited no or poor reductase activity in the presence of the various thioredoxin reductases prompted us to investigate whether they possess glutaredoxin-like activity using glutathione as a reductant. The typical HED (2-hydroxyethyl disulfide) assay was used to measure the ability of all these Trxs to catalyze the reduction of a mixed disulfide formed between glutathione and HED (Fig. 4). Trx-like2.1 and -lilium2.2 reduced the β-mercaptoethanol-glutathione adduct with an efficiency comparable to poplar GrxC1 (CGYC active site) and GrxC2 (CPFC) used as controls. Interestingly, the monocysteinic Trx-like2.1 C45S variant was almost as efficient as the WT form, supporting the fact that, in Trx-like2.1, the second cysteine is dispensable as for most dithiol Grxs active in this assay (Bushweller et al., 1992). Other Trxs (Trxh1, Trx-lilium1.2, WT and mutated Trxs-lilium3) did not display any recordable activity, indicating that they cannot either reduce glutathionylated molecules or use glutathione for their regeneration.

**Midpoint redox potentials of atypical Trxs**

Next, we have investigated whether differences in the midpoint redox potentials could explain the different capacities of these atypical Trxs to reduce insulin disulfide bonds and their ability to be regenerated or not by GSH or thioredoxin reductases. The midpoint redox potentials of these Trxs were found to be slightly different, ranging from -239 to -265 mV (Table I). Despite having an adequate redox potential of -255 mV, the inability of Clot to reduce insulin indicates that other factors, such as the presence of an acidic residue in the active site (WCPDC), might explain the absence of reductase activity. Trx-like2.1 has the lowest redox potential, with a value of -265 mV. The mutations achieved in the Trx-like2.1 R43G/K44P variant, which improved its capacity to reduce insulin, also affected its redox potential (-250 mV) but in an opposite way compared to our expectation. Poplar Trxs-lilium had a redox potential ranging from -239 to -247 mV, very similar to those determined for their Arabidopsis orthologs (Dangoor et al., 2009). In the case of poplar Trx-lilium3, the mutations introduced into the active site strongly affected its redox potential in the expected way, with decreases to -266 and -264 mV for the S84W and S87P single mutants and to -268 mV for the S84W/S87P double mutant. Nevertheless, although the redox potential of Trx-lilium3 S84W/S87P became more electronegative and similar to conventional plant Trxs h, this change did not improve its capacity to reduce insulin.
Regeneration of physiological target proteins

As some Arabidopsis Trx-like and Trx-lilium orthologs were shown to weakly activate *A. thaliana* NADP-malate dehydrogenase (NADP-MDH) (Dangoor et al., 2009), an enzyme regulated by the formation of intramolecular disulfides, the capacity of these wild-type and mutated poplar plastidial Trxs to reduce the *Sorghum bicolor* NADP-MDH protein was investigated. Among WT Trxs, only Trx-lilium2.2 was able to activate this enzyme, but with a lower efficiency as compared to Trxh1 (Fig. 6). Interestingly, the Trx-like2.1 R43G/K44P mutant acquired the capacity to activate SbNADP-MDH with an efficiency comparable to Trxlilium2.2, further showing the importance of active site sequence for protein partner recognition.

Next, to assess the specificity of these atypical Trxs towards other putative physiological target proteins, their ability to regenerate various Tpxs or MSRs was measured in coupled assays in the presence of a NADPH/NTR or a NADPH/GR/GSH reduction system (Table II). We selected a cytosolic and two plastidial Prxs named PtPrxIIB, PtPrxIIE and PtPrxQ respectively as well as two plastidial Gpxs, PtGpx1 and PtGpx3, and three plastidial MSRs, PtMSRA4, AtMSRB2 and AtMSRB1. The catalytic and recycling mechanisms of these enzymes are well characterized. PrxIIB, PrxIIE and MSRB1 are enzymes that use a single cysteine residue in their catalytic cycle (Rouhier et al., 2002; Tarrago et al., 2009). The sulfenic acid formed upon catalysis is either reduced by glutathione or directly by Trxs (Tarrago et al., 2009; Tarrago et al., 2010). PtPrxQ, PtGpx1, PtGpx3, AtMSRB2 and PtMSRA4 are enzymes that form an intramolecular disulfide in the course of their catalysis (Rouhier et al., 2004; Navrot et al., 2006; Rouhier et al., 2007; Tarrago et al., 2009). In this case, the disulfide is usually uniquely reduced by Trxs but not by glutathione or Grxs. Trxh1, which is able to reduce all target proteins except AtMSRB1, was used as a control. Whereas all other Trxs were unable to reduce the tested target proteins, WT Trx-like2.1 as well as the C45S variant supported the activity of PtPrxIIE, PtPrxIIB and AtMSRB1 in the presence of a GSH reducing system (Table II). The presence of Trx-like2.1 was crucial since GSH alone was not able to regenerate the target proteins, except for PtPrxIIE which was slightly reduced by GSH. Remarkably, the activities measured with Trx-like2.1 C45S were in the same range than those obtained with the WT form (even two and three times better for AtMSRB1 and PtPrxIIE respectively), indicating that the second cysteine of Trx-like2.1
active site is not required for this GSH-dependent reaction. Although Trx-like2.1 is reduced by NTR, it did not provide electrons to PrxIIB and IIE using this regeneration system, while Trx h1 did. Regarding the Trx-like2.1 R43G/K44P variant (WCGPC active site), the two substitutions in the active site were sufficient for the protein to acquire, together with the NADPH/NTR system, the capacity to reduce most enzymes whose catalytic mechanism involves the formation of an intramolecular disulfide (PtPrxQ, PtGpx1 and 3, PtMSRA, but not AtMSRB2). Interestingly, this variant conserved the capacity to reduce both PtPrxIIB and PtPrxIIE, but in the presence of the NADPH/NTR reducing system instead of the NADPH/GR/GSH one. Note also that it lost the capacity to regenerate AtMSRB1 regardless the reducing system. These results suggest that, in the case of the two Prxs, the sulfenic acid can be directly reduced by the catalytic cysteine of Trx-like2.1 R43G/K44P variant, but not in the case of AtMSRB1, the reduction of which requires the presence of GSH.

The specific reduction of proteins using for catalysis a single cysteine, which is glutathionylated in the course of its regeneration mechanism, suggests that Trx-like2.1 should become itself transiently glutathionylated. To confirm the possibility to form a glutathione adduct, in vitro glutathionylation assays have been performed with the Trx-like2.1 C45S variant and the redox state of the protein was subsequently analyzed by mass spectrometry analyses. An untreated Trx-like2.1 C45S had the expected molecular weight of 14,020 Da, if we consider that the N-terminal methionine has been cleaved, which is most likely the case since the second residue is an alanine (Fig. 7). A small additional peak corresponding to a molecular mass of 14,326 Da and consistent with the presence of a glutathione adduct was also detected, indicating that a small part of the protein was glutathionylated in E. coli. An oxidation treatment with GSNO, performed on a pre-reduced Trx-like2.1 C45S, led to the formation of three protein species, a major form with a molecular mass of 14,326 Da and two minor forms of 14,020 and 14,632 Da, compatible with the presence of either an unmodified protein or of a protein with one (+ 306 Da) or two glutathione (+ 712 Da) molecules. Expectedly, the two protein species with glutathione adducts disappeared by incubating the protein with DTT (data not shown). Based on the presence of two cysteines in the Trx-like2.1 C45S variant (the catalytic cysteine and the additional non conserved Cys67), trypsic digestion helped us establishing that the peptide containing the catalytic cysteine (ELSQPIIIDWMASWCR) was predominantly glutathionylated (data not shown).
DISCUSSION

The plastidial Trx equipment of photosynthetic organisms

Clot, Trx-like and -lilium isoforms belong to atypical Trx classes found in all higher plants and contain non conventional active site signatures (Chibani et al., 2009). At the beginning of this work, limited information was available for these proteins regarding their localization, expression, reduction systems and possible partners. A. thaliana Trxs-lilium, also referred to as ACHT (for atypical cysteine and histidine rich thioredoxins), and the two Trx-like2 members were known to be located in plastids (Cain et al., 2009; Dangoor et al., 2009). The chloroplastic localization of poplar Trx-like2.1, established here using a translational GFP fusion approach, is in accordance with the finding that the Arabidopsis orthologs are imported in the stroma in in vitro chloroplast import assays (Cain et al., 2009). Contrary to poplar Trx-like2.1, the predictions for poplar Trx-like2.2 and -like2.3 were less ambiguous and indicated the presence of a plastidial targeting sequence, but this will have to be experimentally proven. With 2 members in A. thaliana and 3 in P. trichocarpa, Trxs-like2 extend the already very large number of plastidial Trxs. Overall, there are at least 20 and 24 potential plastidial Trxs in A. thaliana and P. trichocarpa, respectively, all types (Trxs f, Trx x, Trxs m, Trxs y, Trx z, CDSP32, HCF164, Trxs-lilium, Trxs-like2, NTRc) being present in both species (Lennartz et al., 2001; Collin et al., 2003; Collin et al., 2004; Cain et al., 2009; Chibani et al., 2009; Dangoor et al., 2009; Meng et al., 2010). The Trxs-m and -lilium display the highest numbers of isoforms, 4 and 8 for Trx-m in Arabidopsis and poplar respectively and 5 Trxs-lilium in both species. All these proteins, with the exception of HCF164, are assumed to be in the stromal fraction. This multiplicity of plastidial Trxs is likely related to different spatio-temporal expression patterns, to the reduction of specific target proteins or to the use of specific reducing pathways.

Transcript expression patterns of plastidial Trxs

Transcripts coding for Trxs-like and Trxs-lilium have been detected in all poplar organs tested (Fig. 2) suggesting that the diversity of plastidial Trxs is probably not explained by differential expression at the organ level, although for example, transcripts of Trx-like2.1 and Trx-like2.2 seem to be mainly present in photosynthetic organs and female flowers. In Arabidopsis, Dangoor and colleagues reported a preferential expression of Trx-lilium2.2 in green
tissues (Dangoor et al., 2009). In addition, a more detailed analysis using the Genevestigator tool (Hruz et al., 2008) revealed that, in A. thaliana, some atypical Trxs are, however, expressed in a very specific manner as a function of tissue age or cell type. Indeed, Arabidopsis Trxs-lilium1.1, 1.2 and 1.3 are expressed at a very high level in apical meristem, seed suspensor and anther, respectively, the transcript abundance of these three Trxs being lower and comparable in all other tissues. Regarding AtTrx-like1, a high and specific expression is detected in the abscission zone of anthers and in imbibed seeds. Note also that most typical plastidial Trxs (m, f, x and y) are specifically expressed in photosynthetic tissues except Trx y1, the transcript level of which is much higher in seeds and roots (Collin et al., 2004). Finally, some Trx genes display substantial variations in their expression in relation with environment. This is the case of CDSP32 and of the cytosolic Trx h5, the expression of which is triggered by environmental conditions leading to oxidative stress (Broin et al., 2000; Laloi et al., 2004). Altogether, these data argue in favor of a specialization of plant Trxs as a function of the organ type, developmental stage and/or environmental conditions, but this can vary depending on the species considered.

**One Trx-like and one Trx-lilium use glutathione for their regeneration**

While belonging to the same protein superfamily, Grxs, which possess YC[P/G/S]Y[C/S] active site sequences, are usually reduced by glutathione whereas Trxs are reduced by Fdx- or NADPH-dependent thioredoxin reductases. In addition, it is commonly accepted that Grxs preferentially reduce protein-glutathione adducts, whereas Trxs are more specific for the reduction of disulfide bonds formed within a single polypeptide or between two polypeptides. However, in the set of atypical Trxs tested here, two proteins, Trx-like2.1 and Trx-lilium2.2, are able to reduce a β-mercaptoethanol-glutathione mixed disulfide, a preferential Grx substrate, with an efficiency comparable (around 0.58 s\(^{-1}\)) to the poplar Grxs tested here, but lower when compared to previous results obtained with two plastidial Grxs (1.21 s\(^{-1}\) for AtGrxC5 and 23.10 s\(^{-1}\) for poplar GrxS12) (Couturier et al., 2011; Zaffagnini et al., 2012). Strikingly, both proteins can also be reduced by A. thaliana NTRB. This is not unprecedented since a thioredoxin-like protein from the parasitic nematode Haemonchus contortus, which displays a CRSC active site sequence, possesses a similar property (Sotirchos et al., 2009). However, considering the plastidial localization of the proteins, their reduction by NTR should not be of physiological importance, unless protein dual targeting exists according to environmental conditions or
developmental stage (this work and Dangoor et al., 2009). On the contrary, the fact that they are reduced neither by NTRc nor by FTR makes these enzymes strictly dependent on the NADPH/GR/GSH system for their regeneration in plastids.

Previous studies already illustrated the cross reactivity existing between the Trx and GSH/Grx reducing pathways, some Grxs being regenerated by thioredoxin reductases (Johansson et al., 2004; Zaffagnini et al., 2008). In addition, some Trxs are reduced by GSH and/or Grx, but not by NTR. As an example, the presence of an additional cysteine residue at position 4 in poplar Trx h4 (WCGPC active site) prevents its recycling by NTR, but renders this enzyme GSH- and Grx-dependent (Koh et al., 2008). Besides, the poplar TrxCxxS3, harboring an unusual WCMPS active site motif, is also recycled by glutathione, but the activity is extremely low, with a difference of ca 3 orders of magnitude with the present study (Gelhaye et al., 2003). In addition, some genetic and biochemical evidence indicates that the two yeast cytosolic Trxs, though reduced by thioredoxin reductases, are apparently able to catalyze the deglutathionylation of some protein substrates (Greetham et al., 2010). On the other hand, the finding that, in knock-out plants for the cytosolic and mitochondrial NTRs, the cytosolic thioredoxin h3 is only partially oxidized, and that buthionine sulfoximine (BSO), a specific inhibitor of glutathione biosynthesis, leads to full oxidation, indicates a direct or indirect reduction of the Trx by GSH in vivo (Reichheld et al., 2007).

Based on sequence similarity with Grxs, Clot proteins, first identified in Drosophila as required for the biosynthesis of drosopoterin, an eye pigment, were assumed to be glutathione-dependent enzymes (Giordano et al., 2003). Surprisingly, no significant activity was detected for the Clot proteins, either from poplar or Arabidopsis (data not shown). The orthologs from Saccharomyces cerevisiae (also referred to as ScGrx8) or from mammals (referred to as TRP14), which possess the same active site, are also not or very poorly able to reduce insulin and a faint activity in the HED assay was detected for ScGrx8 (Jeong et al., 2004; Eckers et al., 2009). However, it has been shown that TRP14 can reduce a disulfide in the dynein light chain LC8, contributing to the inhibitory activity of LC8 toward the nuclear factor κB (Jung et al., 2008). Similarly, plant Clot proteins might have very specific protein partners.

**Putative physiological targets of atypical Trxs**
For the two proteins exhibiting a GSH- and/or NTR-dependent activity, Trx-like2.1 and Trx-lilium2.2, we have investigated their capacity to regenerate known plastidial members of the Tpx and MSR families. While Trx-lilium2.2 did not exhibit any significant activity, Trx-like2.1 was able to specifically regenerate the established GSH/Grx-dependent proteins (PrxIIE and MSRB1), whose catalytic mechanism relies on a single redox active cysteine, but not the strictly Trx-dependent targets (PrxQ, Gpxs, MSRA4 and MSRB2), whose catalytic mechanism involves two or three cysteines (Fig. 8). The fact that the second active site cysteine of Trx-like2.1 is not essential for the reaction, led us to propose a regeneration mechanism, similar to the one employed by Grxs. Indeed, they generally use a monothiol mechanism where the second active site cysteine is not required, although it can modulate (either increase or decrease) protein reactivity (Bushweller et al., 1992; Couturier et al., 2009; Couturier et al., 2011). Thus, from a biochemical point of view, Trx-like2.1 constitutes a redundant system with plastidial Grxs. Nevertheless, if we compare the turnover numbers of the reactions, it is generally 5 to 16 times less efficient. The k\text{cat} of the reactions catalyzed by Prx IIE and MSRB1 were 0.04 s\(^{-1}\) and 0.03 s\(^{-1}\) respectively with Trx-like2.1, whereas the values obtained for the same enzymes with poplar GrxS12 or AtGrxC5 were 0.66 s\(^{-1}\) and 0.17 s\(^{-1}\) or 0.5 s\(^{-1}\) and 0.35 s\(^{-1}\) respectively (Vieira Dos Santos et al., 2007; Gama et al., 2008; Couturier et al., 2011).

Regarding the catalytic mechanism, the sulfenic acid initially formed on the Prx or MSR proteins probably reacts with GSH forming a glutathionylated intermediate, which is subsequently attacked by the catalytic cysteine of Trx-like2.1 (Supplementary Fig. 4) (Vieira Dos Santos et al., 2007; Tarrago et al., 2009). The latter becomes itself glutathionylated, a possibility demonstrated by the in vitro glutathionylation treatment of the Trx-like2.1 C45S. In the presence of GSH, it is most likely that the glutathionylated Trx form is resolved by another GSH molecule. This is different from the peculiar CDSP32, which can support the activity of AtMSRB1 without GSH, by directly reducing the sulfenic acid formed on the catalytic cysteine (Tarrago et al., 2010).

However, it is also clear from our redox titration of Trx-like2.1 that an intramolecular disulfide can be formed. The midpoint redox potential determined for this disulfide was -265 mV at pH 7.0, a value comparable to certain obtained with other cytosolic or plastidial Trxs and which should be adequate for the reduction of Prxs and MSRs forming an intramolecular disulfide. Since Trxs known to reduce these proteins usually exhibit a WCGPC active site, the
reactivity of a Trx-like2.1 variant was examined. Contrary to our expectation, the redox midpoint potential is increased in this variant (-250 mV). However, this mutated Trx was able to reduce most of these target proteins in the presence of a NTR reducing system. This result suggests that, at least for Trx-like2.1, the presence of two basic residues in the active site sequence, replacing the usual hydrophobic residues, is of high importance for target protein recognition. However, this is not true for the interaction with NTR since WT and mutated Trx-like2.1 proteins displayed similar catalytic efficiencies. A mutational study performed with *E. coli* Trx1 showed that the presence of a positive charge decreases its catalytic efficiency, most likely by affecting protein-protein interactions, although in this case, the mutation did not change the midpoint redox potential (Lin and Chen, 2004). The fact that the redox potential is probably not the major determinant for the interaction of these atypical Trxs with their protein partners is also illustrated by the following results. Although having a redox potential of -255 mV, Clot is not reduced by AtNTRB. Thus, it might be that the presence of an acidic residue in the active site motif (WCPDC) prevents such interaction, while it could favor protein-protein interactions with other yet unknown targets. This observation also raises the question of the *in vivo* reduction of this cytosolic protein. In the case of Trx-lilium3, we have observed that changing the active site from SCGSC to WCGPC decreased the midpoint redox potential from -242 mV to -268 mV. This result is consistent with a study conducted on a *Staphylococcus aureus* Trx, showing that replacement of the active site proline 31 by a serine or a threonine changed the redox potential from -268 mV for the WT protein to -236 and -244 mV for the two mutated proteins (Roos et al., 2007). Nevertheless, although the redox potential of Trx-lilium3 S84W/S87P became more electronegative and similar to conventional plant Trxs h, this change did not improve its capacity to reduce insulin.

All Trxs-lilium, though forming three independent subgroups, have very similar active site sequences (formed by small and uncharged amino acids) and midpoint redox potentials and they all have the capacity to reduce disulfide bonds as indicated by their insulin reductase activity. However, Trx-lilium2.2 was the only one able to activate SbNADP-MDH (less efficiently than Trx h1) and was unable to support the activity of the tested Prxs and MSRs. In addition, only Trx-lilium2.2 exhibited GSH- and NTR-dependent activities, suggesting that some specificity exists among Trx-lilium isoforms. Some differences might arise from the presence of
an N-terminal extension of about 25 amino acids in Trx-lilium1.2 and a C-terminal extension of about 50 amino acids in Trx-lilium3.

**CONCLUSION**

This study reports the biochemical characterization of poplar isoforms belonging to three atypical Trx classes, namely Clot, Trx-like and Trx-lilium. The mutational analysis of the active site sequences of these atypical Trxs indicates that the dipeptide separating the two cysteines of the active site is an important factor for providing particular redox properties and substrate specificities toward both the reducing systems and the target proteins. In particular, the positively charged nature of the Trx-like2.1 active site is a major determinant for its capacity to reduce specific MSR and Tpx at the expense of GSH. While all proteins, except Clot, exhibit disulfide reductase activity with insulin, only two proteins (Trx-lilium2.2 and Trx-like2.1) exhibited a detectable activity with the potential plastidial partners that were tested. Hence, the question of the nature of the physiological targets and roles of all these proteins should now be addressed using other approaches, such as the study of knock-out and knock-down plants and *in vivo* or *in vitro* methods to identify protein partners. For instance, beside their usual disulfide reductase activity, it has recently been shown that some plastidial Trx f and m have chaperone properties (Sanz-Barrio et al., 2012).
MATERIALS AND METHODS

Intracellular localization via GFP Fusion

The full-length coding sequences of PtClot, PtTrx-like1 and PtTrx-like2.1 were cloned into the NcoI and BamHI sites of pCK-GFP S65C using the primers detailed in supplemental Table I. The sequences, fused to the N-terminal part of GFP, are 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. Chloroplasts were visualized by the natural fluorescence of chlorophyll.

RT-PCR

Semi-quantitative RT-PCR was used to estimate the transcript expression in various poplar organs i.e. roots, young and mature leaves, stems, petioles, fruits, stamen and female catkins. Total RNA was isolated with the RNeasy Plant Mini kit (Qiagen) from 100 mg of frozen tissue. To remove contaminating DNA, the samples were treated with DNase I (Qiagen). A total of 1 µg of RNA was converted to cDNA using reverse transcriptase (Qiagen). The PCR program used was as follows: 94°C for 3 min and 35 cycles of 94°C for 30s, 52°C for 45s, and 72°C for 90s. The primers used for these RT-PCR experiments are those designed for cloning the sequence of the mature forms of the proteins (supplemental Table I).

Cloning and site-directed mutagenesis

The sequences encoding the mature forms of Trx-like2.1, -lilium2.2 and -lilium3 were amplified from a Populus tremula x tremuloides leaf cDNA library, of Clot and Trx-lilium1.2 from a Populus trichocarpa x deltoides root cDNA library and of Trx-like1 from a Populus tremula x tremuloides flower cDNA library, using the primers described in supplemental Table I. In the case of Trx-like1 and Clot, the amplified sequence corresponded to the full-length protein. For Trx-like2.1, -lilium1.2, -lilium2.2 and -lilium3, the putative plastidial targeting sequences were removed and the amplified fragment coded for proteins devoid of the first 73, 82, 79 and 69 amino acids respectively. After digestion with NcoI and BamHI, PCR fragments were inserted into the pET-3d expression plasmid. For this cloning, owing to the use of a NcoI restriction site,
a codon for an alanine was added after the ATG start codon in the case of Clot, Trx-like1, Trx-like2.1 and Trx-lilium3. Site-directed mutagenesis was used to create variants for Trx-like2.1 (R43G/K44P and C45S) and for Trx-lilium3 (S84W, S87P and S84W/S87P) using two complementary mutagenic primers described in supplemental Table I. The amino acid numbering is based on the sequence of the mature forms expressed as recombinant proteins. The introduction of the mutation in the DNA sequence was verified by sequencing.

**Production and purification of recombinant Trxs**

The recombinant plasmids obtained were used to transform the BL21(DE3) pSBET strain of *E. coli*. The bacteria were grown to a final volume of approximately 2.4 L at 37°C, and protein production was induced during exponential growth phase by adding 100 µM isopropyl-b-β-thiogalactoside (Euromedex). The bacteria were harvested by centrifugation at 4,400 g for 20 min and then resuspended in buffer A (Tris-HCl 30 mM, pH 8.0, EDTA 1 mM, NaCl 200 mM). Cells were disrupted by sonication, and all Trxs were found in the soluble fraction after centrifugation at 20,000 g for 30 min, except Trx-like1 which was completely insoluble. The soluble fraction was then precipitated with ammonium sulfate successively up to 40 and 80% of the saturation and the precipitated proteins collected by centrifugation (20,000 g, 15 min). The protein pellet was redissolved in buffer A and the sample was loaded onto ACA 44 gel filtration column (Biosepra) equilibrated with buffer A. The proteins of interest were identified using SDS PAGE and Coomassie blue staining. The fractions containing the protein were pooled, dialyzed, and loaded on a diethylaminoethyl (DEAE) sepharose column (Sigma) equilibrated with buffer A without NaCl. The proteins were eluted using a 0 to 0.4 M NaCl gradient, selected based on the highest purity, dialyzed, concentrated and finally stored in buffer A at -20°C until further use. Final purity was checked on 15% SDS-PAGE.

**Determination of the midpoint redox potentials**

Oxidation-reduction titrations were carried out at ambient temperature by measuring fluorescence resulting from the reaction between protein thiol groups and mBBr (Sigma) as previously described (Hirasawa et al., 1999). The reaction mixtures contained 50 µg protein in 100 mM HEPES buffer pH 7.0, containing defined mixtures of oxidized and reduced DTT to set the ambient potential (*Eₐ*). Equilibration times of either 2 or 3h were used. The redox potentials
(\(E_m\)) were calculated by fitting the curve to the Nernst equation for a single two-electron redox couple using GraphPad Prism version 4.0 and using an \(E_m\) value of -327 mV for DTT at pH 7.0.

**Reductase activity**

The insulin reduction assay was carried out in a 500 \(\mu\)L reaction mixture containing 100 mM phosphate pH 7.9, 2 mM EDTA, 0.75 mg ml\(^{-1}\) bovine insulin, 500 \(\mu\)M DTT and 10 \(\mu\)M Trx at 30°C. The reduction of insulin was monitored as the increase in turbidity at 650 nm due to insulin precipitation. Non-enzymatic reduction of insulin by DTT was monitored in the absence of Trx.

The ability of Trxs to catalyze the reduction of DTNB (5,5'-dithio-bis-2-nitrobenzoic acid) was measured at 25°C by monitoring the increase in absorbance at 412 nm caused by the release of TNB-. The reaction medium contained 30 mM Tris-HCl pH 8.0, 2 mM EDTA, 200 \(\mu\)M NADPH, 0.5 \(\mu\)M \textit{A. thaliana} NTRB, 100 \(\mu\)M DTNB and varying concentrations of Trxs ranging from 0.25 to 20 \(\mu\)M. Control experiments were performed under the same condition but in absence of Trx.

**Reduction of Trxs by Fdx/FTR system**

The reduction of oxidized plastidial Trxs by a NADPH/FNR/Fdx/FTR system was assessed by SDS-PAGE separation after alkylation with mPEG-maleimide (Laysan Bio, Inc) as described earlier (Chibani et al., 2011).

**Hydroxyethyldisulfide (HED) activity assay**

A 500 \(\mu\)L mixture containing 200 \(\mu\)M NADPH, 0.5 unit GR, 500 \(\mu\)M GSH and 700 \(\mu\)M HED was prepared in 100 mM Tris-HCl pH 8.0 and 1 mM EDTA. After 2 min incubation, 250 nM Trx or Grx was added. For all proteins, this concentration is within the linear response range of the enzyme. The decrease in absorbance at 340 nm was followed using a Cary 50 spectrophotometer (Agilent technologies). HED activity was determined after subtracting the spontaneous reduction rate observed in the absence of Trx or Grx and expressed as nmole of NADPH oxidized min\(^{-1}\) nmole enzymes\(^{-1}\).

**Reduction or regeneration of target proteins**
The peroxidase activities of poplar PrxIIE, PrxIIB, PrxQ, Gpx1 and Gpx3 were measured spectrophotometrically by following NADPH oxidation at 340 nm in the presence of 10 µM Trxs and either the NADPH/NTR system or the NADPH/GR/GSH system. The 500 µL reaction mixture contained 30 mM Tris-HCl pH 8.0, 1 mM EDTA, 200 µM NADPH, 250 µM H₂O₂, 300 nM PrxIIE, IIB, PrxQ, Gpx1 or Gpx3 and either 0.8 µM AtNTRB or 0.5 unit GR and 500 µM GSH. The reaction was started by adding the thiol-peroxidases. The methionine sulfoxide reductase activities of poplar MSRA4, AtMSRB1 and AtMSRB2 were measured in the same conditions except that MSRs (5 µM PtMSRA, 2.5 µM AtMSRB1) replaced Tpxs and that the substrate was 2 mM N-acetyl-MetO instead of H₂O₂. The rate of NADPH oxidation was determined after subtracting the spontaneous rate in the absence of Prx, Gpx or MSR.

The activation of the Sorghum bicolor NADP-MDH was carried out as described in (Jacquot et al., 1981). An incubation mixture contained 500 µM DTT, 10 µM Trxs and 10 µg of recombinant SbMDH (0.75 mg ml⁻¹) in 100 mM Tris-HCl pH 8.0 buffer. Every 5 min, an aliquot of 20 µL was added to a standard assay mixture containing 100 mM Tris-HCl pH 8.0, 189 µM oxaloacetate, and 800 µM NADPH. The activity was measured by following the decrease in absorbance at 340 nm.

Glutathionylation of Trx-like2.1 C45S and mass spectrometry analysis

For glutathionylation experiments, 100 µM of pre-reduced Trx-like2.1 C45S was incubated with 5 mM GSNO for 30 min at 25°C. Pre-reduction was performed by incubating the protein with an excess of DTT (usually 10 mM) for 30 min and subsequent desalting on G25 columns. Three samples (untreated, GSNO-treated and GSNO-treated subsequently reduced by DTT) were then analyzed using a Bruker microTOF-Q spectrometer (Bruker Daltonics) equipped with an Apollo II electrospray ionization source and a procedure similar to (Couturier et al., 2011). Trypsin digestion and subsequent analyses by reverse-phase liquid chromatography-electrospray ionization-tandem mass spectrometry using a capillary HPLC system coupled to a quadrupole time-of-flight mass spectrometer (CapLC Q-TOF Ultima; Waters Corp.) were performed as described in (Backstrom et al., 2007).

Accession numbers
DNA sequences for these poplar atypical Trxs have been deposited in GenBank under the following accession numbers: JQ407766 for Trx-like1, JQ407767 for Trx-like2.1, JQ407770 for Trx-lilium1.2, JQ407768 for Trx-lilium2.2, JQ407769 for Trx-lilium3 and JQ407771 for Clot.

ACKNOWLEDGEMENTS

Technical support from François Dupire for mass analyses is gratefully acknowledged.
LITERATURE CITED


Lin TY, Chen TS (2004) A positive charge at position 33 of thioredoxin primarily affects its interaction with other proteins but not redox potential. Biochemistry 43: 945-952


FIGURE LEGENDS

Figure 1. Sub-cellular localization of poplar Clot (A), Trx-like1 (B) and Trx-like2.1 (C) in tobacco cells.
The entire open reading frames were fused upstream of the 5’ end of a GFP coding sequence. 1: cells under visible light; 2: fluorescence of the GFP construct; 3: fluorescence of chlorophyll (red); 4: merged images.

Figure 2. Transcript expression profiles in poplar organs.
RT-PCR were performed from total RNAs extracted from roots (R), stems (S), young leaves (Yl), mature leaves (Ml), petioles (P), stamens (St), female catkins (Fc) and fruits (Fr). Trxh1 was used as a control as it has been shown to be constitutively expressed in all organs tested (Rouhier et al., 2006). For these experiments, 35 PCR cycles have been used to amplify each Trx transcript from cDNA obtained from 1 µg of total RNA.

Figure 3. Reduction of insulin by Trx-like and -lilium proteins.
Insulin reduction was measured using a DTT-based assay and 10 µM of Clot and Trx-like2.1 (A) or of Trxs-lilium (B) by measuring the turbidity at 650 nm caused by the precipitation of reduced insulin. Each trace is a representative experiment of two to three repetitions.

Figure 4. Measurement of typical thioredoxin or glutaredoxin activity.
Black bars represent the catalytic efficiency ($k_{cat}/K_{Trx}$) of the tested proteins in a typical thioredoxin assay measuring the capacity to reduce DTNB at the expense of AtNTRB. White bars represent the activity (expressed as nmol oxidized NADPH nmol$^{-1}$ enzyme min$^{-1}$) of the tested proteins in a typical glutaredoxin assay measuring the capacity to reduce a β-mercaptoethanol-glutathione adduct using a glutathione recycling system. Only the proteins for which an appreciable activity was detected have been represented. The data are represented as mean ± SD of at least two separate experiments.

Figure 5. Assessment of poplar Trx-like2.1 reduction by Synechocystis ferredoxin-thioredoxin reductase.
The redox state of Trx-like2.1 (A) or Trx-2 (B) was analyzed by non reducing SDS-PAGE following oxidizing or reducing treatments and alkylation. Lanes 1: Trxs untreated and not alkylated. Lanes 2 to 5 represents mPEG-mal alkylated proteins: lanes 2: untreated Trxs; lanes 3: Trxs incubated with 500 µM DTTox, lanes 4: Trxs incubated with 100 µM DTTred. M: molecular weight marker (from top to the bottom 75, 50, 37, 25, 20 and 15 kDa), lanes 5: Trxs incubated for 15 min with 20 µM NADPH, 20 nM FNR, 0.5 µM Fdx and 0.5 µM FTR. Note that FNR, Fdx and FTR were only slightly detectable by Coomassie blue staining due to the low concentrations used. As previously observed, the shift arising from the alkylation of the thiol groups of the two active site cysteines of Trx (lanes 4 in A and B) was larger than expected (ca 12 kDa instead of 4 kDa) (Chibani et al., 2011).

Figure 6. Activation of SbNADP-MDH by wild-type and mutated Trxs-like and -lilium. NADP-MDH activation was achieved in the presence of 500 µM DTT and 10 µM of wild-type and mutated Trxs. Every 5 min over a 20 min period, an aliquot was used to measure NADP-MDH activity by following NADPH oxidation at 340 nm. The data are represented as mean ± SD of at least two separate experiments.

Figure 7. ESI-TOF mass spectra of glutathionylated Trx-like2.1 C45S. ESI m/z spectra of an untreated (A) or GSNO-treated (B) Trx-like2.1 C45S sample have been obtained using a Bruker microTOF-Q spectrometer in denaturing conditions. From the multiply charged ion spectra initially obtained, we have focused on the peak with 15 charges on the ion. The deconvolution of the ESI spectra indicated that the ion with a m/z of ca 935 amu (atomic mass unit) corresponded to a molecular mass of 14020 Da, the one with a m/z of ca 956 amu to a molecular mass of 14326 Da and the one with a m/z of ca 976 amu to a molecular mass of 14632 Da. The intensity of the signal is represented as an arbitrary unit. However, assuming that the different forms of the protein were identically ionized, the abundance of each species in the sample is well correlated to the intensity of each species on the ESI mass spectra.

Figure 8. Model depicting the reducing pathways for plastidial Trxs and their specificity toward known physiological targets of the Tpx and MSR families.
Except for MSRA, which was only tested with Trx-z, all other reductant/target protein couples have generally been exhaustively tested in previous studies. The conclusions represented here, summarize results presented in (Broin et al., 2002; Collin et al., 2003; Collin et al., 2004; Rouhier et al., 2004; Rey et al., 2005; Moon et al., 2006; Navrot et al., 2006; Perez-Ruiz et al., 2006; Vieira Dos Santos et al., 2007; Couturier et al., 2009; Pulido et al., 2010; Tarrago et al., 2010; Chibani et al., 2011; Couturier et al., 2011). New insights provided by this study, i.e. reduction of Trx-lilium2 and Trx-like2.1 by GSH and regeneration of PrxIIIE and MSRB1 by Trx-like2.1, are highlighted by the use of grey boxes. Dotted arrows indicate that reduction of these Trxs by FTR is possible but not experimentally determined.
<table>
<thead>
<tr>
<th>Proteins</th>
<th>Active site sequences</th>
<th>Redox potentials (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clot</td>
<td>WCPDC</td>
<td>-255± 2</td>
</tr>
<tr>
<td>Trx-like2.1</td>
<td>WCRKC</td>
<td>-265 ± 1</td>
</tr>
<tr>
<td>Trx-like2.1R43G/K44P</td>
<td>WCGPC</td>
<td>-250 ± 2</td>
</tr>
<tr>
<td>Trx-lilium1.2</td>
<td>GCGGC</td>
<td>-247 ± 2</td>
</tr>
<tr>
<td>Trx-lilium2.2</td>
<td>WCASC</td>
<td>-239 ± 2</td>
</tr>
<tr>
<td>Trx-lilium3</td>
<td>SCGSC</td>
<td>-242 ± 1</td>
</tr>
<tr>
<td>Trx-lilium3 S87P</td>
<td>SCGPC</td>
<td>-263 ± 1</td>
</tr>
<tr>
<td>Trx-lilium3 S84W</td>
<td>WCGSC</td>
<td>-266 ± 2</td>
</tr>
<tr>
<td>Trx-lilium3 S84W/S87P</td>
<td>WCGPC</td>
<td>-268 ± 1</td>
</tr>
</tbody>
</table>

**Table I. Redox midpoint potentials of WT and mutated Trx variants.**

For mutated Trxs, the mutation appears in bold characters. The values represent a mean ± S.D. of three separate experiments. Individual titration representations are provided as supplementary Figure 3.
<table>
<thead>
<tr>
<th>Targets</th>
<th>Trx h1</th>
<th>Trx-like2.1</th>
<th>Trx-like2.1</th>
<th>Trx like2.1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NTS</td>
<td>NGS</td>
<td>NTS</td>
<td>NGS</td>
</tr>
<tr>
<td>PtPrxIIB</td>
<td>7.7 ± 0.5</td>
<td>-</td>
<td>16.3 ± 1.1</td>
<td>-</td>
</tr>
<tr>
<td>PtPrxIIE</td>
<td>11.6 ± 1.5</td>
<td>-</td>
<td>16.3 ± 1.1</td>
<td>-</td>
</tr>
<tr>
<td>PtPrxQ</td>
<td>10.7 ± 0.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PtGpx1</td>
<td>10.2 ± 0.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PtGpx3</td>
<td>10.2 ± 0.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PtMSRA4</td>
<td>2.6 ± 0.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AtMSRB2</td>
<td>16.2 ± 1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AtMSRB1</td>
<td>-</td>
<td>-</td>
<td>1.9 ± 0.1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table II. Regeneration of thiol peroxidase or methionine sulfoxide reductase family members by WT and mutated Trx-like2.1 in the presence of a NADPH/NTR system (NTS) or a NADPH/GR/GSH system (NGS).

Data are expressed as nmole of oxidized NADPH min⁻¹ nmole enzyme⁻¹. The hyphen indicates that no significant activity has been detected.
Figure 1. Sub-cellular localization of poplar Clot (A), Trx-like1 (B) and Trx-like2.1 (C) in tobacco cells.
The entire open reading frames were fused upstream of the 5’ end of a GFP coding sequence. 1: cells under visible light; 2: fluorescence of the GFP construct; 3: fluorescence of chlorophyll (red); 4: merged images.
Figure 2. Transcript expression profiles in poplar organs.
RT-PCR were performed from total RNAs extracted from roots (R), stems (S), young leaves (Yl), mature leaves (Ml), petioles (P), stamens (St), female catkins (Fc) and fruits (Fr). Trxh1 was used as a control as it has been shown to be constitutively expressed in all organs tested (Rouhier et al., 2006). For these experiments, 35 PCR cycles have been used to amplify each Trx transcript from cDNA obtained from 1 µg of total RNA.
Figure 3. Reduction of insulin by Trx-like and -lilium proteins. Insulin reduction was measured using a DTT-based assay and 10 µM of Clot and Trx-like2.1 (A) or of Trxs-lilium (B) by measuring the turbidity at 650 nm caused by the precipitation of reduced insulin. Each trace is a representative experiment of two to three repetitions.
Figure 4. Measurement of typical thioredoxin or glutaredoxin activity. Black bars represent the catalytic efficiency ($k_{cat}/K_{Trx}$) of the tested proteins in a typical thioredoxin assay measuring the capacity to reduce DTNB at the expense of AtNTRB. White bars represent the activity (expressed as nmol oxidized NADPH nmol$^{-1}$ enzyme min$^{-1}$) of the tested proteins in a typical glutaredoxin assay measuring the capacity to reduce a β-mercaptoethanol-glutathione adduct using a glutathione recycling system. Only the proteins for which an appreciable activity was detected have been represented. The data are represented as mean ± SD of at least two separate experiments.
Figure 5. Assessment of poplar Trx-like2.1 reduction by *Synechocystis* ferredoxin-thioredoxin reductase.

The redox state of Trx-like2.1 (A) or Trx-z (B) was analyzed by non-reducing SDS-PAGE following oxidizing or reducing treatments and alkylation. Lanes 1: Trxs untreated and not alkylated. Lanes 2 to 5 represent mPEG-mal alkylated proteins: lanes 2: untreated Trxs; lanes 3: Trxs incubated with 500 µM DTTox, lanes 4: Trxs incubated with 100 µM DTTred. M: molecular weight marker (from top to the bottom 75, 50, 37, 25, 20 and 15 kDa), lanes 5: Trxs incubated for 15 min with 20 µM NADPH, 20 nM FNR, 0.5 µM Fdx and 0.5 µM FTR. Note that FNR, Fdx and FTR were only slightly detectable by Coomassie blue staining due to the low concentrations used. As previously observed, the shift arising from the alkylation of the thiol groups of the two active site cysteines of Trx (lanes 4 in A and B) was larger than expected (ca 12 kDa instead of 4 kDa) (Chibani et al., 2011).
Figure 6. Activation of SbNADP-MDH by wild-type and mutated Trxs-like and -lilium. NADP-MDH activation was achieved in the presence of 500 µM DTT and 10 µM of wild-type and mutated Trxs. Every 5 min over a 20 min period, an aliquot was used to measure NADP-MDH activity by following NADPH oxidation at 340 nm. The data are represented as mean ± SD of at least two separate experiments.
Figure 7. ESI-TOF mass spectra of glutathionylated Trx-like2.1 C45S.

ESI m/z spectra of an untreated (A) or GSNO-treated (B) Trx-like2.1 C45S sample have been obtained using a Bruker microTOF-Q spectrometer in denaturing conditions. From the multiply charged ion spectra initially obtained, we have focused on the peak with 15 charges on the ion. The deconvolution of the ESI spectra indicated that the ion with a m/z of ca. 935 amu (atomic mass unit) corresponded to a molecular mass of 14020 Da, the one with a m/z of ca. 956 amu to a molecular mass of 14326 Da and the one with a m/z of ca. 976 amu to a molecular mass of 14632 Da. The intensity of the signal is represented as an arbitrary unit. However, assuming that the different forms of the protein were identically ionized, the abundance of each species in the sample is well correlated to the intensity of each species on the ESI mass spectra.
Figure 8. Model depicting the reducing pathways for plastidial Trxs and their specificity toward known physiological targets of the Tpx and MSR families.

Except for MSRA, which was only tested with Trx-z, all other reductant/target protein couples have generally been exhaustively tested in previous studies. The conclusions represented here, summarize results presented in (Broin et al., 2002; Collin et al., 2003; Collin et al., 2004; Rouhier et al., 2004; Rey et al., 2005; Moon et al., 2006; Navrot et al., 2006; Perez-Ruiz et al., 2006; Vieira Dos Santos et al., 2007; Couturier et al., 2009; Pulido et al., 2010; Tarrago et al., 2010; Chibani et al., 2011; Couturier et al., 2011). New insights provided by this study, i.e. reduction of Trx-lilium2 and Trx-like2.1 by GSH and regeneration of PrxIIE and MsrB1 by Trx-like2.1, are highlighted by the use of grey boxes. Dotted arrows indicate that reduction of these Trxs by FTR is possible but not experimentally determined.