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Biochemical properties of poplar thioredoxin z

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ABSTRACT

Trx-z is a chloroplastic thioredoxin, exhibiting a usual WCGPC active site, but whose biochemical properties are unknown. We demonstrate here that Trx-z supports the activity of several plastidial antioxidant enzymes, such as thiol-peroxidases and methionine sulfoxide reductases, using electrons provided by ferredoxin–thioredoxin reductase. Its disulfide reductase activity requires the presence of both active site cysteines forming a catalytic disulfide bridge with a midpoint redox potential of −251 mV at pH 7. These in vitro biochemical data suggest that, besides its decisive role in the regulation of plastidial transcription, Trx-z might also be involved in stress response.
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1. Introduction

Thioredoxins (Trxs) constitute a multigenic family of generally small ubiquitous proteins of 12–14 kDa with a conserved WC[G/P]PC active site involved in dithiol–disulfide exchange reactions. Plant Trxs are classified into different groups depending on sequence similarity, gene structure and sub-cellular localization, i.e., cytosol, nucleus, chloroplast and mitochondria. Chloroplastic Trxs comprise proteins named Trx-f, -m, -x, -y, -like, -lilium, chloroplastic NADPH-thioredoxin reductase (NTRc), CDSP32 and HCF164 [1,2]. Recently, the formerly named CiTrx for Cf-9 interacting thioredoxin, also referred to as Trx-z or Trx-p, was found to be localized in plastids [3–5]. Most stromal Trxs are thought to be reduced by a ferredoxin–thioredoxin reductase (FTR) with electrons provided by light and relayed by ferredoxins (Fdxs), but this has been experimentally demonstrated only for Trxs-f and -m [6,7]. On the other hand, the hybrid protein named NTRc, which is constituted by a NTR module fused to a Trx module, uses NADPH as a source of reducing power [8]. In other organelles, Trxs are usually reduced by NADPH via NADPH-thioredoxin reductases (NTRs) [9].

In terms of functions, Trxs-f and -m primarily act as activators of enzymes involved in carbon metabolism [6,7], whereas Trxs-x, -y, -like -lilium, CDSP32 and NTRc would contribute to the response to oxidative stress, in particular through the regeneration of antioxidant enzymes such as peroxiredoxins (Prx) and methionine sulfoxide reductases (MSR) [8,10–16]. Besides, NTRc seems to regulate several other processes, including chlorophyll biosynthesis, the shikimate pathway and starch synthesis [17,18]. Trx-z or CiTrx was initially thought to be an adaptor protein connecting Cf-9 (receptor-like protein) and the ACIK1 protein kinase during the Cf-9/Avr9-induced defense response in tomato, with a putative cytosolic localization [19,20]. However, the recent characterization of a Trx-z knock-out *Arabidopsis thaliana* mutant showed that it displays a severe albino phenotype and abnormal chloroplast development [3–5]. This phenotype is likely related to Trx-z involvement in plastid-encoded polymerase (PEP)-dependent gene expression in chloroplasts, forming a complex with PEP subunits and fructokinase-like proteins (FLNs) [3–5].

Although *A. thaliana* Trx-z was shown to possess disulfide reductase activity and to interact with FLNs in a redox-dependent manner, there are no biochemical data concerning its capacity to reduce other physiological target proteins [3]. We have studied here the capacity of a poplar Trx-z to regenerate known antioxidant plastidial target proteins and to accept electrons from either FTR or NTRc.

Abbreviations: Fdx, ferredoxin; FNR, ferredoxin:NADP+ reductase; FTR, ferredoxin–thioredoxin reductase; MSR, methionine sulfoxide reductase; NTR, NADPH-thioredoxin reductase

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2. Materials and methods

2.1. Cloning, site-directed mutagenesis, production and purification of recombinant proteins

The sequence encoding poplar Trx-z (POPTR_0001s06590 in the Phytozome portal, http://www.phytozome.net/), amplified from *Populus trichocarpa* root cDNAs has been cloned into pET-15b using the two following primers (5′CCCCCCCCCATATGGGAAAGTACATTAGAGAA3′ and 5′CCCCGGATCCTCACATTTCATTGTTAAT3′, restriction sites underlined). Site-directed mutagenesis using two complementary mutagenic primers (5′ACATGGTGTGGACCC**TCT**GTTTTGAGGCCCAA3′ and 5′TTGGGCCATCAAAAC**AGA**GGGTCCACACCATGT3′, mutagenized codon in bold) was used to produce a variant named Trx-z C111S, mutated on the second active site cysteine.

The recombinant plasmids (pET15bTrx-z and pET15bTrx-z C111S) were used to transform the *Escherichia coli* BL21(DE3) strain. The culture and purification conditions were as described in [21]. After dialysis against TE buffer (30 mM Tris–HCl, pH 8.0, 1 mM EDTA), protein concentrations were determined using a molar extinction coefficient at 280 nm of 13 075 M^{-1} cm^{-1} for Trx-z and of 12 950 M^{-1} cm^{-1} for Trx-z C111S. Other recombinant proteins (glutathione peroxidases, peroxiredoxins and MSRs) were purified in the laboratory following procedures described previously [10,22–26].

2.2. Titration of free thiol groups

The number of free thiol groups in untreated or reduced Trx-z was determined spectrophotometrically using DTNB as described previously [27].

2.3. Disulfide reductase activity

Insulin reduction was measured using 10 μM Trx as in [27]. The non-enzymatic reduction of insulin by DTT was used as a control. The ability of Trxs to catalyze the reduction of DTNB (5,5′-dithio-bis-2-nitrobenzoic acid) in presence of *A. thaliana* NTRB (AtNTRB) was measured at 25 °C by monitoring the increase in absorbance at 412 nm caused by the release of thionitrobenzoate (TNB^-). The reaction medium contained 30 mM Tris–HCl, pH 8.0, 2 mM EDTA, 200 μM NADPH, 0.5 μM AtNTRB, 100 μM DTNB and 0–40 μM Trxs. The activity measured in absence of Trxs was subtracted.

2.4. Determination of the midpoint redox potential and cysteine pK_a

Oxidation–reduction titrations were carried out at ambient temperature by measuring fluorescence resulting from the reaction between protein thiol groups and monobromobimane (mBBr), whereas cysteine pK_a was measured using 2-pyridyldithiobimane following procedures described previously [24,27].

2.5. Regeneration of target proteins by Trx-z

The capacity of Trx-z to support the activity of plastidial target proteins (poplar PrxIIE, PrxQ, Gpx1, Gpx3 or MSRA4 and *A. thaliana* 2-Cys Prx, MSRB1 or MSRB2) was measured spectrophotometrically by following NADPH oxidation at 340 nm. The 500 μl reaction mixture contained 30 mM Tris–HCl, pH 8.0, 1 mM EDTA, 200 μM NADPH, 0.8 μM AtNTRB and variable Trx-z concentrations (from 2 to 80 μM). The peroxidase activity was measured using 500 μM H_2O_2 (100 μM for At2-Cys Prx) and 3 μM of the various thiol-peroxidases. The MSR activity was measured using 9 μM PtMSRA4, 5 μM AtMSRB1 or 880 nM AtMSRB2 and 2 mM *N*-acetyl-MetO as substrate. The capacity of AtNTRc to reduce Trx-z was tested by replacing AtNTRB in an assay comprising AtMSRB2. For all these tests, activity was corrected by subtracting the NADPH oxidation observed in the absence of thiol-peroxidases or MSRs. The k_{cat} and K_m values for Trx-z (K_{Trx}) of thiol-peroxidases and MSRs have been calculated by non-linear regression using the program GraphPad Prism software version 4.03.

2.6. Reduction of Trx-z by a Fdx/FTR system

About 20 μM of oxidized Trx-z was reduced by incubating the protein with 20 μM NADPH, 40 nM of recombinant *Chlamydomonas reinhardtii* ferredoxin:NADP+ reductase (FNR) and 1 μM of recombinant Fdx and FTR from *Synechocystis* for 15 min at room temperature. The 50 μl reaction was then precipitated on ice for 30 min with one volume of 20% trichloroacetic acid (TCA). After centrifugation (10 min at 13 000×*g*) and washing with 2% TCA, the pellet was resuspended into 100 mM Tris–HCl, pH 8.0, 1% SDS containing 20 mM of methoxyl-PEG maleimide of 2 kDa (mPEG maleimide) which alkylates free thiol groups. The protein mixture was then separated on non-reducing 15% SDS–PAGE.

Alternatively, the reduction of oxidized Trx-z by FTR was measured spectrophotometrically by following NADPH oxidation at 340 nm in a coupled system. The 500 μl reaction mixture contained 30 mM Tris–HCl, pH 8.0, 200 μM NADPH, 200 nM FNR, 5 μM Fdx, 5 μM FTR, 10 μM Trx-z, 880 nM AtMSRB2 and 2 mM *N*-acetyl-MetO. Control experiments were performed by omitting each of the components in individual assays.

3. Results and discussion

3.1. Reductase activity of poplar Trx-z

In order to investigate the biochemical properties of Trx-z, a mature form of 117 amino acids, devoid of the targeting sequence, was expressed in *E. coli* as a His-tagged protein and its ability to catalyze insulin reduction was first measured. Trx-z is able to efficiently reduce insulin with reduction rates comparable to spinach Trxs-m and -f (SoTrx-m and SoTrx-f) (Fig. 1). A monocysteinic variant, Trx-z C111S, mutated on the second active site cysteine, is much less efficient pointing to the importance of the resolving cysteine for disulfide reductase activity. The residual activity is likely attributed to the capacity of the catalytic cysteine to perform a nucleophilic attack on the insulin disulfide bridge forming an

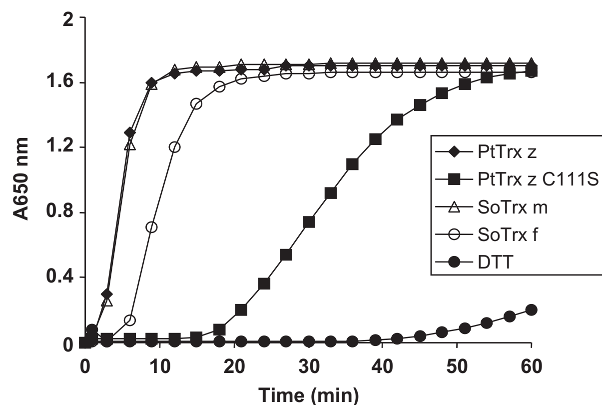

Fig. 1. Reduction of insulin by Trx-z. Insulin reduction was assessed using 10 μM of various Trxs by measuring the turbidity at 650 nm caused by the precipitation of reduced insulin.

intermediate covalent adduct subsequently reduced either by DTT or by the catalytic cysteine of another Trx-z molecule. Next, the ability of Trx-z to be reduced by a NADPH Trx reductase was examined using DTNB as a final electron acceptor, as this might be a useful tool for measuring its capacity to regenerate a variety of target proteins. Contrary to SoTrxs-f and -m and to Trx-z C111S, which are not able to reduce DTNB using the reducing power of NADPH and AtNTRB (data not shown), the k_{cat} of the reaction catalyzed by Trx-z is 1.5 s^{-1}, whereas the apparent affinity of AtNTRB for Trx-z is 22 μM. The kinetic parameters of the reaction are comparable to those obtained with some poplar Trx h [28,29]. Together, these results indicate that both active site cysteines of Trx-z are necessary for its reduction by AtNTRB and that in case Trx-z is targeted elsewhere than in plastids, i.e., in the cytosol, as suggested by the study of Rivas and colleagues, it can well be reduced by AtNTRA or AtNTRB [20].

3.2. Redox properties

Thiol titration of the "as-purified" Trx-z indicated that no thiol groups can be detected and thus that the protein is oxidized. As two thiols can be measured, as expected, for a pre-reduced Trx-z and as the protein does not form any dimer in non-reducing SDS–PAGE (data not shown), we conclude that an intramolecular disulfide is formed inside a monomer in the oxidized state. Using the classical fluorescent mBBr method, we have measured a redox midpoint potential (E_m) value of −251 ± 5 mV at pH 7 for the catalytic disulfide of Trx-z (Fig. 2), which is slightly lower than the values determined for three Arabidopsis Trxs-lilium (E_m values comprised between −237 and −240 mV) but slightly higher than those of other cytosolic and plastidial Trxs (E_m values usually comprised between −275 and −330 mV) [11,13,16,30,31]. In addition, using the Trx-z C111S variant, we have determined a pK_a value of 6.4 for the catalytic cysteine Cys108 (Fig. 3), a value comparable to those determined for other Trxs, generally around 7 [32].

3.3. Regeneration of plastidial target proteins

Using the capacity of Trx-z to be regenerated by AtNTRB, we have explored for the first time its ability to support the activity of the whole set of plastidial thiol-peroxidases (PtPrxIIE, PtPrxQ, At2-Cys Prx, PtGpx1, PtGpx3) or MSRs (PtMSRA4, AtMSRB1, AtMSRB2) in a coupled assay by following NADPH oxidation. The catalytic efficiencies of all target proteins as well as their apparent K_m values for Trx-z are summarized in Table 1. Interestingly, among all enzymes tested, Trx-z does not efficiently regenerate both At2-Cys Prx and AtMSRB1 as well as another known Trx target enzyme of the carbon metabolism, the NADP malate dehydrogenase from sorgho (data not shown). The absence of activity with AtMSRB1 was somehow expected because this enzyme is regenerated through the direct reduction of the sulfenic acid formed after the reduction of methionine sulfoxide by the glutathione/Grx couple or by the peculiar CDSP32 and not by dithiol/disulfide exchange like the majority of other MSRs [16,26,33]. However, the inability of Trx-z to regenerate an oxidized At2-Cys Prx is more puzzling. Despite slightly unfavourable E_m values (−315 mV for At 2-Cys Prx vs −250 mV for Trx-z), the reason for this absence of activity is likely not related to the redox potential as Trx-z can reduce PtPrxQ which possesses an E_m value of −325 mV [24,34]. It is nevertheless still possible that contrary to poplar Trx-z, *A. thaliana* Trx-z can support the activity of *A. thaliana* 2-Cys Prx. However, from the analysis of knock-out plants for NTRc or CDSP32, 2-Cys Prx seems to preferentially rely on these two proteins for its reduction [35,36].

The catalytic parameters measured under steady-state conditions for other thiol-peroxidases or MSR lie in the same range previously described, with k_{cat} values comprised between 0.04 and 1.16 s^{-1}, K_{Trx} ranging from 5 to 25 μM and thus k_{cat}/K_{Trx} comprised between 10^3 and 10^4 M^{-1} s^{-1} (Table 1) [11,16,24–26]. Among thiol-peroxidases, the lowest catalytic efficiency (k_{cat}/K_{Trx}) was observed for PtPrxIIE. This is presumably explained by the lower efficiency of the Trx system compared to the Grx system observed for this thiol-peroxidase [22]. On the other hand, the highest catalytic efficiencies have been obtained with PtGpx3 and PtPrxQ. This result is very interesting as these enzymes previously displayed a marked selectivity toward their electron donors, being

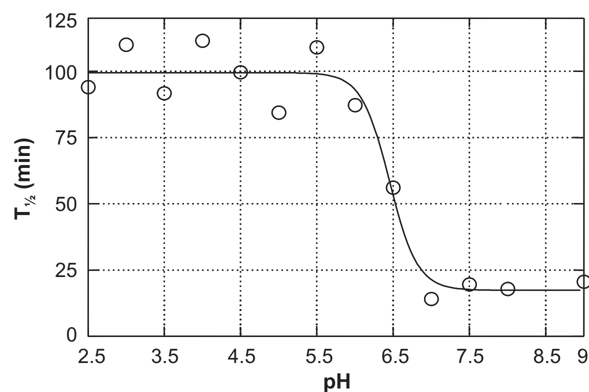

Fig. 3. pK_a determination of Trx-z catalytic cysteine. The reaction of Cys108 of the Trx-z C111S variant with PDT-bimane was monitored at 343 nm at pH values ranging from 2.5 to 9. $T_{1/2}$ represents the time to reach half-maximal reactivity as monitored by half-maximal release of pyridyl-2-thione. Each data point represents the average of at least three experiments.

Table 1
Kinetic parameters of plastidial thiol-peroxidases and methionine sulfoxide reductases measured under steady-state conditions using Trx-z as an electron donor. Each data point used to obtain the substrate saturating curves represents the average of at least two experiments.

	K_{Trx} (μM)	k_{cat} (s^{-1})	k_{cat}/K_{Trx} ($\times 10^3$ M^{-1} s^{-1})
PtPrxIIE	24.9 ± 3.0	0.050 ± 0.002	2.0
PtPrxQ	7.3 ± 1.9	0.130 ± 0.008	17.0
PtGpx1	11.0 ± 2.0	0.070 ± 0.007	6.3
PtGpx3	7.5 ± 2.6	0.150 ± 0.018	20.0
PtMSRA4	5.0 ± 0.7	0.040 ± 0.001	7.9
AtMSRB2	23.3 ± 3.9	1.160 ± 0.066	49.0

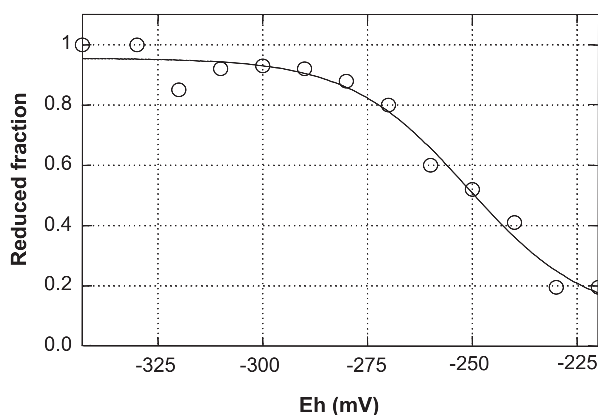

Fig. 2. Redox titration of poplar Trx-z. The titration was carried out using a total DTT concentration of 2 mM in the redox buffer and with a redox equilibration time of 2 h. Free protein thiols are labeled by mBBr. Values are the means ± S.D. of three replicates.

exclusively (PtGpx3) or preferentially (PtPrxQ) reduced by Trxs-y [23,24]. Among MSRs, although the best affinity is observed for the PtMSRA4-Trx-z couple, the k_{cat}/K_{Trx} is much better (around 6 times) for the AtMSRB2-Trx-z couple resulting from a better k_{cat} value.

3.4. Physiological reduction of Trx-z

Being located in plastids, we wished to determine which of GSH, NTRc or FTR could be the physiological electron source of Trx-z. We took advantage of the fact that GSH and NTRc are unable to directly support the activity of AtMSRB2 to test whether Trx-z can regenerate AtMSRB2 at their expense [26]. However, no activity was detected, indicating that none of these two systems can efficiently provide the reducing power necessary to Trx-z (data not shown). Then, in order to test the reduction of Trx-z by FTR, we have adapted a four-component electron donor/recycling system consisting of NADPH as primary source, *Chlamydomonas* FNR, *Synechocystis* Fdx and FTR [34]. This reconstituted system is very useful as it avoids using the complicated light reconstituted system. The functionality (i.e., electron transfer capacity) of the first three components (NADPH/FNR/Fdx) of the system was confirmed by measuring the reduction of horse heart cytochrome c, used as the final electron acceptor (data not shown). Using this coupled assay and AtMSRB2 as target protein, we have indeed observed the capacity of FTR to reduce Trx-z, which was deduced from the linear and time-prolonged NADPH oxidation observed at 340 nm (Fig. 4A). At this Trx-z concentration (10 μM), the activity observed is similar to the one determined using AtNTRB. Alternatively, the reduction of oxidized Trx-z by the NADPH/FNR/Fdx/FTR system was assessed in non-reducing SDS–PAGE after alkylation of free thiol groups by mPEG maleimide (Fig. 4B). Compared to the oxidized protein (lanes 2 and 3), the addition of reduced DTT (lane 4) or NADPH/FNR/Fdx/FTR system (lane 5) generated a shift in protein migration corresponding to the alkylation of the free thiol groups liberated, confirming that oxidized Trx-z can indeed be reduced by the chloroplastic Fdx/FTR system.

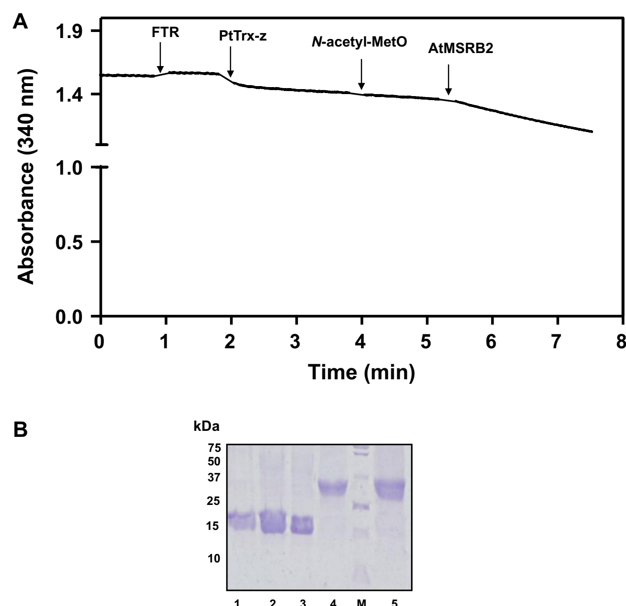

Fig. 4. Reduction of Trx-z by FTR. (A) FTR-dependent recycling of AtMSRB2 by Trx-z. The initial reaction mixture contained 200 μM NADPH, 200 nM FNR and 5 μM Fdx. After equilibration, 5 μM FTR, 10 μM Trx-z, 2 mM *N*-acetyl-MetO and finally 880 nM AtMSRB2 were successively added as indicated by arrows. As expected, a slight NADPH consumption is visible after adding 10 μM of oxidized Trx-z. No activity was detected when omitting any of the components. (B) Alkylation of Trx-z upon reducing treatments analyzed by non-reducing SDS–PAGE. Lane1: untreated Trx-z. Lanes 2–5 represents mPEG-mal alkylated proteins – lane 2: untreated Trx-z; lane 3: Trx-z incubated with 500 μM DTT_{ox}; lane 4: Trx-z incubated with 100 μM DTT_{red}; M: molecular weight marker; lane 5: Trx-z 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 are not detectable by coomassie blue staining due to the low concentrations used. The shift observed following the alkylation of the two thiol groups of Trx z is larger than expected (ca. 15 kDa instead of 4 kDa), but similar shifts have been often observed with many other proteins.

4. Conclusions

Previous studies on Trx-z showed that this protein is involved in the regulation of plant disease resistance by interacting with the cytoplasmic part of the tomato Cf-9 resistance protein and that this regulation is independent of the Trx reductase activity [19,20]. The proposed cytosolic localization of Trx-z is supported by the size of the protein detected by western blot which is consistent with a full-length protein without any cleaved N-terminal plastidial targeting sequence. More recently, it has been shown that Trx-z is localized in chloroplast and belongs to a PEP complex, together with fructokinase-like proteins [3,5]. The Arabidopsis knockout mutant of Trx-z displays an albino phenotype and the seedlings rapidly die on soil or on agar plates in the absence of sucrose [3–5]. The molecular characterization of this mutant indicated that chloroplast development was affected because of the deregulation of the PEP-dependent transcription machinery [3]. In this complex, Trx-z interacts with FLNs in a redox-dependent manner.

Considering these two studies, a different targeting for Trx-z in response to biotic or abiotic constraints might be realistic since as argued by Arsova and colleagues, other similar cases, such as the one of the NRIP1 protein, exist [37]. If this is true, the capacity of Trx-z to be reduced by both FTR and NTRB makes a lot of sense.

Besides these two roles, we propose, based on our in vitro biochemical data, that Trx-z might also be involved in protecting plastids against oxidative damage by regenerating various antioxidant enzymes belonging to the thiol-peroxidase or MSR families. However, the severe developmental phenotype of the Trx-z mutant prevents an exploration of this possibility in vivo.

Being reduced by the light/FTR system, Trx-z could constitute an alternative, though maybe partially redundant, to other previously described plastidial Trxs. Trx-x, NTRc and CDSP32 have been found to be good reductants of 2-Cys Prx, whereas Trxs-y were the most efficient electron donors to PrxQ and Gpx [10–12,15,23,24,33]. Concerning the MSR family, the reductant specificity has only been explored for AtMSRB1 and AtMSRB2. While CDSP32 constitutes a good reductant for MSRB1, the specificity is different for MSRB2 for which Trxs-m1, -y2 and -f1 proved to be efficient [16,26].

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