New insights into the reduction systems of plastidial thioredoxins point out the unique properties of thioredoxin z from Arabidopsis

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ABSTRACT

In plants, thioredoxins (TRX) constitute a large protein disulfide oxido-reductase family comprising ten plastidial members in *Arabidopsis thaliana* (Arabidopsis) subdivided in five types. The f and m-types regulate enzymes involved mainly in carbon metabolism whereas the x, y and z types have an antioxidant function. The reduction of TRXm and f in chloroplasts is performed in the light by ferredoxin:thioredoxin reductase (FTR) that uses photosynthetically reduced ferredoxin (Fd) as a reductant. The reduction system of Arabidopsis TRXx, y and z has never been demonstrated. Recently, a gene encoding an atypical plastidial NADPH-dependent TRX reductase (NTRC) was found. In the present study, gene expression analysis revealed that both reductases are expressed in all organs of Arabidopsis and could potentially serve as electron donors to plastidial TRX. This ability was tested *in vitro* either with purified NTRC in presence of NADPH or with a light-driven reconstituted system comprising thylakoids and purified Fd and FTR. Our results demonstrate that FTR reduces the x and y TRX isoforms but not the recently identified TRXz. Moreover, we show that NTRC cannot be an efficient alternative reducing system neither for TRXz nor for the other plastidial TRX. Our data reveal that TRXf, m, x and y, known as redox regulators in the chloroplast, have also the ability to reduce, *in vitro,* TRXz. Overall, the present study points out the unique properties of TRXz among plastidial TRX.

Key words: Thioredoxin, FTR, NTRC, Redox signaling, Plastid, Arabidopsis

INTRODUCTION

Thioredoxins (TRX) belong to a large multigenic family of oxidoreductases found in all free living organisms. These small soluble proteins (12-14 kDa) share a characteristic fold, a conserved active site WCG/PPC sequence, and a low redox potential that confers on them strong reductive properties. The two cysteines of the active site are involved in thiol/disulfide exchanges with their target proteins.

The first plant thioredoxins (m and f-types) were identified as regulators of chloroplastic enzymes related to carbon metabolism (Buchanan, 1980). Genomic revolution revealed that plant TRX isoforms are encoded by multi-gene families (Meyer et al., 2005; Nuruzzaman et al., 2008). In *Arabidopsis thaliana* (Arabidopsis), twenty TRX isoforms are found, divided into three subgroups: cytosolic, mitochondrial and plastidial. Sequence similarities and intron positions revealed that plastidial isoforms comprised nine members in Arabidopsis, subdivided into four types, the f, m, x and y-types (Mestres-Ortega and Meyer, 1999; Meyer et al., 2005), with four isoforms in the m-type, 2 in the f-type, 2 in the y-type, 1 in the x-type. Recently, two independent studies identified a tenth plastidial thioredoxin, TRXz (Arsova et al., 2010; Meng et al., 2010).

This multiplicity of TRX isoforms raised the question of their redundancy or possible functional specialization. Complementation studies on a TRX-deficient yeast strain hypersensitive to oxidative stress showed that Arabidopsis TRXf, m and x displayed different efficiencies (Issakidis-Bourguet et al., 2001). Afterwards, biochemical *in vitro* studies revealed specificity of chloroplastic TRX towards their target enzymes. Indeed, TRXf and m redox regulate the activity of enzymes mainly involved in the primary metabolism, whereas the more recently identified isoforms x,

y and z can serve as hydrogen donors for antioxidant enzymes such as peroxiredoxins (PRX), suggesting their role in oxidative stress defence (Collin et al., 2003, 2004; Lamkemeyer et al., 2006; Née et al., 2009; Chibani et al., 2011). Moreover, recent proteomic studies revealed a multiplicity (nearly 100) of putative chloroplastic targets involved in a wide range of processes (Michelet et al., 2006; Lemaire et al., 2007; Viera Dos Santos et al., 2007; Valerio et al. 2010). New putative TRX targets were also found in isolated organelles from wheat endosperm (Balmer et al., 2006) and in Arabidopsis root extracts (Marchand et al., 2010).

Once oxidized, TRX can be re-reduced by specific reductases. In the light, the photosynthetic electron transfer chain reduces ferredoxin (Fd) which can donate electrons to various enzymes including Fd:TRX reductase (FTR). This key enzyme of the Fd/TRX regulatory system has been shown to reduce efficiently TRXm and f in a reconstituted light activation system (Droux et al., 1987). In the cytosol and mitochondria of plant cells, TRX are reduced by an NADPH-dependent TRX reductase (NTR) (Jacquot et al., 1994; Laloi et al., 2001; Reichheld et al., 2005). A new gene encoding an atypical plastidial NTR (named NTRC) was identified in Arabidopsis, rice and *Medicago truncatula* (Serrato et al., 2004; Pérez-Ruiz et al., 2006; Alkhalfioui et al., 2007). It encodes a bipartite protein containing an NTR domain fused to a TRX domain. NTRC was shown to be an efficient redox system for chloroplast protection against oxidative damage and proposed as a possible alternative plastidial TRX reducing system (Serrato et al., 2004; Pérez-Ruiz et al., 2006).

The recent finding of three new plastidial TRX types (x, y and z) as well as the presence of plastidial TRX isoforms in non-photosynthetic organs (Collin et al., 2004; Balmer et al., 2006; Traverso et al., 2008) raised the question of their reducing system(s). One can wonder whether NTRC can reduce plastidial TRX in non-

photosynthetic tissues, or be a reducing system for the x, y and z isoforms. A direct biochemical test can assess the ability of a given reductase to reduce a given TRX. However, the interaction is possible only if the two partners share the same spatial and temporal localization. Thus, in the present work, the expression patterns of the ten genes encoding the plastidial TRX from Arabidopsis together with the four genes encoding the plastidial TRX reductase subunits were first examined using quantitative RT-PCR. Gene expression profiles revealed that both FTR and NTRC could potentially serve as reductants for all TRX in plastids. This ability was tested directly *in vitro* using NTRC in presence of NADPH, or FTR in a light-driven reconstituted Fd/TRX system. Here we demonstrate that FTR can reduce TRXx and y but not TRXz. Moreover, we show that NTRC cannot be an alternative reducing system for plastidial TRX, but that reduced plastidial thioredoxins are able to reduce TRXz *in vitro*.

MATERIALS AND METHODS

Reagents

All biochemical reagents were purchased from Sigma-Aldrich, unless otherwise mentioned.

Plant Material

Arabidopsis thaliana wild-type (Columbia ecotype) were grown on soil in the greenhouse under natural growth conditions (20°C day/18°C night temperature, 60% day/55% night hygrometry, light intensity 80-100 μE m^{-2} s^{-1}) and watered every 2 days.

Peas (*Pisum sativum* var. merveille de Kelvedon) were grown in the greenhouse on vermiculite, watered daily and harvested 3 weeks after sowing.

RNA Isolation and cDNA Synthesis

Total RNA were isolated from 100 mg of frozen plant material (except for seeds: 30 mg) using the NucleoSpin® RNA Plant kit from Macherey-Nagel, according to manufacturer's instructions. For RNA extraction from dry seeds 5% (w/v) polyvinylpyrrolidone was added to the extraction buffer. The reverse transcription step was performed with up to 1 μg of total RNA, using the SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen) with oligo(dT) primer.

Quantitative RT-PCR Analysis

Gene expression profiling was conducted by real-time quantitative RT-PCR using gene-specific primer pairs and procedures detailed in Table S1.

Expression, Production and Purification of Recombinant Proteins

Proteins and related purification procedures are described in Table S2.

2-Cys PRX Activity Assays

PRX activity was assayed in 30 mM Tris-HCl buffer, pH 7.9, in the presence of 20 µM 2-Cys PRXA from Arabidopsis, 400 µM DTT, 600 µM H_2O_2 and 10 µM TRX. Remaining hydrogen peroxide was quantified colorimetrically at 560 nm using Peroxoquant reagent (Perbio Science). Alternatively, 2-Cys PRX activity was determined as oxidation of NADPH followed at 340 nm in a reaction mixture containing 100 mM phosphate buffer, pH 7.2, 10 mM EDTA, 1 mM H_2O_2, 10 µM PRX, 200 nM NTRC and 150 µM NADPH.

Radiolabeling of SH groups in TRX z

A sample of TRXz preparation (60µg) was alkylated with [^{14}C]iodoacetamide (IAM) (0.5 MBeq/µmol, 2 mM), without treatment, or after an oxidizing (trans-4,5-dihydroxy-1,2-dithiane (DTTox) 10 mM, 15 min) or a reducing treatment (dithiotreitol (DTTred) 100 mM, 15 min). After trichloroacetic acid precipitation and separation by SDS-PAGE (Droux et al., 1987), proteins were Coomassie blue stained

and labeling was visualized by exposure of the dried gel to a low energy imaging screen, for two weeks (personal molecular Imager FX system, BioRad).

Reduction of TRXx, y and z by the Fd/FTR System

The reconstituted light activation system comprised, in a total volume of 50 µL, washed thylakoids equivalent to 10-20 µg chlorophyll, prepared as described in Droux et al. (1987), 100 mM Tris-HCl, pH 7.9, 2-5 µM Fd, 10 µM FTR, 25 µM TRX and 5 µM subunit mutant NADP-MDH. Aliquots were withdrawn as a function of time of illumination (120 µE m^{-2} s^{-2}) and, for TRXx and y, tested for NADP-MDH activation level. For TRXz (NADP-MDH omitted) thiol redox status was estimated by redox western. Alternatively to the light-dependent system, 10 µM TRXz was incubated for 15 min in the presence of 20 µM NADPH, 40 nM FNR and 1 µM FTR and 1 µM Fd according to Chibani et al., (2011). Free thiol groups were alkylated for 25 min, with 25 mM N-ethyl maleimide (NEM) and 30 mM iodoacetic acid (IAA). Alkylated proteins were subjected to non-reducing SDS-PAGE and the reduction state of TRXz was evaluated from its monomer/dimer status by Western blot.

Chemical Activation of NADP-Malate Dehydrogenase

Δ15-NADP-MDH (shortened by 15 amino acids at the N-terminus) whose biochemical characteristics are identical to those of the full-length wild-type MDH (Johansson et al., 1999) and mutant C207A/C365A/C377A NADP-MDH were used. Enzymes (5 µM) were incubated at room temperature, with 25 µM TRX plus 1 mM DTT, or 5 or 100 µM TRXz plus 100 mM DTT or DTT alone. The enzymatic activity

was measured in 100 mM Tris-HCl, pH 7.9, 180 µM NADPH, 750 µM oxaloacetate. Disappearance of NADPH was followed spectrophotometrically at 340 nm.

Immunodetection of TRX z by Western Blot

Samples were separated by non-reducing 12% SDS-PAGE and transferred onto nitrocellulose membrane (iBlot Gel Transfer Device, Invitrogen). Detection was performed using either a commercial anti *Strep*-tag II monoclonal antibody conjugated to horseradish peroxidase (IBA) at a dilution of 1:10,000 or a primary specific anti-TRXz antibody at a dilution of 1:2,500 and a secondary horseradish peroxidase conjugated antibody at a dilution of 1:50,000, and revealed by enhanced chemiluminescence (ECL, GE Healthcare). The specificity of the anti-TRXz antibody was checked towards all the other plastidial TRX by Western blot (Figure S1).

Reduction of plastidial TRX by NTRC and NTRA tested spectrophotometrically

The ability of Arabidopsis cytosolic NTRA or plastidial NTRC to reduce TRX was measured by the 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB, Pierce) reduction assay. The reaction mixture (1 mL) contained 20 nM NTRC or NTRA, 150 µM NADPH, 1 mM DNTB and 10 μM TRX in 100 mM $NaPO_4$, pH 8.0 and 1 mM EDTA. All measurements were carried out at 25°C by monitoring the increase in absorbance at 412 nm caused by the release of thionitrobenzoate (TNB^-).

Reduction of TRXz by the GSH/GRX system, NTRC or plastidial TRX

TRXz was pre-oxidized by 10 mM DTTox during 20 min. DTTox was removed on a PD Spin Trap G-25 column (GE Healthcare). Oxidized TRXz (10 µM) was incubated for 20 min at room temperature with various reductants: either 1, 5 or 10 mM reduced glutathione (GSH) or 300 µM NADPH, 6 µg/mL bakers yeast glutathione reductase (GR), 5 mM GSH and 10 µM chloroplastic glutaredoxin GRXS16. NTRC (2 µM) was tested with 500 µM NADPH. Plastidial TRX (f1, m1, x or y1; 10 µM) were also tested in presence of 1 mM DTT. Redox state of TRXz was evaluated from its monomer/dimer status by redox western.

RESULTS

Transcript levels reveal that both NTRC and FTR are expressed along with plastidial TRX, including in non-photosynthetic organs

Using real-time quantitative PCR the presence of mRNAs encoding plastidial TRX was detected in all organs of Arabidopsis at different levels depending on the isoform (Figure 1A). Globally, genes encoding m-type TRX (except m3) and TRX-x were more abundantly expressed, especially in leaves and young seedlings. Genes coding for TRXf1 and f2 and TRXy2, though expressed at a lower level, showed similar expression profiles. The gene coding for TRXz was expressed at the lowest level and its expression was restricted to leaves, flowers and young siliques. Noteworthy, Attrxm3 and Attrxy1 expression patterns differed from those of other plastidial Arabidopsis trx genes, being predominantly expressed in non-photosynthetic tissues *e. g.* mature siliques, roots and dry seeds. As previously suggested (Collin et al., 2004) the gene encoding TRXy1 is the most strongly expressed in seeds among all plastidial Arabidopsis TRX. Overall, these results are consistent with transcriptomic data obtained from the "Arabidopsis eFP Browser" (Figure S2).

The expression levels of the genes encoding the variable subunits FTRA1 and FTRA2 of FTR (Keryer et al., 2004) were close to the one encoding NTRC (Figure 1B). The gene encoding the catalytic FTRB subunit was expressed in all organs tested including non-photosynthetic organs. FTRB transcripts were quite abundant as compared to FTRA1 and FTRA2. Expression profiles of both genes encoding FTR variable subunits were more strongly expressed in leaves, young seedlings and mature

siliques. Similarly, NTRC showed a wide expression profile with a higher level in green tissues.

These findings prompted us to test both FTR and NTRC in their ability to reduce plastidial TRX. TRXx and y have been characterized in our laboratory (Collin et al., 2003, 2004) and showed specific antioxidant functions. However, their reduction system was still unknown as well as the one of Arabidopsis TRXz.

TRXz is a functional oxidoreductase with target specificities

Among the well-known TRX-dependent chloroplastic enzymes, NADP-MDH is probably the most studied enzyme (Ruelland and Miginiac-Maslow, 1999). It is devoid of activity in its oxidized state and gets activated in presence of reduced TRXf or m, except m3 (Collin et al., 2003). We expressed TRXz in its mature form (N-terminal transit peptide removed) in *E. coli* as a *Strep-tag*ged protein. It failed to activate wild-type sorghum NADP-MDH in the presence of DTT (data not shown).

Activation of NADP-MDH involves the reduction of two regulatory disulfides located in sequence extensions at the N- and C-terminus (Issakidis et al., 1994). We engineered a mutant form of NADP-MDH where only the N-terminal disulfide remains (C207A/C365A/C377A mutant, Ruelland et al., 1997). This mutant has a basal activity and is more easily activated, due to a less negative redox potential. It is activated by plastidial TRX isoforms that do not activate the wild type enzyme, such as TRXx and y (Collin et al, 2003; 2004). Unlike TRXm1 used as a positive control, TRXz proved inefficient (even at 100 µM) towards mutant NADP-MDH, in the presence of a high (100 mM) DTTconcentration necessary to reach a complete reduction of this TRX. Thus, TRXz is unable to activate NADP-MDH (Table 1).

We also tested the capacity of TRXz to mediate proton donation to 2-Cys PRX by following the reduction of H_2O_2 by PRX in the presence of DTT. 2-Cys PRX is known to be reduced efficiently by TRXx, to a lesser extent by TRXy, and not by TRXm3 (Collin et al., 2003; 2004). Figure 2 shows that, indeed TRXx is the best reductant for 2-Cys PRX, Trxm3 being quite inefficient. TRXz was as efficient as TRXy1 validating that our preparation is redox active. Thus, unlike poplar TRXz (Chibani et al., 2011), Arabidopsis TRXz can donate electrons to homologous 2-Cys PRX.

FTR reduces efficiently TRXx and y but cannot reduce TRXz

FTR is the known reductant of plastidial TRXf and m but its capacity to reduce the more recently identified Arabidopsis TRX isoforms x, y and z was never studied. We tested the capacity of FTR to reduce plastidial Arabidopsis TRX in a reconstituted system comprising illuminated pea thylakoids, Arabidopsis ferredoxin 2 (Fd) and spinach FTR (Droux et al., 1987). In this system, TRXx, y1 and y2 were able to activate mutant NADP-MDH with efficiencies comparable to TRXf1 (Figure 3). In the dark, or when a component of the activation mixture was omitted, no NADP-MDH activation was observed (Figure 3 and data not shown). These data provide evidence that TRXx and y can be efficiently reduced by FTR. Since TRXz is not able to activate mutant NADP-MDH, its reduction was followed by redox western. Indeed, we observed that TRXz forms dimers when oxidized and gets monomerized upon reduction by DTT. We checked that monomerization was correlated with the appearance of free thiols on the protein using radiolabeling with the thiol-alkylating agent iodoacetamide (^{14}C-IAM). A strong and specific labeling was observed for the

monomer in its reduced form, most probably corresponding to the reduction of the active-site disulfide (Figure 4). As there was no labeling of the dimer, it should correspond to oxidized protein. This hypothesis is strengthened by the absence of labeling of the oxidized monomer, corresponding to the initial redox state of our TRXz purified preparation and after a treatment with DTTox (Figure 4). This observation allowed us to use the monomerization/dimerization behavior of the protein as a test of reduction/oxidation. We tested TRXz reduction by FTR in the reconstituted light system described above (Figure 5). Upon illumination, no monomerization of TRXz was observed. In the same experiment, the light-driven reduction of TRXf1 (evidenced by NADP-MDH activation) proved efficient (data not shown). Inability of FTR to reduce TRXz was confirmed using an *in vitro* reconstituted "dark" system consisting of NADPH, FNR, Fd and FTR (Figure S5B, lane 9). These results provide evidence that FTR can be the physiological reductase of plastidial TRXx and y, with comparable efficiencies to TRXf. Noteworthy; TRXz is the first plastidial TRX which is not reduced by their classical reductase.

NTRC is not an efficient alternative reducing system for plastidial TRX

Most plastidial TRX genes are expressed in green tissues but, as mentioned above, some isoforms, such as TRXy1, are predominantly present in non-photosynthetic tissues where the mRNA corresponding to NTRC could be detected. Moreover, as FTR cannot reduce TRXz, NTRC could possibly be its physiological substitute. We produced and purified the Arabidopsis recombinant NTRC. Its functionality was confirmed by its capacity to mediate an efficient degradation of H_2O_2 by 2-Cys PRXA (Figure S3). We then assayed the reduction of the five types of plastidial TRX by NTRC using DTNB as a final electron acceptor, as this is a useful tool for measuring TRX oxido-reductase capacity. At 20 nM, NTRC alone, probably due to its own TRX

domain, displays a TRX reductase activity with a k_{cat} value (s^{-1}) of 4.47 ± 0.59. This activity increases 2-3 fold in presence of plastidial TRX (Figure S4A). However, this reduction rate corresponds to the basal rate observed with the cytosolic NTRA for TRXf1 that is five-fold lower than the reduction rate of the cytosolic TRXh3 (Figure S4B). Moreover, when the ability of NTRC to reduce TRXz was tested by following its monomerization by redox western, no increase of the monomeric form at the expense of the dimers was observed (Figure S5A). The faint intermediate molecular weight bands, more abundant in aged preparations (data not shown), most probably correspond to the dimer's conformational variants as they are recognized by the anti-TRXz antibody and disappear upon reduction. We conclude that TRXz cannot be efficiently reduced by NTR.

TRXz is not reduced by the GSH/GRX system

Previous data have demonstrated that a subgroup of cytosolic TRX, which are not reduced by NTR, can be reduced by glutaredoxin (GRX) which is itself reduced by glutathione (GSH) (Gelhaye et al., 2003). In this system, known as the GSH/GRX system, the transmission of the redox signal is mediated from NADPH to GSH *via* glutathione reductase (GR). None of TRXf, m, x and y are reduced by GSH (unpublished results). However, Chibani et al. (2011) have recently found close values for the redox midpoint potentials (E_m) of TRXz and GSH (Noctor, 2006). The ability of TRXz to be reduced by GR, GSH and/or a plastidial GRX (GRXS16; Bandyopadhyay et al., 2008) was tested by following its reduction through its monomerization. No monomerization of TRXz occurred with any of these

components (Fig. S5B). Thus we conclude that none of the components of the GSH/GRX system can reduce TRXz *in vitro*.

Plastidial TRX can reduce TRXz

The E_m value for the catalytic disulfide of TRXz was found to be -311 mV at pH 7.9 (Chibani et al., 2011), which is less negative than the E_m values of the other plastidial TRX ranging from -365 to -335mV (Collin et al., 2003; 2004). Therefore, we investigated the ability of other plastidial TRX to reduce TRXz (Figure 6). While 1 mM DTT could not directly reduce TRXz, in presence of TRXf or m, the amount of monomeric TRXz was largely increased at the expense of the dimeric form (Figure 6A). In comparison, TRXx and y exhibited a significant, though lower, efficiency (Figure 6B). These data demonstrate that TRXz can be reduced by other plastidial TRX *in vitro*.

DISCUSSION

The main goal of the present work was to test *in vitro* the ability and specificity of plastidial reductases to reduce the various plastidial TRX. As a preliminary, we show, using quantitative RT-PCR, that the ten genes encoding plastidial TRX together with the three genes encoding FTR subunits and the gene encoding NTRC were expressed in the same organs, at the same time (Figure 1) and thus could be partners. These data will need to be further completed with a study at the protein level and gene expression analyses in different cell types. The detection of a messenger RNA suggesting the presence of the translated corresponding protein, these findings prompted us to test both FTR and NTRC in their capacities to reduce plastidial TRX and particularly the more recently identified TRXx, y and z isoforms from Arabidopsis.

FTR is the well-known reductase for TRXf and m isoforms, TRXx, y and z were found as efficient proton donors to peroxiredoxins (Collin et al., 2003, 2004; present work: Figure 2) but the upstream reduction of TRX by following PRX activity in a reconstituted Fd/FTR/TRX light activation system cannot be easily tested. Indeed, reactive oxygen species (ROS) production by illuminated thylakoids interferes with the activity test. However, the ability of FTR in reducing TRXx and y could be tested by following the activation of an engineered mutant NADP-MDH enzyme which can be reduced by these TRX isoforms (Figure 3). Our results demonstrate that TRXx and y are reduced by FTR with efficiencies comparable to those of the m and f-type isoforms (Droux et al., 1987). Thus, in the light, FTR is clearly the reducing system for plastidial TRXf, m, x and y in Arabidopsis. However, the recent finding of f and m-TRX in non-photosynthetic organs raised the question of their reducing system in heterotrophic plastids (Traverso et al., 2008). Moreover, the presence of a complete FTR system in amyloplasts was demonstrated (Balmer et al., 2006). In roots, FNR

reduces type III Fd which is more easily reduced than leaf isoforms, as it displays a less negative E_m (Hanke et al., 2004). This property favors the electron transfer from NADPH to FTR via FNR and Fd. The functionality of this system was already tested by König et al. (2002) for TRXf and m but still needs to be tested for other plastidial TRX isoforms, especially the ones expressed in non-photosynthetic tissue such as TRXm3 and y1.

The ability of FTR in reducing TRXz could be assessed in the reconstituted light system by following the reduction state of TRXz through its monomerization. Indeed, TRXz could not activate NADP-MDH, either wild-type or mutant, but the labeling experiment (Figure 4) showing that only the reduced monomer can be derivatized offered us a simple way to observe the reduction of this TRX. Our data show that FTR cannot reduce TRXz in the light (Figure 5) in contrast with all the other plastidial TRX. This finding is not consistent with the work of Chibani et al. (2011) where a heterologous NADPH-driven FTR reduction system was used. These authors assessed the reduction of poplar TRXz by a shift of migration during non-reducing SDS-PAGE following the alkylation with methoxyl-PEG maleimide. A shift of *ca.* 15 kDa, instead of 4 kDa as expected for the alkylation of two thiol groups, was obtained. This form could correspond to the dimer we observed in our experiments. A dimerization/monomerization upon oxidation/reduction was previously observed for TRXf which contains a third Cys residue responsible for this behavior (Michelet et al., 2005). In the mature Arabidopsis TRXz, no additional Cys residue is found, indicating that dimerization is linked to the specific properties of this isoform.

Noteworthy, in our light activation experiment, when TRXz was mixed with thylakoid membranes, no monomeric form was observed at all (Figure 5), even in the dark, while our TRXz preparations always appeared as a mixture of monomers and

dimers, even in the NADPH/FNR/FTR/Fd reconstituted system (Figure S4). Moreover, the amount of TRXz seemed to be lower in the dark (time 0) and to increase upon illumination (Figure 5). Therefore, TRXz might interact (directly or indirectly) with thylakoid membranes in the dark and could be released in the light. Such a phenomenon was recently reported for FNR (Benz et al., 2010) and would deserve a more detailed study.

The NTR domain of NTRC being active in the reduction of the TRX domain, it has been proposed as a possible alternative plastidial TRX reducing system (Serrato et al., 2004). In the present work, we tested Arabidopsis NTRC with homologous TRX isoforms belonging to the five plastidial types. We found that, *in vitro*, NTRC cannot reduce efficiently any plastidial TRX. This confirms the results obtained by others (Serrato et al., 2004; Pérez-Ruiz et al., 2006; Chibani et al., 2011) testing heterologous proteins.

Our data show that FTR is the only reductase capable to reduce TRXf, m, x and y in plastids and reveal that even though TRXz is expressed mainly in green tissues, it is not reduced by any known plastidial TRX reduction system. Interestingly, we found that other plastidial TRX can reduce TRXz. This is thermodymically feasible as these TRX have a redox midpoint potential (E_m) less negative than the E_m of TRXz (Collin et al., 2003; 2004; Chibani et al., 2011). This is the first example, to our knowledge, of a TRX reducing another TRX. Overall, these biochemical data underscoring that TRXz stands out from the other plastidial isoforms are consistent with the severe albino phenotype of the Arabidopsis *trxz* knock-out mutant (Arsova et al., 2010; Meng et al., 2010) contrasting with the mutants of the other plastidial TRX that do not exhibit a visible phenotype under standard growth conditions (Laugier et al., 2012).

TRXz was previously identified in a proteomic study as a component of transcriptionally active chromosome in plastids (pTAC; Pfalz et al., 2006) and more recently as a subunit of a complex formed with the plastid-encoded RNA polymerase (PEP; Schröter et al., 2010). *trxz* mutant or silenced plants are affected in the expression of the chloroplast genome, particularly the genes transcribed by the PEP (Arsova et al., 2010; Schröter et al., 2010). This strongly suggests the implication of TRXz in the transcription of the plastome. TRXz interacts with several targets, including 2 Cys-PRX, FNR and two proteins with similarities to plastid fructokinase-like (FLN) proteins (Schröter et al., 2010) previously identified as a component of pTAC (Pfalz et al., 2006) but with no fructokinase activity (Arsova et al., 2010). It has been shown that TRXz acts as an adaptor protein between the receptor-like protein Cf-9 and the Ser/Thr kinase ACIK1 in the cytosol of tomato (Nekrasov et al., 2006). Recently, Schröter et al. (2010) proposed that TRXz could play a similar adaptor role as a subunit of the PEP complex for regulation of plastid gene expression. TRXz would be an additional key player of photosynthetic gene expression (Dietz and Pfannschmidt, 2011).

Overall, this points to a unique role of TRXz, which indeed possesses TRX activity. TRXz appears as an important sensor of the redox status of the chloroplast being potentially oxidized by 2-Cys PRX (sensing reactive oxygen species) and reduced by other TRX (sensing light indirectly) revealing its potential important role in the redox homeostasis of the chloroplast.

Supplementary material

Supplementary data are available at *JXB* online.

Table S1: List of specific primers used for RT-qPCR and experimental procedure.

Table S2: Summary of the different proteins used and purification procedures.

Figure S1: Anti-TRXz antibody specificity.

Figure S2: Expression patterns of plastidial thioredoxins and reductases from the « Arabidopsis eFP browser » website.

Figure S3: Reduction of H_2O_2 by 2-Cys PRX using NTRC as an electron donor.

Figure S4: Reduction of plastidial TRX by NTRs.

Figure S5: Reduction of TRXz by various reductants.

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Table 1

Table 1. Activation test of mutant NADP-MDH by TRXz.

Mutant NADP-MDH (0.9 µg) was incubated with 100 mM DTT and various concentrations of TRXz. Activation by TRXm1 was used as positive control. Activity was measured spectrophotometrically at 340 nm through the oxaloacetate-dependent disappearance of NADPH. Activation treatments were made at room temperature for 20 min in presence of DTT (100 mM) alone or with TRX (5 µM unless otherwise mentioned).

	Initial rates of reduction (µmoles oxidized NADPH .mg^{-1} NADP-MDH.sec^{-1})
DTT alone	0.71 ± 0.06
TRX m1	1.36 ± 0.09
TRX z	0.73 ± 0.07
100 µM TRX z	0.79 ± 0.10

Legends for figures

Figure 1. Expression of plastidial TRX and TRX reductases in Arabidopsis.

RT-qPCR analyses were performed on total RNA isolated from young seedlings, leaves, flowers, young and mature siliques, seeds and roots. Transcript levels are the result of three independent experiments and were normalized by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) using the PP2A gene as standard (Czechowski et al., 2005). A, expression patterns of plastidial TRX genes, trxf1 transcripts level in leaves used as calibrator. B, expression patterns of ntrc and ftr subunits genes, ntrc transcripts level in leaves used as calibrator.

Figure 2. Efficiency of TRXz in the reduction of 2-Cys PRX compared to other plastidial TRX.

The enzyme 2-Cys PRXA (20 μM) was incubated in the presence of 400 µM DTT, 600 µM H_2O_2 and 10 µM TRX. As a control, 2-Cys PRX was incubated without TRX (DTT). Data are the mean of 3 independent experiments. Error bars correspond to standard deviation.

Figure 3. Activation of NADP-MDH by plastidial TRXf, x and y in the light, in a Fd/TRX reconstituted system.

Mutant NADP-MDH was illuminated at 120 µE m^{-2} s^{-1} in the presence of freshly prepared thylakoids (equivalent to 20 µg chlorophyll), 5 µM ferredoxin, 2 µM FTR, and 25 µM TRXf1 (f1, black circles), TRXy1 (y1, black diamonds), TRXy2 (y2,

black triangles) or TRXx (x, black squares). TRXy2 was also tested in the dark (open triangles). Experiments were reproduced 3 times, a representative set of data is shown.

Figure 4. Redox and oligomerization state of TRXz.

TRXz was alkylated with ^{14}C-IAM after an oxidizing (DTTox 10 mM) or a reducing treatment (DTTred 100 mM). The samples were separated by non-reducing SDS-PAGE. Proteins and radiolabeling were respectively visualized by Coomassie blue staining and exposure of the dried gel to a low energy imaging screen.

Figure 5. Reduction test of TRXz by FTR in a reconstituted light-driven Fd/TRX system.

Experimental conditions were as described in legend of Fig. 3. As controls, oxidized TRXz (10 µM) was incubated with (lanes 2 and 3) or without (lane 1) 100 mM DTT. For the reduction test of TRXz by FTR, TRXz (25 µM) was incubated in the presence of thylakoids, 5 µM Fd and 10 µM FTR in the light. Aliquots were withdrawn periodically, the proteins were alkylated, subjected to non-reducing SDS-PAGE, electrotransferred onto nitrocellulose membrane and probed with anti *Strep*-tag antibody. Experiment was reproduced 4 times, a representative experiment is shown.

Figure 6. Reduction of TRXz by other plastidial TRX.

TRXz (10 µM) was incubated without (lane 1) or with DTT (1, 10 or 100 mM, lane 2, 7, 8 respectively) or in presence of 10 µM of another plastidial TRX (f1, m1: panel A; x, y1: panel B) without (lane 3, 5) or with DTT (1 mM, lane 4, 6), for 15 min at room temperature. Proteins were then alkylated and subjected to non-reducing SDS-PAGE, electrotransferred onto nitrocellulose membrane and probed with an anti-TRXz antibody. Experiments were reproduced four times, a representative experiment is shown.

Figure 1

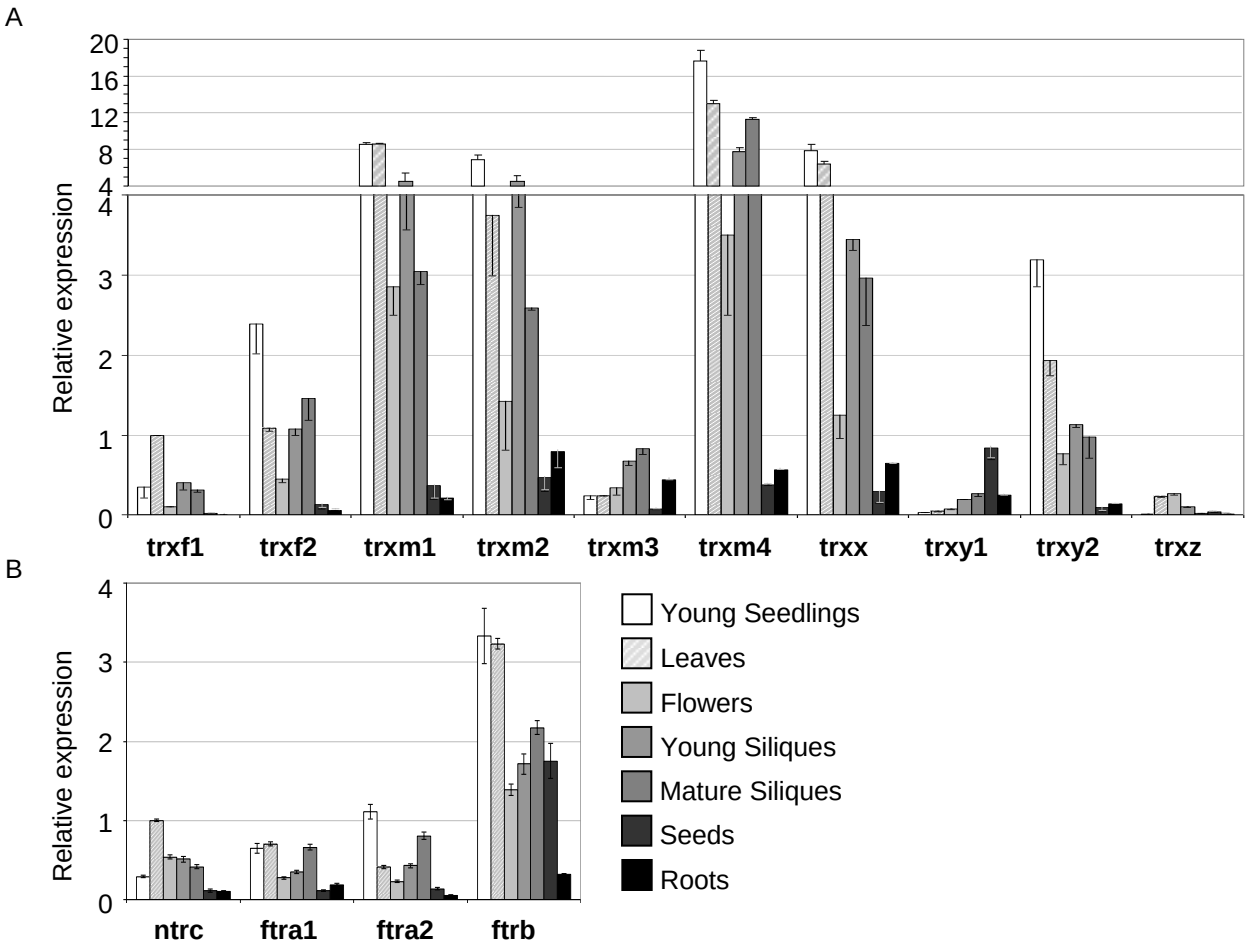

A
Relative expression
20
16
12
8
4
4
3
2
1
0
trxf1
trxf2
trxm1
trxm2
trxm3
trxm4
trxx
trxy1
trxy2
trxz
B
Relative expression
4
3
2
1
0
ntrc
ftra1
ftra2
ftrb
Young Seedlings
Leaves
Flowers
Young Siliques
Mature Siliques
Seeds
Roots

Figure 2

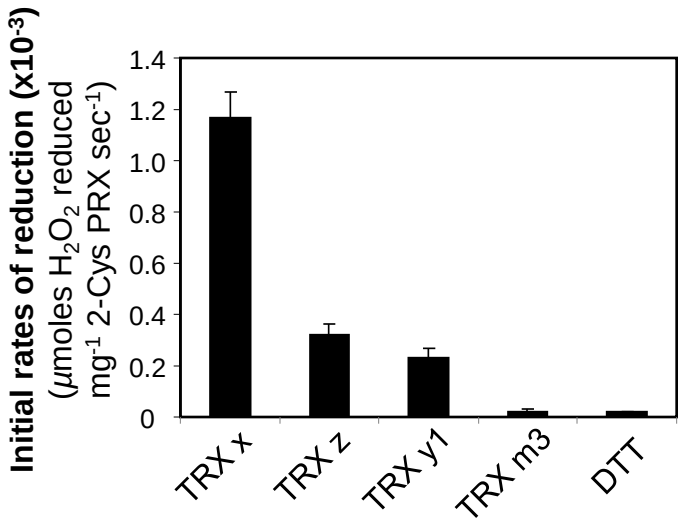

Initial rates of reduction (x10-3)
(µmoles H2O2 reduced
mg-1 2-Cys PRX sec-1)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
TRX x
TRX z
TRX y1
TRX m3
DTT

Figure 3

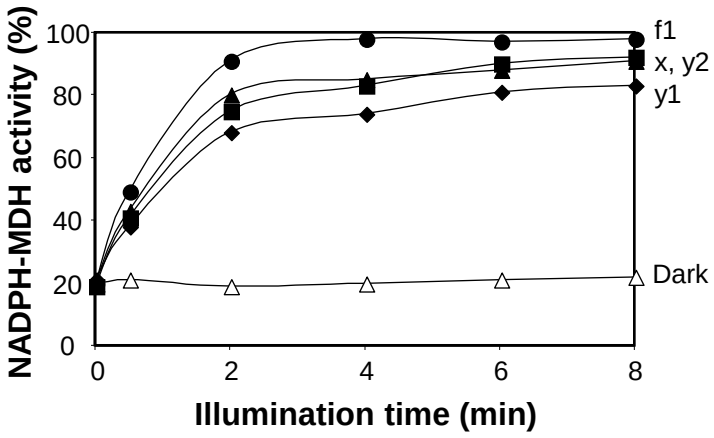

NADPH-MDH activity (%)
100
80
60
40
20
0
0
2
4
6
8
Illumination time (min)
f1
x, y2
y1
Dark

Figure 4

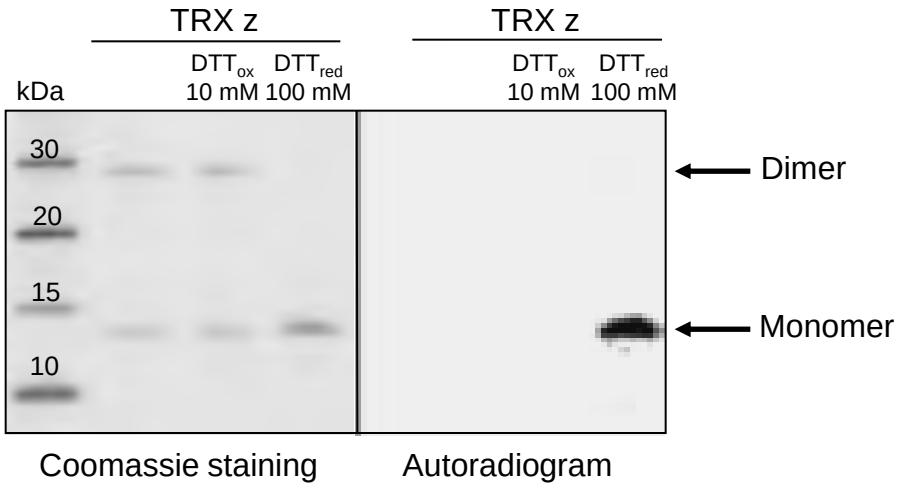

TRX z
TRX z
DTT$_{ox}$ 10 mM
DTT$_{red}$ 100 mM
DTT$_{ox}$ 10 mM
DTT$_{red}$ 100 mM
kDa
30
20
15
10
Dimer
Monomer
Coomassie staining
Autoradiogram

Figure 5

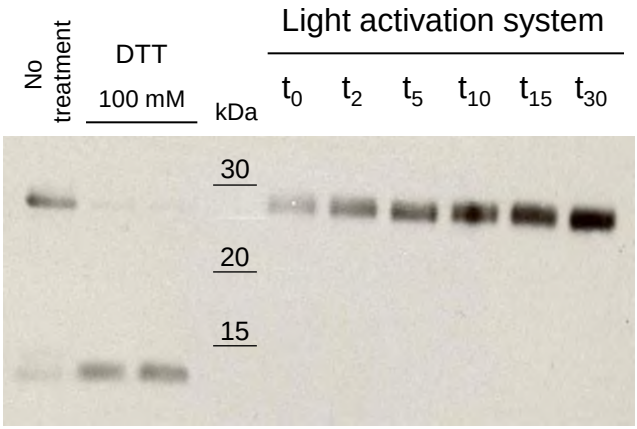
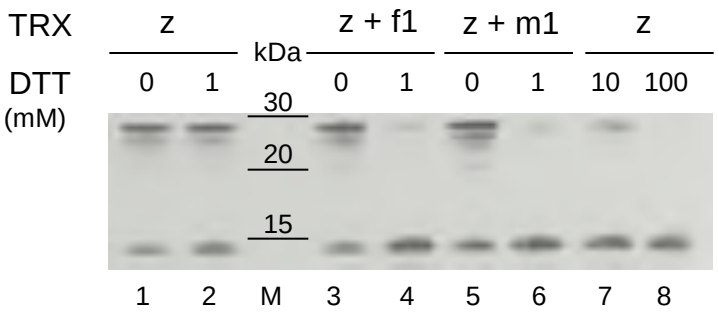
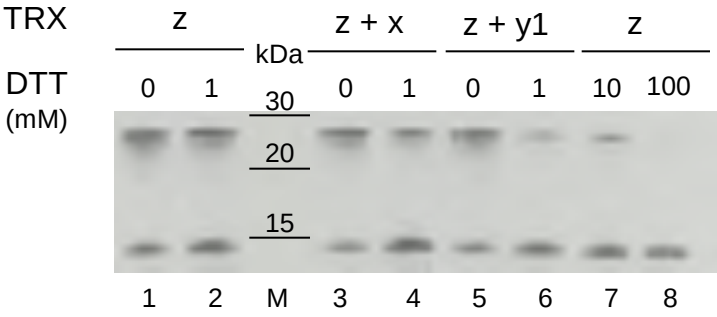

No treatment
DTT
100 mM
kDa
Light activation system
t_0
t_2
t_5
t_{10}
t_{15}
t_{30}
30
20
15

Figure 6

A


TRX
z
z + f1
z + m1
z
kDa
DTT
(mM)
0
1
0
1
0
1
10
100
30
20
15
1
2
M
3
4
5
6
7
8

B


TRX
z
z + x
z + y1
z
kDa
DTT
(mM)
0
1
0
1
0
1
10
100
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20
15
1
2
M
3
4
5
6
7
8

Figure S1

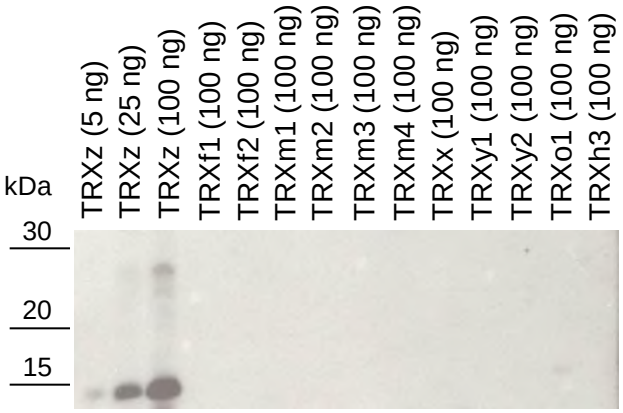
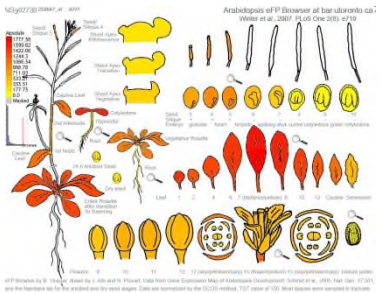


Figure S1. Anti-TRX z antibody specificity.
Anti TRX z antibody was tested against all the plastidial (f1, 2; m1, 2, 3, 4; x; y1, 2; z), a mitochondrial (o3) and a cytosolic (h3) TRX. After separation by SDS-PAGE, proteins were electrotransferred onto nitrocellulose membrane and probed with the anti-TRXz antibody.

Figure S2



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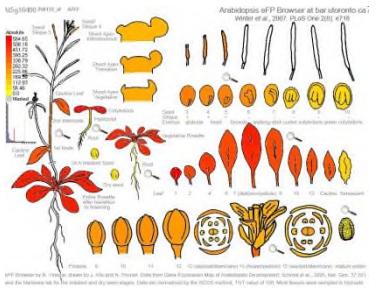

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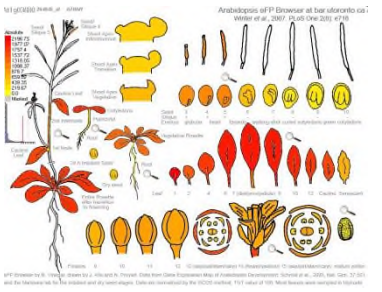

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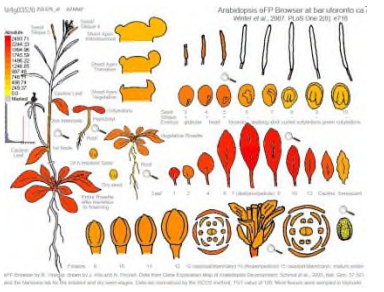

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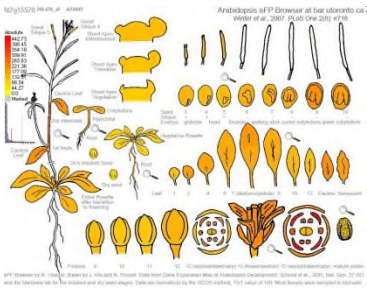

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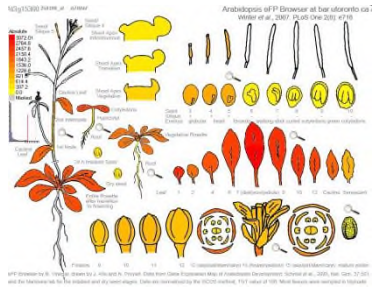

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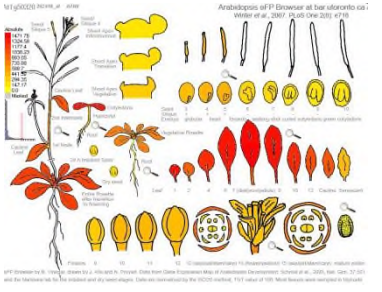

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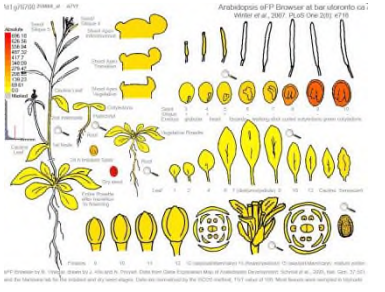

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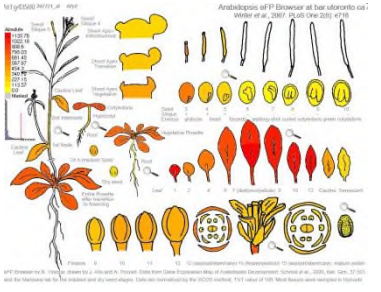

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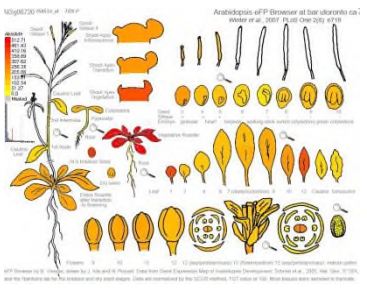

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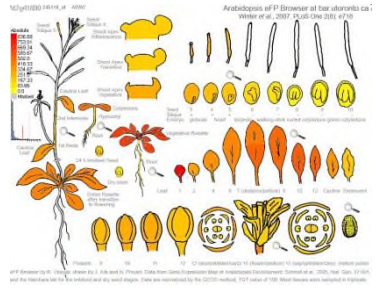

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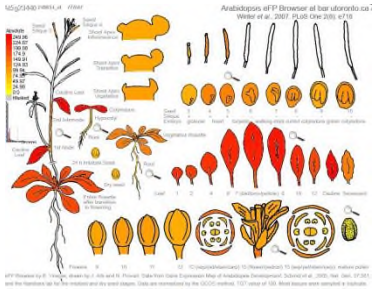

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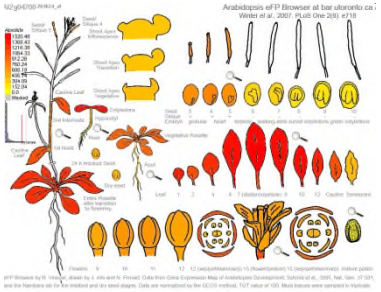

ftrb

Figure S2. Expression pattern of the trxf1, f2, m1, m2, m3, m4, x, y1, y2 and z and the ntrc, ftra1 and ftrb genes from *Arabidopsis thaliana*. Data were obtained from the « Arabidopsis eFP browser » (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi). ftra2 could not be analyzed due to the absence of the corresponding specific probe on Affymetrix Arabidopsis microarray.

Figure S3

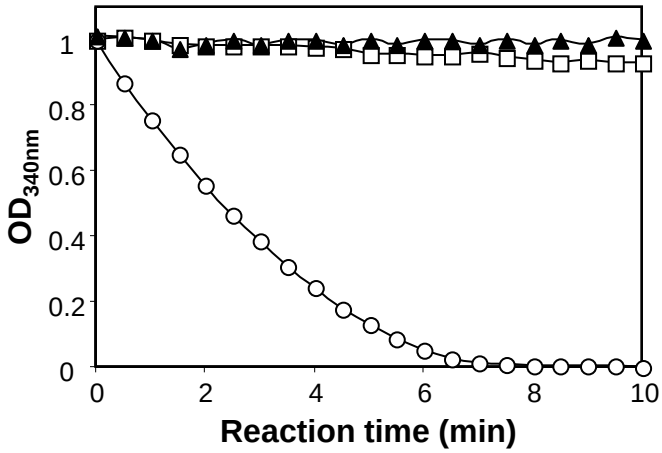

Figure S3. Reduction of hydrogen peroxide by 2-Cys PRX using NTRC as an electron donor.
Arabidopsis NTRC (200 nM) was incubated in presence of 150 µM NADPH and its capacity as an electron donor to 2-Cys PRX A (20 μM) was tested through its capacity to reduce H_2O_2 (1 mM). Reaction was spectrophotometrically monitored by the disappearance of the reduced cofactor at 340 nm. Data were normalized to the initial OD. Open squares: NTRC alone, open circles: NTRC plus 10 µM 2-Cys PRX, black triangles: 2-Cys PRX without NTRC. Experiments were reproduced 3 times, a representative set of data is shown.

Figure S4

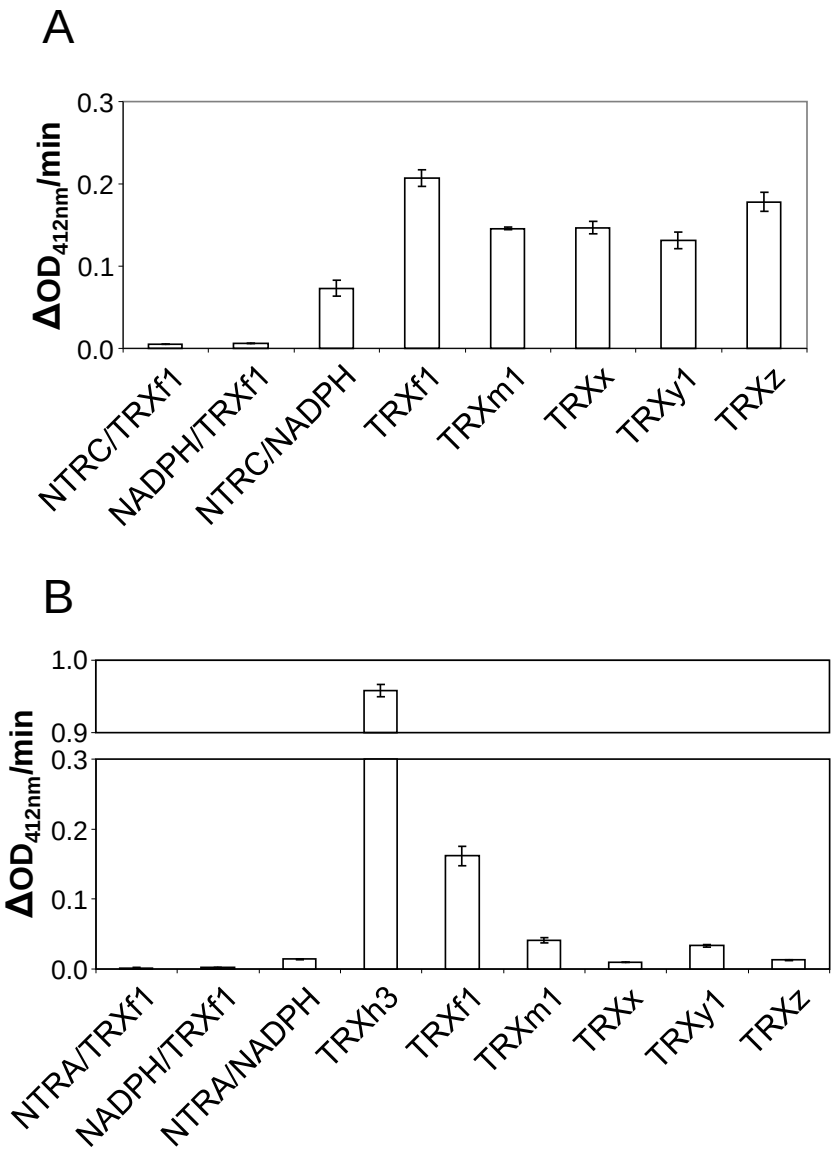

Figure S4. Reduction test of plastidial TRX by NADPH-dependent TRX reductases.
NTRC (20 nM) or NTRA (20 nM) was incubated in the presence of 150 µM NADPH and 1 mM DTNB with 10 µM TRX. The reaction of DTNB with free thiols was followed spectrophotometrically at 412 nm through the appearance of TNB^- upon time. As controls, we tested TRXf1 in the absence of NADPH and NTRC or NTRA; the basal activity of NTRC or NTRA was measured in presence of NADPH without TRX. Data are the mean of 3 independent experiments. Error bars correspond to standard deviation.

Figure S5

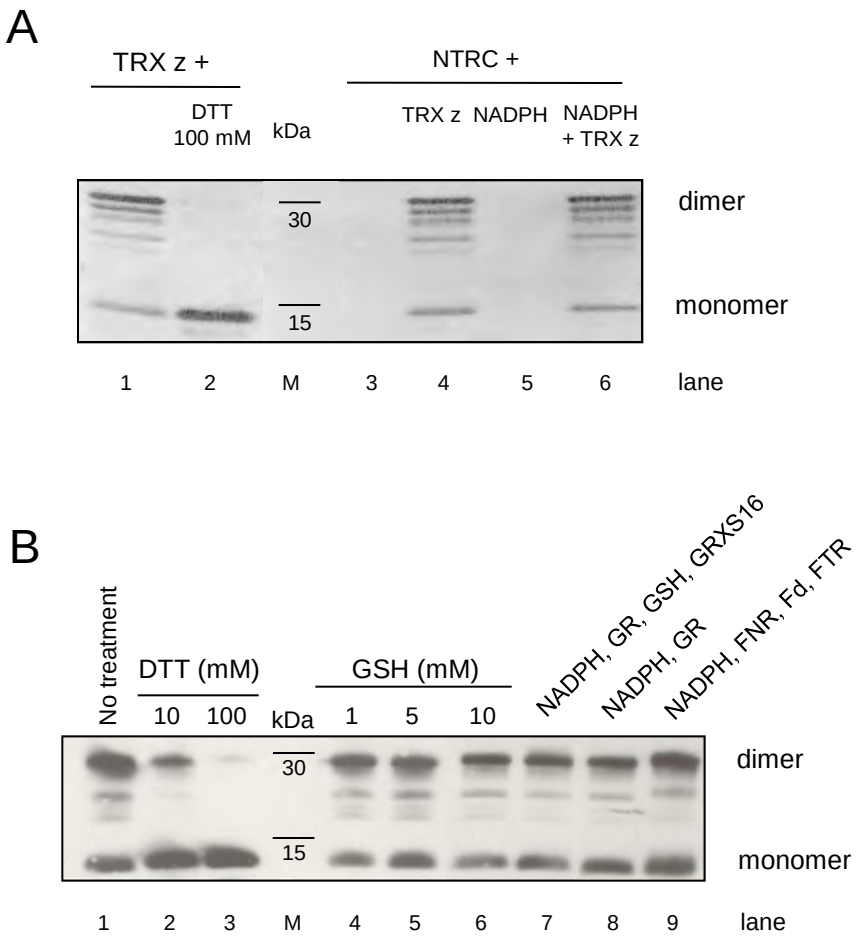

Figure S5. Reduction of TRXz by NTRC, GSH, GRX, GR or FTR studied by redox western.
A- Reduction by NTRC: As a control for reduction, oxidized TRXz (10 µM; lane 1) was fully reduced with 100 mM DTT (lane 2). Reduction of TRXz by NTRC was tested in the absence (lane 4) or the presence (lane 6) of NADPH.
B- Reduction by GSH, GRX, GR or FTR: As a control for reduction, oxidized TRXz (10 µM; lane 1) was incubated with 10 or 100 mM DTT (lanes 2 and 3). Reduction of TRXz was tested either with reduced glutathione (GSH) (1, 5 or 10 mM; lanes 4 to 6) or 300 µM NADPH, 6 µg/mL bakers yeast glutathione reductase (GR), 5 mM GSH and 10 µM of chloroplastic glutaredoxin GRXS16 (lane 7) or 300 µM NADPH and 6 µg/mL GR (lane 8) or 20 µM NADPH, 40 nM FNR, 1 µM Fd and 1 µM FTR (lane 9).
After a 15 min treatment at room temperature, proteins were alkylated and subjected to non reducing SDS-PAGE, electrotransferred onto nitrocellulose membrane and probed either with the anti-TRXz specific antibody (panel A) or with the anti Strep-tag antibody (panel B). The reduction of TRXz was followed through its monomerization. Experiments were reproduced 3 times, a representative experiment is shown.

New insights into the reduction systems of plastidial thioredoxins point out the unique properties of thioredoxin z from Arabidopsis[1]

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ABSTRACT

In plants, thioredoxins (TRX) constitute a large protein disulfide oxido-reductase family comprising ten plastidial members in *Arabidopsis thaliana* (Arabidopsis) subdivided in five types. The f and m-types regulate enzymes involved mainly in carbon metabolism whereas the x, y and z types have an antioxidant function. The reduction of TRXm and f in chloroplasts is performed in the light by ferredoxin:thioredoxin reductase (FTR) that uses photosynthetically reduced ferredoxin (Fd) as a reductant. The reduction system of Arabidopsis TRXx, y and z has never been demonstrated. Recently, a gene encoding an atypical plastidial NADPH-dependent TRX reductase (NTRC) was found. In the present study, gene expression analysis revealed that both reductases are expressed in all organs of Arabidopsis and could potentially serve as electron donors to plastidial TRX. This ability was tested *in vitro* either with purified NTRC in presence of NADPH or with a light-driven reconstituted system comprising thylakoids and purified Fd and FTR. Our results demonstrate that FTR reduces the x and y TRX isoforms but not the recently identified TRXz. Moreover, we show that NTRC cannot be an efficient alternative reducing system neither for TRXz nor for the other plastidial TRX. Our data reveal that TRXf, m, x and y, known as redox regulators in the chloroplast, have also the ability to reduce, *in vitro,* TRXz. Overall, the present study points out the unique properties of TRXz among plastidial TRX.

Key words: Thioredoxin, FTR, NTRC, Redox signaling, Plastid, Arabidopsis

INTRODUCTION

Thioredoxins (TRX) belong to a large multigenic family of oxidoreductases found in all free living organisms. These small soluble proteins (12-14 kDa) share a characteristic fold, a conserved active site WCG/PPC sequence, and a low redox potential that confers on them strong reductive properties. The two cysteines of the active site are involved in thiol/disulfide exchanges with their target proteins.

The first plant thioredoxins (m and f-types) were identified as regulators of chloroplastic enzymes related to carbon metabolism (Buchanan, 1980). Genomic revolution revealed that plant TRX isoforms are encoded by multi-gene families (Meyer et al., 2005; Nuruzzaman et al., 2008). In *Arabidopsis thaliana* (Arabidopsis), twenty TRX isoforms are found, divided into three subgroups: cytosolic, mitochondrial and plastidial. Sequence similarities and intron positions revealed that plastidial isoforms comprised nine members in Arabidopsis, subdivided into four types, the f, m, x and y-types (Mestres-Ortega and Meyer, 1999; Meyer et al., 2005), with four isoforms in the m-type, 2 in the f-type, 2 in the y-type, 1 in the x-type. Recently, two independent studies identified a tenth plastidial thioredoxin, TRXz (Arsova et al., 2010; Meng et al., 2010).

This multiplicity of TRX isoforms raised the question of their redundancy or possible functional specialization. Complementation studies on a TRX-deficient yeast strain hypersensitive to oxidative stress showed that Arabidopsis TRXf, m and x displayed different efficiencies (Issakidis-Bourguet et al., 2001). Afterwards, biochemical *in vitro* studies revealed specificity of chloroplastic TRX towards their target enzymes. Indeed, TRXf and m redox regulate the activity of enzymes mainly involved in the primary metabolism, whereas the more recently identified isoforms x,

y and z can serve as hydrogen donors for antioxidant enzymes such as peroxiredoxins (PRX), suggesting their role in oxidative stress defence (Collin et al., 2003, 2004; Lamkemeyer et al., 2006; Née et al., 2009; Chibani et al., 2011). Moreover, recent proteomic studies revealed a multiplicity (nearly 100) of putative chloroplastic targets involved in a wide range of processes (Michelet et al., 2006; Lemaire et al., 2007; Viera Dos Santos et al., 2007; Valerio et al. 2010). New putative TRX targets were also found in isolated organelles from wheat endosperm (Balmer et al., 2006) and in Arabidopsis root extracts (Marchand et al., 2010).

Once oxidized, TRX can be re-reduced by specific reductases. In the light, the photosynthetic electron transfer chain reduces ferredoxin (Fd) which can donate electrons to various enzymes including Fd:TRX reductase (FTR). This key enzyme of the Fd/TRX regulatory system has been shown to reduce efficiently TRXm and f in a reconstituted light activation system (Droux et al., 1987). In the cytosol and mitochondria of plant cells, TRX are reduced by an NADPH-dependent TRX reductase (NTR) (Jacquot et al., 1994; Laloi et al., 2001; Reichheld et al., 2005). A new gene encoding an atypical plastidial NTR (named NTRC) was identified in Arabidopsis, rice and *Medicago truncatula* (Serrato et al., 2004; Pérez-Ruiz et al., 2006; Alkhalfioui et al., 2007). It encodes a bipartite protein containing an NTR domain fused to a TRX domain. NTRC was shown to be an efficient redox system for chloroplast protection against oxidative damage and proposed as a possible alternative plastidial TRX reducing system (Serrato et al., 2004; Pérez-Ruiz et al., 2006).

The recent finding of three new plastidial TRX types (x, y and z) as well as the presence of plastidial TRX isoforms in non-photosynthetic organs (Collin et al., 2004; Balmer et al., 2006; Traverso et al., 2008) raised the question of their reducing system(s). One can wonder whether NTRC can reduce plastidial TRX in non-

photosynthetic tissues, or be a reducing system for the x, y and z isoforms. A direct biochemical test can assess the ability of a given reductase to reduce a given TRX. However, the interaction is possible only if the two partners share the same spatial and temporal localization. Thus, in the present work, the expression patterns of the ten genes encoding the plastidial TRX from Arabidopsis together with the four genes encoding the plastidial TRX reductase subunits were first examined using quantitative RT-PCR. Gene expression profiles revealed that both FTR and NTRC could potentially serve as reductants for all TRX in plastids. This ability was tested directly *in vitro* using NTRC in presence of NADPH, or FTR in a light-driven reconstituted Fd/TRX system. Here we demonstrate that FTR can reduce TRXx and y but not TRXz. Moreover, we show that NTRC cannot be an alternative reducing system for plastidial TRX, but that reduced plastidial thioredoxins are able to reduce TRXz *in vitro*.

MATERIALS AND METHODS

Reagents

All biochemical reagents were purchased from Sigma-Aldrich, unless otherwise mentioned.

Plant Material

Arabidopsis thaliana wild-type (Columbia ecotype) were grown on soil in the greenhouse under natural growth conditions (20°C day/18°C night temperature, 60% day/55% night hygrometry, light intensity 80-100 μE m^{-2} s^{-1}) and watered every 2 days.

Peas (*Pisum sativum* var. merveille de Kelvedon) were grown in the greenhouse on vermiculite, watered daily and harvested 3 weeks after sowing.

RNA Isolation and cDNA Synthesis

Total RNA were isolated from 100 mg of frozen plant material (except for seeds: 30 mg) using the NucleoSpin® RNA Plant kit from Macherey-Nagel, according to manufacturer's instructions. For RNA extraction from dry seeds 5% (w/v) polyvinylpyrrolidone was added to the extraction buffer. The reverse transcription step was performed with up to 1 μg of total RNA, using the SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen) with oligo(dT) primer.

Quantitative RT-PCR Analysis

Gene expression profiling was conducted by real-time quantitative RT-PCR using gene-specific primer pairs and procedures detailed in Table S1.

Expression, Production and Purification of Recombinant Proteins

Proteins and related purification procedures are described in Table S2.

2-Cys PRX Activity Assays

PRX activity was assayed in 30 mM Tris-HCl buffer, pH 7.9, in the presence of 20 µM 2-Cys PRXA from Arabidopsis, 400 µM DTT, 600 µM H_2O_2 and 10 µM TRX. Remaining hydrogen peroxide was quantified colorimetrically at 560 nm using Peroxoquant reagent (Perbio Science). Alternatively, 2-Cys PRX activity was determined as oxidation of NADPH followed at 340 nm in a reaction mixture containing 100 mM phosphate buffer, pH 7.2, 10 mM EDTA, 1 mM H_2O_2, 10 µM PRX, 200 nM NTRC and 150 µM NADPH.

Radiolabeling of SH groups in TRX z

A sample of TRXz preparation (60µg) was alkylated with [^{14}C]iodoacetamide (IAM) (0.5 MBeq/µmol, 2 mM), without treatment, or after an oxidizing (trans-4,5-dihydroxy-1,2-dithiane (DTTox) 10 mM, 15 min) or a reducing treatment (dithiotreitol (DTTred) 100 mM, 15 min). After trichloroacetic acid precipitation and separation by SDS-PAGE (Droux et al., 1987), proteins were Coomassie blue stained

and labeling was visualized by exposure of the dried gel to a low energy imaging screen, for two weeks (personal molecular Imager FX system, BioRad).

Reduction of TRXx, y and z by the Fd/FTR System

The reconstituted light activation system comprised, in a total volume of 50 µL, washed thylakoids equivalent to 10-20 µg chlorophyll, prepared as described in Droux et al. (1987), 100 mM Tris-HCl, pH 7.9, 2-5 µM Fd, 10 µM FTR, 25 µM TRX and 5 µM subunit mutant NADP-MDH. Aliquots were withdrawn as a function of time of illumination (120 µE m^{-2} s^{-2}) and, for TRXx and y, tested for NADP-MDH activation level. For TRXz (NADP-MDH omitted) thiol redox status was estimated by redox western. Alternatively to the light-dependent system, 10 µM TRXz was incubated for 15 min in the presence of 20 µM NADPH, 40 nM FNR and 1 µM FTR and 1 µM Fd according to Chibani et al., (2011). Free thiol groups were alkylated for 25 min, with 25 mM N-ethyl maleimide (NEM) and 30 mM iodoacetic acid (IAA). Alkylated proteins were subjected to non-reducing SDS-PAGE and the reduction state of TRXz was evaluated from its monomer/dimer status by Western blot.

Chemical Activation of NADP-Malate Dehydrogenase

Δ15-NADP-MDH (shortened by 15 amino acids at the N-terminus) whose biochemical characteristics are identical to those of the full-length wild-type MDH (Johansson et al., 1999) and mutant C207A/C365A/C377A NADP-MDH were used. Enzymes (5 µM) were incubated at room temperature, with 25 µM TRX plus 1 mM DTT, or 5 or 100 µM TRXz plus 100 mM DTT or DTT alone. The enzymatic activity

was measured in 100 mM Tris-HCl, pH 7.9, 180 µM NADPH, 750 µM oxaloacetate. Disappearance of NADPH was followed spectrophotometrically at 340 nm.

Immunodetection of TRX z by Western Blot

Samples were separated by non-reducing 12% SDS-PAGE and transferred onto nitrocellulose membrane (iBlot Gel Transfer Device, Invitrogen). Detection was performed using either a commercial anti *Strep*-tag II monoclonal antibody conjugated to horseradish peroxidase (IBA) at a dilution of 1:10,000 or a primary specific anti-TRXz antibody at a dilution of 1:2,500 and a secondary horseradish peroxidase conjugated antibody at a dilution of 1:50,000, and revealed by enhanced chemiluminescence (ECL, GE Healthcare). The specificity of the anti-TRXz antibody was checked towards all the other plastidial TRX by Western blot (Figure S1).

Reduction of plastidial TRX by NTRC and NTRA tested spectrophotometrically

The ability of Arabidopsis cytosolic NTRA or plastidial NTRC to reduce TRX was measured by the 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB, Pierce) reduction assay. The reaction mixture (1 mL) contained 20 nM NTRC or NTRA, 150 µM NADPH, 1 mM DNTB and 10 μM TRX in 100 mM $NaPO_4$, pH 8.0 and 1 mM EDTA. All measurements were carried out at 25°C by monitoring the increase in absorbance at 412 nm caused by the release of thionitrobenzoate (TNB^-).

Reduction of TRXz by the GSH/GRX system, NTRC or plastidial TRX

TRXz was pre-oxidized by 10 mM DTTox during 20 min. DTTox was removed on a PD Spin Trap G-25 column (GE Healthcare). Oxidized TRXz (10 µM) was incubated for 20 min at room temperature with various reductants: either 1, 5 or 10 mM reduced glutathione (GSH) or 300 µM NADPH, 6 µg/mL bakers yeast glutathione reductase (GR), 5 mM GSH and 10 µM chloroplastic glutaredoxin GRXS16. NTRC (2 µM) was tested with 500 µM NADPH. Plastidial TRX (f1, m1, x or y1; 10 µM) were also tested in presence of 1 mM DTT. Redox state of TRXz was evaluated from its monomer/dimer status by redox western.

RESULTS

Transcript levels reveal that both NTRC and FTR are expressed along with plastidial TRX, including in non-photosynthetic organs

Using real-time quantitative PCR the presence of mRNAs encoding plastidial TRX was detected in all organs of Arabidopsis at different levels depending on the isoform (Figure 1A). Globally, genes encoding m-type TRX (except m3) and TRX-x were more abundantly expressed, especially in leaves and young seedlings. Genes coding for TRXf1 and f2 and TRXy2, though expressed at a lower level, showed similar expression profiles. The gene coding for TRXz was expressed at the lowest level and its expression was restricted to leaves, flowers and young siliques. Noteworthy, Attrxm3 and Attrxy1 expression patterns differed from those of other plastidial Arabidopsis trx genes, being predominantly expressed in non-photosynthetic tissues *e. g.* mature siliques, roots and dry seeds. As previously suggested (Collin et al., 2004) the gene encoding TRXy1 is the most strongly expressed in seeds among all plastidial Arabidopsis TRX. Overall, these results are consistent with transcriptomic data obtained from the "Arabidopsis eFP Browser" (Figure S2).

The expression levels of the genes encoding the variable subunits FTRA1 and FTRA2 of FTR (Keryer et al., 2004) were close to the one encoding NTRC (Figure 1B). The gene encoding the catalytic FTRB subunit was expressed in all organs tested including non-photosynthetic organs. FTRB transcripts were quite abundant as compared to FTRA1 and FTRA2. Expression profiles of both genes encoding FTR variable subunits were more strongly expressed in leaves, young seedlings and mature

siliques. Similarly, NTRC showed a wide expression profile with a higher level in green tissues.

These findings prompted us to test both FTR and NTRC in their ability to reduce plastidial TRX. TRXx and y have been characterized in our laboratory (Collin et al., 2003, 2004) and showed specific antioxidant functions. However, their reduction system was still unknown as well as the one of Arabidopsis TRXz.

TRXz is a functional oxidoreductase with target specificities

Among the well-known TRX-dependent chloroplastic enzymes, NADP-MDH is probably the most studied enzyme (Ruelland and Miginiac-Maslow, 1999). It is devoid of activity in its oxidized state and gets activated in presence of reduced TRXf or m, except m3 (Collin et al., 2003). We expressed TRXz in its mature form (N-terminal transit peptide removed) in *E. coli* as a *Strep-tag*ged protein. It failed to activate wild-type sorghum NADP-MDH in the presence of DTT (data not shown).

Activation of NADP-MDH involves the reduction of two regulatory disulfides located in sequence extensions at the N- and C-terminus (Issakidis et al., 1994). We engineered a mutant form of NADP-MDH where only the N-terminal disulfide remains (C207A/C365A/C377A mutant, Ruelland et al., 1997). This mutant has a basal activity and is more easily activated, due to a less negative redox potential. It is activated by plastidial TRX isoforms that do not activate the wild type enzyme, such as TRXx and y (Collin et al, 2003; 2004). Unlike TRXm1 used as a positive control, TRXz proved inefficient (even at 100 µM) towards mutant NADP-MDH, in the presence of a high (100 mM) DTTconcentration necessary to reach a complete reduction of this TRX. Thus, TRXz is unable to activate NADP-MDH (Table 1).

We also tested the capacity of TRXz to mediate proton donation to 2-Cys PRX by following the reduction of H_2O_2 by PRX in the presence of DTT. 2-Cys PRX is known to be reduced efficiently by TRXx, to a lesser extent by TRXy, and not by TRXm3 (Collin et al., 2003; 2004). Figure 2 shows that, indeed TRXx is the best reductant for 2-Cys PRX, Trxm3 being quite inefficient. TRXz was as efficient as TRXy1 validating that our preparation is redox active. Thus, unlike poplar TRXz (Chibani et al., 2011), Arabidopsis TRXz can donate electrons to homologous 2-Cys PRX.

FTR reduces efficiently TRXx and y but cannot reduce TRXz

FTR is the known reductant of plastidial TRXf and m but its capacity to reduce the more recently identified Arabidopsis TRX isoforms x, y and z was never studied. We tested the capacity of FTR to reduce plastidial Arabidopsis TRX in a reconstituted system comprising illuminated pea thylakoids, Arabidopsis ferredoxin 2 (Fd) and spinach FTR (Droux et al., 1987). In this system, TRXx, y1 and y2 were able to activate mutant NADP-MDH with efficiencies comparable to TRXf1 (Figure 3). In the dark, or when a component of the activation mixture was omitted, no NADP-MDH activation was observed (Figure 3 and data not shown). These data provide evidence that TRXx and y can be efficiently reduced by FTR. Since TRXz is not able to activate mutant NADP-MDH, its reduction was followed by redox western. Indeed, we observed that TRXz forms dimers when oxidized and gets monomerized upon reduction by DTT. We checked that monomerization was correlated with the appearance of free thiols on the protein using radiolabeling with the thiol-alkylating agent iodoacetamide (^{14}C-IAM). A strong and specific labeling was observed for the

monomer in its reduced form, most probably corresponding to the reduction of the active-site disulfide (Figure 4). As there was no labeling of the dimer, it should correspond to oxidized protein. This hypothesis is strengthened by the absence of labeling of the oxidized monomer, corresponding to the initial redox state of our TRXz purified preparation and after a treatment with DTTox (Figure 4). This observation allowed us to use the monomerization/dimerization behavior of the protein as a test of reduction/oxidation. We tested TRXz reduction by FTR in the reconstituted light system described above (Figure 5). Upon illumination, no monomerization of TRXz was observed. In the same experiment, the light-driven reduction of TRXf1 (evidenced by NADP-MDH activation) proved efficient (data not shown). Inability of FTR to reduce TRXz was confirmed using an *in vitro* reconstituted "dark" system consisting of NADPH, FNR, Fd and FTR (Figure S5B, lane 9). These results provide evidence that FTR can be the physiological reductase of plastidial TRXx and y, with comparable efficiencies to TRXf. Noteworthy; TRXz is the first plastidial TRX which is not reduced by their classical reductase.

NTRC is not an efficient alternative reducing system for plastidial TRX

Most plastidial TRX genes are expressed in green tissues but, as mentioned above, some isoforms, such as TRXy1, are predominantly present in non-photosynthetic tissues where the mRNA corresponding to NTRC could be detected. Moreover, as FTR cannot reduce TRXz, NTRC could possibly be its physiological substitute. We produced and purified the Arabidopsis recombinant NTRC. Its functionality was confirmed by its capacity to mediate an efficient degradation of H_2O_2 by 2-Cys PRXA (Figure S3). We then assayed the reduction of the five types of plastidial TRX by NTRC using DTNB as a final electron acceptor, as this is a useful tool for measuring TRX oxido-reductase capacity. At 20 nM, NTRC alone, probably due to its own TRX

domain, displays a TRX reductase activity with a k_{cat} value (s^{-1}) of 4.47 ± 0.59. This activity increases 2-3 fold in presence of plastidial TRX (Figure S4A). However, this reduction rate corresponds to the basal rate observed with the cytosolic NTRA for TRXf1 that is five-fold lower than the reduction rate of the cytosolic TRXh3 (Figure S4B). Moreover, when the ability of NTRC to reduce TRXz was tested by following its monomerization by redox western, no increase of the monomeric form at the expense of the dimers was observed (Figure S5A). The faint intermediate molecular weight bands, more abundant in aged preparations (data not shown), most probably correspond to the dimer's conformational variants as they are recognized by the anti-TRXz antibody and disappear upon reduction. We conclude that TRXz cannot be efficiently reduced by NTR.

TRXz is not reduced by the GSH/GRX system

Previous data have demonstrated that a subgroup of cytosolic TRX, which are not reduced by NTR, can be reduced by glutaredoxin (GRX) which is itself reduced by glutathione (GSH) (Gelhaye et al., 2003). In this system, known as the GSH/GRX system, the transmission of the redox signal is mediated from NADPH to GSH *via* glutathione reductase (GR). None of TRXf, m, x and y are reduced by GSH (unpublished results). However, Chibani et al. (2011) have recently found close values for the redox midpoint potentials (E_m) of TRXz and GSH (Noctor, 2006). The ability of TRXz to be reduced by GR, GSH and/or a plastidial GRX (GRXS16; Bandyopadhyay et al., 2008) was tested by following its reduction through its monomerization. No monomerization of TRXz occurred with any of these

components (Fig. S5B). Thus we conclude that none of the components of the GSH/GRX system can reduce TRXz *in vitro*.

Plastidial TRX can reduce TRXz

The E_m value for the catalytic disulfide of TRXz was found to be -311 mV at pH 7.9 (Chibani et al., 2011), which is less negative than the E_m values of the other plastidial TRX ranging from -365 to -335mV (Collin et al., 2003; 2004). Therefore, we investigated the ability of other plastidial TRX to reduce TRXz (Figure 6). While 1 mM DTT could not directly reduce TRXz, in presence of TRXf or m, the amount of monomeric TRXz was largely increased at the expense of the dimeric form (Figure 6A). In comparison, TRXx and y exhibited a significant, though lower, efficiency (Figure 6B). These data demonstrate that TRXz can be reduced by other plastidial TRX *in vitro*.

DISCUSSION

The main goal of the present work was to test *in vitro* the ability and specificity of plastidial reductases to reduce the various plastidial TRX. As a preliminary, we show, using quantitative RT-PCR, that the ten genes encoding plastidial TRX together with the three genes encoding FTR subunits and the gene encoding NTRC were expressed in the same organs, at the same time (Figure 1) and thus could be partners. These data will need to be further completed with a study at the protein level and gene expression analyses in different cell types. The detection of a messenger RNA suggesting the presence of the translated corresponding protein, these findings prompted us to test both FTR and NTRC in their capacities to reduce plastidial TRX and particularly the more recently identified TRXx, y and z isoforms from Arabidopsis.

FTR is the well-known reductase for TRXf and m isoforms, TRXx, y and z were found as efficient proton donors to peroxiredoxins (Collin et al., 2003, 2004; present work: Figure 2) but the upstream reduction of TRX by following PRX activity in a reconstituted Fd/FTR/TRX light activation system cannot be easily tested. Indeed, reactive oxygen species (ROS) production by illuminated thylakoids interferes with the activity test. However, the ability of FTR in reducing TRXx and y could be tested by following the activation of an engineered mutant NADP-MDH enzyme which can be reduced by these TRX isoforms (Figure 3). Our results demonstrate that TRXx and y are reduced by FTR with efficiencies comparable to those of the m and f-type isoforms (Droux et al., 1987). Thus, in the light, FTR is clearly the reducing system for plastidial TRXf, m, x and y in Arabidopsis. However, the recent finding of f and m-TRX in non-photosynthetic organs raised the question of their reducing system in heterotrophic plastids (Traverso et al., 2008). Moreover, the presence of a complete FTR system in amyloplasts was demonstrated (Balmer et al., 2006). In roots, FNR

reduces type III Fd which is more easily reduced than leaf isoforms, as it displays a less negative E_m (Hanke et al., 2004). This property favors the electron transfer from NADPH to FTR via FNR and Fd. The functionality of this system was already tested by König et al. (2002) for TRXf and m but still needs to be tested for other plastidial TRX isoforms, especially the ones expressed in non-photosynthetic tissue such as TRXm3 and y1.

The ability of FTR in reducing TRXz could be assessed in the reconstituted light system by following the reduction state of TRXz through its monomerization. Indeed, TRXz could not activate NADP-MDH, either wild-type or mutant, but the labeling experiment (Figure 4) showing that only the reduced monomer can be derivatized offered us a simple way to observe the reduction of this TRX. Our data show that FTR cannot reduce TRXz in the light (Figure 5) in contrast with all the other plastidial TRX. This finding is not consistent with the work of Chibani et al. (2011) where a heterologous NADPH-driven FTR reduction system was used. These authors assessed the reduction of poplar TRXz by a shift of migration during non-reducing SDS-PAGE following the alkylation with methoxyl-PEG maleimide. A shift of *ca.* 15 kDa, instead of 4 kDa as expected for the alkylation of two thiol groups, was obtained. This form could correspond to the dimer we observed in our experiments. A dimerization/monomerization upon oxidation/reduction was previously observed for TRXf which contains a third Cys residue responsible for this behavior (Michelet et al., 2005). In the mature Arabidopsis TRXz, no additional Cys residue is found, indicating that dimerization is linked to the specific properties of this isoform.

Noteworthy, in our light activation experiment, when TRXz was mixed with thylakoid membranes, no monomeric form was observed at all (Figure 5), even in the dark, while our TRXz preparations always appeared as a mixture of monomers and

dimers, even in the NADPH/FNR/FTR/Fd reconstituted system (Figure S4). Moreover, the amount of TRXz seemed to be lower in the dark (time 0) and to increase upon illumination (Figure 5). Therefore, TRXz might interact (directly or indirectly) with thylakoid membranes in the dark and could be released in the light. Such a phenomenon was recently reported for FNR (Benz et al., 2010) and would deserve a more detailed study.

The NTR domain of NTRC being active in the reduction of the TRX domain, it has been proposed as a possible alternative plastidial TRX reducing system (Serrato et al., 2004). In the present work, we tested Arabidopsis NTRC with homologous TRX isoforms belonging to the five plastidial types. We found that, *in vitro*, NTRC cannot reduce efficiently any plastidial TRX. This confirms the results obtained by others (Serrato et al., 2004; Pérez-Ruiz et al., 2006; Chibani et al., 2011) testing heterologous proteins.

Our data show that FTR is the only reductase capable to reduce TRXf, m, x and y in plastids and reveal that even though TRXz is expressed mainly in green tissues, it is not reduced by any known plastidial TRX reduction system. Interestingly, we found that other plastidial TRX can reduce TRXz. This is thermodymically feasible as these TRX have a redox midpoint potential (E_m) less negative than the E_m of TRXz (Collin et al., 2003; 2004; Chibani et al., 2011). This is the first example, to our knowledge, of a TRX reducing another TRX. Overall, these biochemical data underscoring that TRXz stands out from the other plastidial isoforms are consistent with the severe albino phenotype of the Arabidopsis *trxz* knock-out mutant (Arsova et al., 2010; Meng et al., 2010) contrasting with the mutants of the other plastidial TRX that do not exhibit a visible phenotype under standard growth conditions (Laugier et al., 2012).

TRXz was previously identified in a proteomic study as a component of transcriptionally active chromosome in plastids (pTAC; Pfalz et al., 2006) and more recently as a subunit of a complex formed with the plastid-encoded RNA polymerase (PEP; Schröter et al., 2010). *trxz* mutant or silenced plants are affected in the expression of the chloroplast genome, particularly the genes transcribed by the PEP (Arsova et al., 2010; Schröter et al., 2010). This strongly suggests the implication of TRXz in the transcription of the plastome. TRXz interacts with several targets, including 2 Cys-PRX, FNR and two proteins with similarities to plastid fructokinase-like (FLN) proteins (Schröter et al., 2010) previously identified as a component of pTAC (Pfalz et al., 2006) but with no fructokinase activity (Arsova et al., 2010). It has been shown that TRXz acts as an adaptor protein between the receptor-like protein Cf-9 and the Ser/Thr kinase ACIK1 in the cytosol of tomato (Nekrasov et al., 2006). Recently, Schröter et al. (2010) proposed that TRXz could play a similar adaptor role as a subunit of the PEP complex for regulation of plastid gene expression. TRXz would be an additional key player of photosynthetic gene expression (Dietz and Pfannschmidt, 2011).

Overall, this points to a unique role of TRXz, which indeed possesses TRX activity. TRXz appears as an important sensor of the redox status of the chloroplast being potentially oxidized by 2-Cys PRX (sensing reactive oxygen species) and reduced by other TRX (sensing light indirectly) revealing its potential important role in the redox homeostasis of the chloroplast.

Supplementary material

Supplementary data are available at *JXB* online.

Table S1: List of specific primers used for RT-qPCR and experimental procedure.

Table S2: Summary of the different proteins used and purification procedures.

Figure S1: Anti-TRXz antibody specificity.

Figure S2: Expression patterns of plastidial thioredoxins and reductases from the « Arabidopsis eFP browser » website.

Figure S3: Reduction of H_2O_2 by 2-Cys PRX using NTRC as an electron donor.

Figure S4: Reduction of plastidial TRX by NTRs.

Figure S5: Reduction of TRXz by various reductants.

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Table 1

Table 1. Activation test of mutant NADP-MDH by TRXz.

Mutant NADP-MDH (0.9 µg) was incubated with 100 mM DTT and various concentrations of TRXz. Activation by TRXm1 was used as positive control. Activity was measured spectrophotometrically at 340 nm through the oxaloacetate-dependent disappearance of NADPH. Activation treatments were made at room temperature for 20 min in presence of DTT (100 mM) alone or with TRX (5 µM unless otherwise mentioned).

	Initial rates of reduction (µmoles oxidized NADPH .mg^{-1} NADP-MDH.sec^{-1})
DTT alone	0.71 ± 0.06
TRX m1	1.36 ± 0.09
TRX z	0.73 ± 0.07
100 µM TRX z	0.79 ± 0.10

Legends for figures

Figure 1. Expression of plastidial TRX and TRX reductases in Arabidopsis.

RT-qPCR analyses were performed on total RNA isolated from young seedlings, leaves, flowers, young and mature siliques, seeds and roots. Transcript levels are the result of three independent experiments and were normalized by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) using the PP2A gene as standard (Czechowski et al., 2005). A, expression patterns of plastidial TRX genes, trxf1 transcripts level in leaves used as calibrator. B, expression patterns of ntrc and ftr subunits genes, ntrc transcripts level in leaves used as calibrator.

Figure 2. Efficiency of TRXz in the reduction of 2-Cys PRX compared to other plastidial TRX.

The enzyme 2-Cys PRXA (20 μM) was incubated in the presence of 400 µM DTT, 600 µM H_2O_2 and 10 µM TRX. As a control, 2-Cys PRX was incubated without TRX (DTT). Data are the mean of 3 independent experiments. Error bars correspond to standard deviation.

Figure 3. Activation of NADP-MDH by plastidial TRXf, x and y in the light, in a Fd/TRX reconstituted system.

Mutant NADP-MDH was illuminated at 120 µE m^{-2} s^{-1} in the presence of freshly prepared thylakoids (equivalent to 20 µg chlorophyll), 5 µM ferredoxin, 2 µM FTR, and 25 µM TRXf1 (f1, black circles), TRXy1 (y1, black diamonds), TRXy2 (y2,

black triangles) or TRXx (x, black squares). TRXy2 was also tested in the dark (open triangles). Experiments were reproduced 3 times, a representative set of data is shown.

Figure 4. Redox and oligomerization state of TRXz.

TRXz was alkylated with ^{14}C-IAM after an oxidizing (DTTox 10 mM) or a reducing treatment (DTTred 100 mM). The samples were separated by non-reducing SDS-PAGE. Proteins and radiolabeling were respectively visualized by Coomassie blue staining and exposure of the dried gel to a low energy imaging screen.

Figure 5. Reduction test of TRXz by FTR in a reconstituted light-driven Fd/TRX system.

Experimental conditions were as described in legend of Fig. 3. As controls, oxidized TRXz (10 µM) was incubated with (lanes 2 and 3) or without (lane 1) 100 mM DTT. For the reduction test of TRXz by FTR, TRXz (25 µM) was incubated in the presence of thylakoids, 5 µM Fd and 10 µM FTR in the light. Aliquots were withdrawn periodically, the proteins were alkylated, subjected to non-reducing SDS-PAGE, electrotransferred onto nitrocellulose membrane and probed with anti *Strep*-tag antibody. Experiment was reproduced 4 times, a representative experiment is shown.

Figure 6. Reduction of TRXz by other plastidial TRX.

TRXz (10 µM) was incubated without (lane 1) or with DTT (1, 10 or 100 mM, lane 2, 7, 8 respectively) or in presence of 10 µM of another plastidial TRX (f1, m1: panel A; x, y1: panel B) without (lane 3, 5) or with DTT (1 mM, lane 4, 6), for 15 min at room temperature. Proteins were then alkylated and subjected to non-reducing SDS-PAGE, electrotransferred onto nitrocellulose membrane and probed with an anti-TRXz antibody. Experiments were reproduced four times, a representative experiment is shown.