Identification and characterization of a mitochondrial thioredoxin system in plants

Christophe Laloi*, Naganand Rayapuram†, Yvette Chartier*, Jean-Michel Grienenberger†, Géraldine Bonnard†, and Yves Meyer*‡

*Laboratoire Génome et Développement des Plantes, Unité Mixte de Recherche Centre National de la Recherche Scientifique 5096, Université de Perpignan, 52 Avenue de Villeneuve, F-66860 Perpignan, France; and †Institut de Biologie Moléculaire des Plantes du Centre National de la Recherche Scientifique, Université Louis Pasteur, 12 Rue du Général Zimmer, F-67084 Strasbourg, France

Edited by Bob B. Buchanan, University of California, Berkeley, CA, and approved September 20, 2001 (received for review July 5, 2001)

Plants possess two well described thioredoxin systems: a cytoplasmic system including several thioredoxins and an NADPH-dependent thioredoxin reductase and a specific chloroplastic system characterized by a ferredoxin-dependent thioredoxin reductase. On the basis of biochemical activities, plants also are supposed to have a mitochondrial thioredoxin system as described in yeast and mammals, but no gene encoding plant mitochondrial thioredoxin or thioredoxin reductase has been identified yet. We report the characterization of a plant thioredoxin system located in mitochondria. *Arabidopsis thaliana* genome sequencing has revealed numerous thioredoxin genes among which we have identified *AtTRX-o1*, a gene encoding a thioredoxin with a potential mitochondrial transit peptide. *AtTRX-o1* and a second gene, *AtTRX-o2*, define, on the basis of the sequence and intron positions, a new thioredoxin type up to now specific to plants. We also have characterized *AtNTRA*, a gene encoding a protein highly similar to the previously described cytosolic NADPH-dependent thioredoxin reductase AtNTRB but with a putative presequence for import into mitochondria. Western blot analysis of *A. thaliana* subcellular and submitochondrial fractions and *in vitro* import experiments show that AtTRX-o1 and AtNTRA are targeted to the mitochondrial matrix through their cleavable N-terminal signal. The two proteins truncated to the estimated mature forms were produced in *Escherichia coli*; AtTRX-o1 efficiently reduces insulin in the presence of DTT and is reduced efficiently by AtNTRA and NADPH. Therefore, the thioredoxin and the NADPH-dependent thioredoxin reductase described here are proposed to constitute a functional plant mitochondrial thioredoxin system.

Thiol:disulphide oxidoreductases constitute a large protein family characterized by the typical active site CXXC that permits the oxidation, reduction, or isomerization of the disulphide bond of target proteins. Among this family, thioredoxins, with the WCG/PPC motif, present a low redox potential that confers reductive properties. In its reduced state, thioredoxin is able to reduce the disulphide bridges of numerous target proteins. Subsequently, the oxidized thioredoxin is reduced by the flavoenzyme thioredoxin reductase.

Since the discovery of the first *Escherichia coli* thioredoxin, acting as a potent hydrogen donor for ribonucleotide reductase (1), thioredoxins have been described in all prokaryotes and eukaryotes including fungi, invertebrates, vertebrates, and plants. The cytosolic thioredoxin system, a thioredoxin and a homodimeric NADPH-dependent thioredoxin reductase, has been shown to be involved in numerous regulation mechanisms such as the reduction of peroxiredoxins, the activity of some transcription factors, or the signaling of apoptosis (for a recent review see ref. 2). Plants are distinguishable from other organisms by their very complex thioredoxin systems as revealed by sequencing of the *Arabidopsis thaliana* genome. Indeed, at least 20 thioredoxin genes, among which eight cytosolic thioredoxins *h*, have been identified (3). In addition, plants also are characterized by a complex chloroplastic thioredoxin system including different thioredoxin types, thioredoxins *m*, *f*, and *x*, that are reduced by a heterodimeric ferredoxin-dependent thioredoxin reductase. Recently, a complete mitochondrial thioredoxin system has been identified both in the yeast *Saccharomyces cerevisiae* (4) and in mammals (5, 6). In contrast, no mitochondrial thioredoxin system has been described yet in plants, although thioredoxin and thioredoxin reductase activities have been reported in plant mitochondrial extracts (7, 8). As in yeast and mammals, this enzyme system could be involved in protection against oxidative stress.

In this paper we report the identification and characterization of a complete mitochondrial thioredoxin system in *A. thaliana* including a thioredoxin and a thioredoxin reductase. The mitochondrial thioredoxin defines a previously uncharacterized type of thioredoxin by its sequence and the position and the number of introns in the gene, whereas the reductase is highly similar to the previously characterized cytosolic NADPH-dependent thioredoxin reductase (9). Both mitochondrial thioredoxin and thioredoxin reductase genes come from duplication events. We discuss the role of this plant thioredoxin system in mitochondria, knowing that the activities of several major mitochondrial proteins are controlled by disulphide reduction.

Materials and Methods

Isolation of the *A. thaliana* Thioredoxins *AtTRX-o1*, *AtTRX-o2*, and Thioredoxin Reductase *AtNTRA* Genes and cDNAs. On the basis of known genomic and expressed sequence tag (EST) sequences, we designed primers to isolate each gene by PCR on *A. thaliana* (ecotype Columbia) genomic DNA and its corresponding cDNAs by reverse transcription (RT)-PCR from flower bud mRNAs. The PCR products were cloned into the pGEM-T Easy Vector system I (Promega) and sequenced. The identification of transcription initiation sites was determined by the RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) method. RLM-RACE was achieved from flower bud total RNAs as recommended by the provider (Ambion, Austin, TX) with some modifications. RT was achieved with oligo(dT) primers and MMLV reverse transcriptase by using the ProSTAR First Strand RT-PCR kit (Stratagene). For the determination of the 5′ extremity of the large *AtNTRA* transcript, cDNA synthesis was carried out at 55°C with gene-specific primers using the SUPERSCRIPT RT-PCR system (Invitrogen). Nested PCRs were achieved with the provided adapter-specific primers in combination with gene-specific primers. Primer combinations

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: EST, expressed sequence tag; RT, reverse transcription; BAC, bacterial artificial chromosome.

Data deposition: The sequence reported in this paper has been deposited in the GenBank (accession no. AF396650) and GenPept (accession no. AAK83918) databases.

‡To whom reprint requests should be addressed. E-mail: ymeyer@univ-perp.fr.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

can be found in Table 1, which is published as supporting information on the PNAS web site, www.pnas.org.

Expression and Purification of Recombinant Proteins. Partial cDNAs were cloned into the *Nde*I/*Bam*HI sites of the pET16b expression vector (Novagen) to produce AtTRX-o1(Glu-83–Lys-194), AtTRX-o2(Asp-47–Lys-159), and AtNTRA(Glu-55–Asp-383) His-tagged recombinant proteins. *E. coli* BL21 strains were cotransformed with the different constructs and pSBET plasmid, and recombinant protein expression was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37°C. His-tagged recombinant proteins were purified on Ni^{2+} columns as described (10) and then washed with 0.1 M potassium phosphate (pH 7.0) by ultrafiltration on Microcon columns (Amicon–Millipore).

Subcellular Fractionation of *A. thaliana* Protoplasts. *A. thaliana* protoplasts were prepared from 3–4-day-old suspension cell cultures as described (11). Typically, protoplasts obtained from a 500-ml culture were resuspended in 50 ml of extraction buffer (400 mM sucrose, 50 mM Tris·HCl, pH 7.5, 3 mM EDTA, 0.1% BSA, and 2 mM DTT) and disrupted by filtrations through nylon meshes. Cell debris were eliminated by centrifugation for 5 min at 100 $\times$ *g*, and the supernatant was recentrifuged for 10 min at 2,000 $\times$ *g*. The chloroplast-enriched pellet was collected and purified as described (11), and the supernatant was centrifuged at 16,000 $\times$ *g* for 15 min to pellet mitochondria. The supernatant (cytosol) was stored for further analysis. The mitochondrial pellets were purified on 18–23-40% Percoll step gradients. The mitochondria were collected at the 23–40% interface and washed in the extraction buffer without BSA and DTT. All fractions were frozen at −80°C before SDS/PAGE.

Mitoplast Preparation and Submitochondrial Fractionation. Mitoplasts were purified as described (12) with slight modifications. Crude mitochondria obtained as described above were resuspended in the swelling buffer to a final protein concentration of 4 mg/ml. The suspension then was loaded on a 15–32-60% sucrose step gradient and spun for 60 min at 33,000 rpm in a TST41 swing rotor (Kontron, Zurich). Mitoplasts were collected at the 32/60% interface. The membrane and soluble fractions of mitoplasts were recovered in a 100,000 $\times$ *g* pellet and supernatant, respectively, as described (12).

Western Blot and Immunodetection. Proteins were separated by SDS/PAGE and transferred to Immobilon-P membranes (Amersham Pharmacia). Western blots with rabbit polyclonal antibodies against AtTRX-o1 and AtNTRB were performed at a dilution of 1:5,000 and 1:10,000, respectively. Goat anti-rabbit antibodies conjugated to horseradish peroxidase (Amersham Pharmacia) were used as secondary antibodies and revealed with enhanced chemiluminescence reagents (Amersham Pharmacia). Antibodies directed against spinach large subunit of ribulose bisphosphate carboxylase (a gift from B. Camara, Institut de Biologie Moléculaire des Plantes-Centre National de la Recherche Scientifique, Strasbourg, France) and *A. thaliana* thioredoxin *h*3 (AtTRX-h3) were used as control for the chloroplast and cytosolic fractions, respectively. Antibodies directed against tobacco manganese superoxide dismutase (ref. 13; obtained from F. van Breusegem, Gent, Belgium) and wheat subunit 9 of NADH dehydrogenase (14) were used as control matrix and inner membrane protein fractions of mitochondria, respectively.

Import of Radiolabeled Proteins into Isolated Mitochondria. Mitochondria were isolated from potato tubers (*Solanum tuberosum* var. Bintje) with a juice extractor as described (15). Proteins were synthesized from the corresponding cDNA clones in pGEM-T vector by coupled transcription/translation in the presence of [^{35}S]methionine according the supplier's instructions (Promega). Import assays were carried out as described (16).

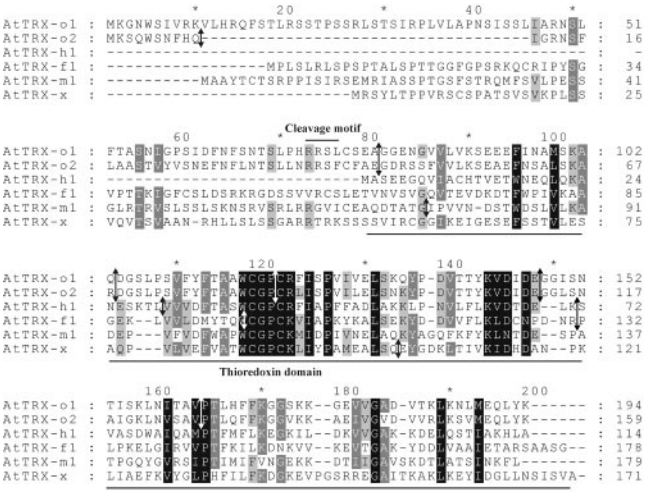


Fig. 1. Multiple sequence alignment of *A. thaliana* AtTRX-o1 (AAC12840) and AtTRX-o2 (AF396650) with different types of *A. thaliana* thioredoxins. The CLUSTALW software (npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html) was used to align AtTRX-h1 (P29448), AtTRX-f1 (Q9XFH8), AtTRX-m1 (O48737), and AtTRX-x (AAF15952). The arrows delimit protein regions encoded by different exons.

Enzymatic Activities. Thioredoxin activity was determined by using the insulin-disulphide reduction assay as described (17). Briefly, recombinant thioredoxin was used at a final concentration of 1, 2.5, 5, or 10 μM in 1 ml of 0.1 M potassium phosphate (pH 7.0), 2 mM EDTA, 0.13 mM bovine insulin, and in first experiments 0.33 mM DTT as reductant. The same buffer without DTT was used as reference. Thioredoxin activity was measured at 20°C and defined as the maximal increase rate of turbidity at OD at 650 nm due to insulin precipitation. The activity of AtTRX-o1 and AtTRX-o2 with thioredoxin reductases and NADPH (0.5 mM) as reductant was assayed with low concentrations of thioredoxin reductase (50 nM), allowing obtention of saturation Michaelis–Menten kinetics and determination of their affinities for the different thioredoxins. Protein concentration was determined by using the Bio-Rad protein assay kit with BSA as standard.

Results

Identification of a Gene Encoding a Thioredoxin with a Putative Mitochondrial Transit Peptide in *A. thaliana*. Looking for thioredoxin sequences in the *A. thaliana* database has led us to identify one gene encoding a thioredoxin with a putative mitochondrial target signal that we have named *AtTRX-o1*. Two partial EST sequences are found in the database (GenBank accession numbers F14287 and F14419). We have isolated a cDNA encoding the complete coding region corresponding to the annotation of the *A. thaliana* genome (GenPept accession number AAC12840). The number and positions of the five introns in the thioredoxin domain differ from all other plant thioredoxin genes that have at most two introns (18) and allow us to define *AtTRX-o1* as a member of a new thioredoxin class. A second *o* gene was found in *A. thaliana* genome, which we named *AtTRX-o2*. A cDNA obtained by RT-PCR showed that the *AtTRX-o2* gene possesses an additional intron in the 5′ coding sequence when compared with *AtTRX-o1* (Fig. 1). Consequently, the N-terminal region of the encoded protein does not have the characteristics of a mitochondrial targeting peptide. The deduced amino acids sequence (GenPept accession number

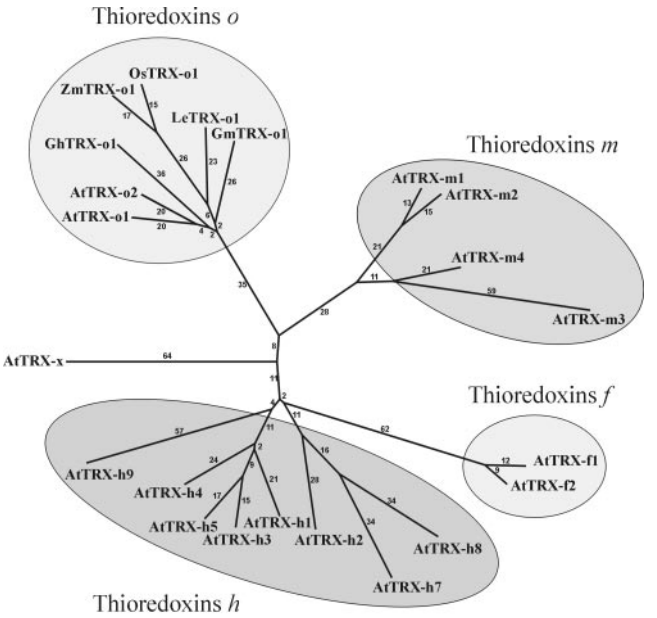


Fig. 2. Phylogenetic tree of *A. thaliana* thioredoxins and plant thioredoxin *o* homologues. The DARWIN software was use to generate the phylogenetic tree of the following thioredoxins. *A. thaliana* thioredoxins *h*: AtTRX-h1 (P29448), AtTRX-h2 (S58123), AtTRX-h3 (S58118), AtTRX-h4 (S58119), AtTRX-h5 (S58120), AtTRX-h7 (AAD39316), AtTRX-h8 (AAG52561), and AtTRX-h9 (AAG51342). *A. thaliana* thioredoxins *f*: AtTRX-f1 (Q9XFH8) and AtTRX-f2 (Q9XFH9). *A. thaliana* thioredoxins *m*: AtTRX-m1 (O48737), AtTRX-m2 (AAF15949), AtTRX-m3 (AAF15950), and AtTRX-m4 (Q9SEU6). *A. thaliana* thioredoxin *x*: AtTRX-x (AAF15952). Plant thioredoxins *o* are translations of ESTs contigs: *G. hirsutum* GhTRX-o1 (GenBank ESTs AI725806 and AI729485); *G. max* GmTRX-o1 (AW423678, AW234806, BE210498, and AI941321); *L. esculentum* LeTRX-o1 (BE462179, AW037483, AW037392, and BE433326); *Z. mays* ZmTRX-o1 (BE345397, AI668273, BE519105, AI737410, and BE050128); *O. sativa* OsTRX-o1 (C27892 and AU100897).

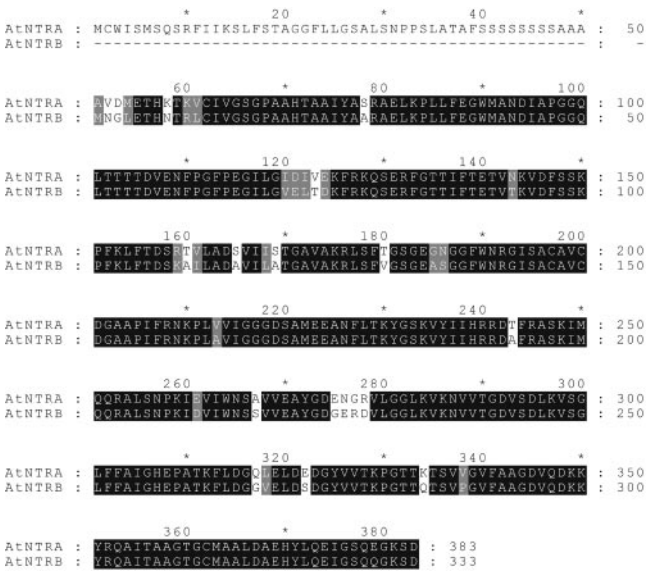


Fig. 3. Multiple sequence alignment of *A. thaliana* AtNTRA (AAB86519) and AtNTRB (CAB54874). Deduced amino acid sequences were aligned with the CLUSTALW program. Identical residues are indicated by black boxes, and similar residues are indicated by gray boxes.

AAK83918) does not correspond to annotation of the *A. thaliana* genome (GenPept accession number AAF98203) because of an incorrect prediction of 3′ splice site of the second intron. *AtTRX-o1* and *AtTRX-o2* genes are found on bacterial artificial chromosome (BAC) clone F19I3 on chromosome II and BAC clone F17F8 on chromosome I of *A. thaliana*, respectively. These two BAC clones belong to two duplicated regions described previously (19). RNA ligase-mediated rapid amplification of cDNA ends experiments have shown that both *AtTRX-o1* and *AtTRX-o2* genes present a unique transcription initiation site producing a 5′-untranslated region of 102 and 129 nucleotides, respectively (data not shown).

Searches in EST databases have revealed 22 ESTs from plants showing high similarity to *AtTRX-o* genes. No similar sequence has been found in other organisms. We could reconstitute cDNAs covering the coding region for *Lycopersicon esculentum*, *Glycine max*, and *Gossypium hirsutum*. Only partial proteins could be deduced from EST sequences for *Zea mays*, *Oryza sativa*, *Saccharum bicolor*, and for *Saccharum sp.* Two different cDNAs are found in both *G. max* and *Triticum aestivum*. This analysis suggests that these thioredoxin genes belong to a small multigenic family present only in plants. We have constructed a phylogenic tree by using the program DARWIN (20) with most *A. thaliana* thioredoxins and some plant thioredoxin sequences homologous to AtTRX-o. It confirms that thioredoxins *o* (*o* for organelle) constitute a new type of plant thioredoxins (Fig. 2).

Identification of a Gene Encoding an NADPH-Dependent Thioredoxin Reductase with a Putative Mitochondrial Transit Peptide. The isolation and characterization of an *A. thaliana* cDNA clone called *ATTHIREDB* and renamed *AtNTRB* (GenBank accession number Z23109), encoding a cytosolic NADPH-dependent thioredoxin reductase, have been reported previously (9). A similar cDNA clone (GenBank accession number Z23108) containing only a partial ORF of 1,148 nucleotides was described. In the *A. thaliana* genome, we have identified the corresponding gene that we renamed *AtNTRA*. Both *AtNTRA* and *AtNTRB* genes possess a unique large intron (726 and 395 nucleotides long, respectively). *AtNTRA* encodes a protein with an N-terminal extension of 50 amino acids when compared with AtNTRB (Fig. 3). In the conserved region, the two proteins share 89% identity. As for *AtTRX-o* genes, *AtNTRA* and *AtNTRB* genes are located on two duplicated regions; *AtNTRA* is found on BAC clone F5J6 on chromosome II, and *AtNTRB* is found on BAC clone F15J1 on chromosome IV. By rapid amplification of cDNA ends experiments two 5′ transcript extremities were found for *AtNTRA*. One is situated only 5 nucleotides upstream of the first ATG and corresponds to ESTs found in the database. The second 5′ extremity is located 114 nucleotides upstream of the ATG.

Both AtTRX-o1 and AtNTRA N-Terminal Domains Present Features of Presequences of Nuclear-Encoded Plant Mitochondrial Proteins. The two N-terminal domains of AtTRX-o1 and AtNTRA are characteristic of mitochondrial targeting sequences (21); they have a high content of basic and hydroxylated residues but few acidic and aromatic residues, and their first 20 amino acids can form an amphiphilic α-helix. Remarkably, serine content is particularly high, a distinct feature of plant presequences (22). Prediction of protein localization by the different programs PSORT (23), PREDOTAR 0.5 (www.inra.fr/Internet/Produits/Predotar/), and TARGETP (24) gives a high probability of mitochondrial localization for both proteins. The three thioredoxins *o* deduced from ESTs from *L. esculentum*, *G. max*, and *G. hirsutum* also present an N-terminal extension and are predicted to be targeted to the mitochondria (Table 2, which is published as supporting information on the PNAS web site). In contrast, because of the additional intron in the 5′-coding region corresponding to the AtTRX-o1 amphiphilic α-helix, cellular localization predictions for AtTRX-o2 remain unclear.

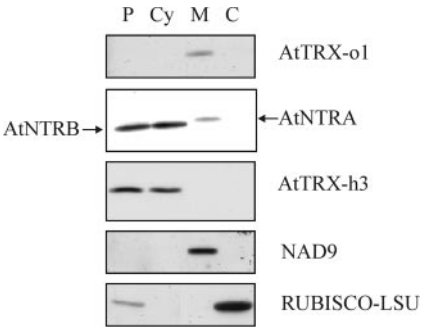


Fig. 4. Subcellular localization of AtTRX-o1 and AtNTRA in *A. thaliana* protoplasts. Western blot analyses of *A. thaliana* total protoplast (P), cytosol (Cy), mitochondrial (M), and chloroplast (C) fractions probed with antibodies directed against AtTRX-o1, AtNTRB, AtTRX-h3, NAD9, and the large subunit of the ribulose 1,5-bisphosphate carboxylase (RUBISCO-LSU) are shown.

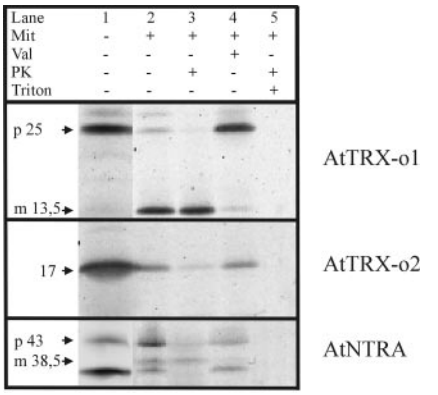


Fig. 5. *In vitro* protein import into isolated mitochondria. AtTRX-o1, AtTRX-o2 and AtNTRA were translated *in vitro* (lane 1) and incubated for 30 min at 25°C with potato mitochondria (lanes 2–5). Mitochondria were treated with valinomycin before import (lane 4) or with Triton X-100 (lane 5) and/or submitted to proteinase K digestion after import (lanes 3 and 5). Migration of the precursor (p) and mature (m) polypeptides is indicated.

Subcellular Localization of AtTRX-o1 and AtNTRA in *A. thaliana*. Antibodies directed against AtTRX-o1(Glu-83–Lys-194) and AtNTRB proteins were used to analyze subcellular fractions of *A. thaliana* protoplasts. The anti-AtTRX-o1 antibody recognizes AtTRX-o1 and AtTRX-o2 recombinant proteins with similar efficiency (data not shown). Anti-AtTRX-o1 antibodies recognize a single 13.5-kDa band present in the mitochondrial fraction but neither in cytosolic nor in chloroplastic fractions prepared from *A. thaliana* protoplasts (Fig. 4). The absence of signal in these fractions demonstrates that anti-AtTRX-o antibodies do not cross react with cytosolic or chloroplastic thioredoxins and are specific for AtTRX-o proteins in *A. thaliana* protoplasts. Conversely, anti-AtTRX-h3 antibodies only recognize a cytosolic protein of a similar apparent molecular mass.

Anti-AtNTRB antibodies recognize both AtNTRA and AtNTRB recombinant proteins (data not shown). Anti-AtNTRB antibodies reveal a 35-kDa band in total protoplast and cytosolic extracts (Fig. 4, lanes 1–2). In the mitochondrial fraction, anti-AtNTRB antibodies react with a 38-kDa protein. No signal was obtained in the chloroplast fraction. The antibodies allow the identification of two thioredoxin reductase proteins, a cytosolic enzyme of 35 kDa, and a mitochondrial one of 38 kDa apparent molecular masses.

The relative amount of cytosolic versus mitochondrial proteins in the total protoplast extract do not allow the detection of mitochondrial proteins as shown for NAD9, a subunit of NADH dehydrogenase. This is not the case with a very abundant chloroplastic protein such as the large subunit of the ribulose bisphosphate carboxylase, which can be detected in total protoplast extract. No contamination could be detected between the different protoplast fractions with the antibodies used (Fig. 4).

AtTRX-o1 and AtNTRA Precursors Are Imported into Mitochondria Through N-Terminal Cleavable Targeting Sequences. Because the antibodies used cannot discriminate between AtTRX-o1 and AtTRX-o2, the origin of the signal observed on Western blots was not assessed. Therefore we tried to import the two radiolabeled proteins *in vitro* into mitochondria. When translated *in vitro*, *AtTRX-o1* and *AtTRX-o2* products are expressed as proteins of 25- and 17-kDa apparent molecular masses, respectively (Fig. 5). After incubation of AtTRX-o1 precursor with mitochondria, a 13.5-kDa band appeared, whereas no processed band is detected in the case of AtTRX-o2. The unprocessed AtTRX-o2 is bound to mitochondria, but the protein is not resistant to proteinase K, indicating that AtTRX-o2 is not imported into mitochondria. Conversely, the 13.5-kDa protein is resistant to proteinase K, and the protection is abolished when mitochondrial membranes are solubilized with Triton before proteinase K treatment. AtTRX-o1 import is inhibited when the electrochemical membrane potential is abolished in the presence of the ionophore valinomycin. The mature AtTRX-o1 protein obtained after *in vitro* import corresponds to the 13.5-kDa protein immunodetected in *A. thaliana* mitochondria. Because mature AtTRX-o1 and AtTRX-h3 have the same apparent molecular masses, a maturation at an "R-2" cleavage motif RRSL present at position 73–76 could be proposed.

Similarly, we tested whether *AtNTRA* encodes a precursor processed after import. Two major proteins of 43 and 36.5 kDa were obtained by *in vitro* coupled transcription/translation of an *AtNTRA* cDNA corresponding to the short RNA form (Fig. 5). Both proteins can be immunoprecipitated by using anti-AtNTRB antibodies (data not shown) and most likely correspond to translation from the first and third ATG. The smaller protein migrates at the same position as the cytosolic protein immunodetected in *A. thaliana*. After incubation of the translation products with mitochondria, a signal corresponding to a 38.5-kDa protein resistant to proteinase K appeared. The protection is abolished when the mitochondria are treated with Triton. The *in vitro* processed protein migrates at the same position as the endogenous *A. thaliana* mitochondrial protein detected by anti-AtNTRB antibodies. Most of the precursor protein and the totality of the 36.5-kDa protein are degraded by proteinase K treatment. AtNTRA import depends on the membrane potential. The 4.5-kDa shift between the precursor and the mature AtNTRA proteins suggests a cleavage site in the serine stretch (Ser-40–48).

AtTRX-o1 and AtNTRA Are Located in the Mitochondrial Matrix. To determine the location of AtTRX-o1 and AtNTRA in mitochondria we prepared mitoplasts and separated the proteins into soluble and membrane fractions. Both AtTRX-o1 and AtNTRA are found in the soluble fraction (Fig. 6). The fact that no salt was used during the procedure indicates that the proteins are truly soluble proteins from the matrix and not proteins bound to either side of the inner mitochondrial membrane.

Activity of AtTRX-o1, AtTRX-o2, and AtNTRA *in Vitro*. AtTRX-o1(Glu-83–Lys-194), AtTRX-o2(Asp-47–Lys-159), and AtNTRA(Glu-55–Asp-383) recombinant proteins were produced in *E. coli*. Both AtTRX-o1 and AtTRX-o2 are able to reduce the insulin-disulfide bridges with DTT as reducing power (data not shown). The AtTRX-o1 precursor protein does not show a greater reducing activity, suggesting that there is no

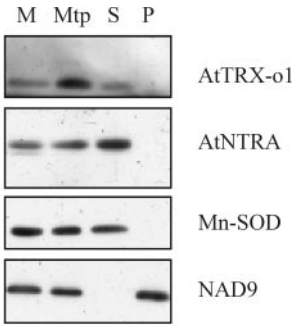


Fig. 6. Submitochondrial localization of AtTRX-o1 and AtNTRA. Western blot analysis of *A. thaliana* total mitochondria (M) and mitoplast (Mtp) fractions probed with anti-AtTRX-o1 and anti-AtNTRB sera is shown. Broken mitoplasts were centrifuged at 100,000 × *g*, giving rise to pellet (P) and supernatant (S) fractions. Western blots of the same protein fractions probed with antibodies against wheat NAD9 and tobacco Mn-SOD are shown on the bottom.

determinant for enzymatic activity in the N-terminal region deleted in the recombinant proteins (data not shown). We also have analyzed the activity of the thioredoxin system constituted by the AtTRX-o1 protein and the AtNTRA thioredoxin reductase in the insulin reduction assay. AtTRX-o1 and AtNTRA efficiently reduce insulin disulfide in an NADPH-dependent process *in vitro*, demonstrating that these two proteins can form an active thioredoxin system (Fig. 7*A*). In addition, we have evaluated the activity of AtTRX-o1 in association with AtNTRB and compared it to thioredoxin systems with AtTRX-o2 and cytosolic AtTRX-h3 (Fig. 7 *A* and *B*). All systems reduced insulin nearly as efficiently except that AtNTRB presents a slightly higher K_m for AtTRX-h3, showing that neither AtNTRA nor AtNTRB have a significantly greater affinity for AtTRX-o1 or AtTRX-o2 (Fig. 7*C*).

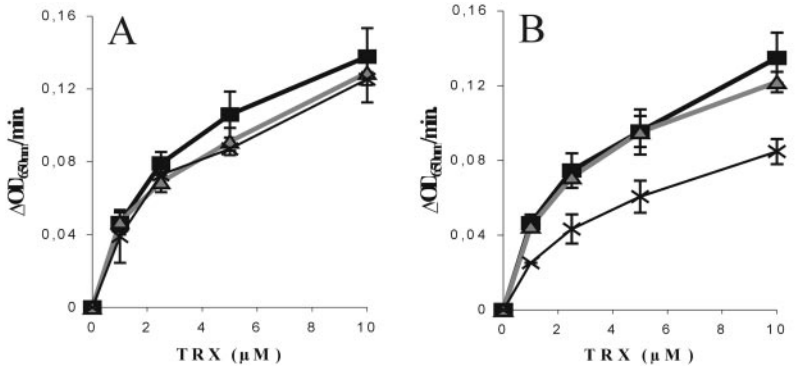
Discussion

Previous work has reported the presence of thioredoxin and thioredoxin reductase activities in plant mitochondrial extracts, but the proteins involved were not characterized (7, 8, 25). In this paper, we describe the identification of two genes, *AtTRX-o1* and *AtNTRA*, respectively encoding a previously uncharacterized type of thioredoxin and a thioredoxin reductase, both with typical N-terminal mitochondrial target signals. *In vitro* import experiments have confirmed that the proteins are targeted to mitochondria and cleaved after import. By Western blot analysis, we have shown that AtTRX-o1 and AtNTRA are located into the mitochondrial matrix but neither in the cytosol nor in the chloroplast of *A. thaliana* cells. Because of similar densities in gradients, mitochondrial preparations may be contaminated by peroxisomes (26). However, under the osmolarity conditions used to prepare the mitoplasts, peroxisomes burst, and their matrix proteins are released (27). Thus, peroxisomal contamination of purified mitoplast is highly unlikely. Nevertheless, the possibility of a dual targeting to both mitochondria and peroxisome, as has been described recently in animals (28–30), remains possible. AtTRX-o1(Glu-83–Lys-194) recombinant protein associated with AtNTRA(Glu-55–Asp-383) reduces insulin *in vitro* in an NADPH-dependent reaction. Thus, AtNTRA and AtTRX-o1 constitute a mitochondrial thioredoxin system functionally characterized in plants.

During this study we discovered that the gene *AtNTRA* is expressed from two transcripts, the smaller one being more abundant (as estimated by the number of ESTs found). Because *AtNTRB* is expressed poorly, we propose that in addition to the mitochondrial precursor protein, a cytosolic protein could be translated from the third AUG codon of the small and more abundant *AtNTRA* mRNA. Such multiple transcription start sites within thioredoxin reductase genes have been reported recently in animals (31). In plants, the presence of double transcription initiation sites has been described for genes encoding cytosolic and mitochondrial aminoacyl-tRNA synthetases (32, 33), and the alternative use of two in-frame initiation codons was described for dual mitochondrial and cytosolic proteins (34).

It has been reported recently that almost all the *A. thaliana* genome results from duplication events (19, 35). Interestingly, *AtTRX-o1* and *AtNTRA* are present in chromosome parts that resulted each from duplication events that were estimated to happen 100 million years ago, i.e., after the divergence of monocots and dicots. *AtTRX-o2*, the paralogue of *AtTRX-o1*, is still present in the *A. thaliana* genome. It encodes a protein with a thioredoxin activity, but an intron in the presequence destroys the ability to drive the protein into the mitochondria. Because all homologous thioredoxin *o* cDNAs, from both monocots and dicots, encode a protein with a potential mitochondrial transit peptide, we assume that the additional intron of *AtTRX-o2* appeared after the duplication event.

The complexity of plant thioredoxin systems raises the question of the cellular localization of thioredoxins and thioredoxin reductases. The analysis of the sequence of the *A. thaliana* genome reveals only one gene showing some similarity to NADPH-dependent thioredoxin reductase, in addition to *AtNTRA* and *AtNTRB*; it encodes a protein without a presequence, and its location remains to be determined. On the contrary, numerous thioredoxin homologues present an N-terminal extension when compared with cytosolic thioredoxins *h*. ESTs encoding homologues of *A. thaliana* mitochondrial thioredoxin *o* are found in all the plants investigated. Most of



C

K_m (µM)	AtTRX-o1	AtTRX-o2	AtTRX-h3
AtNTRA	2.6 ± 0.6	1.8 ± 0.7	2.8 ± 1.3
AtNTRB	2.2 ± 0.1	2.1 ± 0.3	3.0 ± 1.2

Fig. 7. Activity of different *A. thaliana* thioredoxin systems determined by the insulin-disulfide reduction assay. (*A*) AtNTRA and (*B*) AtNTRB: ■, AtTRX-o1; ▲, AtTRX-o2; X, AtTRX-h3. (*C*) K_m values of AtNTRA and AtNTRB for the different recombinant thioredoxins at pH 7.0 and 20°C.

them present a typical mitochondrial presequence. The four thioredoxins *m* are predicted to be chloroplastic (36), which corresponds with the biochemical characterization of at least one member of the family in pea and spinach chloroplasts. The newly identified thioredoxins *ch2* are also probably chloroplastic (3). The predictions are less clear for thioredoxin *x* and for one of the two thioredoxins *f*, although one member of the family has been isolated from pea and spinach chloroplasts. Cellular localization predictions also are complicated by proteins that are dually targeted to mitochondria and chloroplast or mitochondria and cytosol (37, 38) and proteins without N-terminal targeting signal that are addressed to mitochondria (39). Consequently, even in *A. thaliana*, the genome of which has been sequenced fully, the cellular localization of thioredoxins will require additional experimental work. The possibility remains for additional mitochondrial thioredoxins (e.g., thioredoxin *h*) in *A. thaliana*, in particular thioredoxins located in the intermembrane space. Indeed, cytochrome *c* maturation in plant mitochondria is proposed to involve a thiol:disulfide oxidoreductase located in the intermembrane space (40). As its counterpart in Gram-negative bacteria, this enzyme would be part of a cascade leading to the reduction of the cysteines of apocytochrome heme binding motif before heme covalent linkage (41). Thus the existence of such a pathway specific to plant mitochondria (when compared with fungi or animal) could suggest the presence of several thioredoxins in plant mitochondria.

During aerobic respiration in mitochondria, an incomplete reduction of dioxygen may lead to an oxidative stress characterized by the accumulation of reactive oxygen species. Recent work has identified specific peroxidases both in mammals and yeast mitochondria, which are reactive oxygen species scavenging enzymes and the activities of which are thioredoxin-dependent (4). Both yeast Δ*prx1* and Δ*trr2* mutants, respectively disrupted for *PRX1* encoding a mitochondrial thioredoxin-dependent peroxidase and *TRR2* encoding the mitochondrial thioredoxin reductase, are more sensitive to H_2O_2. We have reported recently that the *A. thaliana* cytosolic thioredoxin *h* AtTRX-h3 is able to interact *in vivo* with the yeast thioredoxin-dependent peroxidase YLR109 (10). By a search in databases, we identified four *A. thaliana* TPx homologues of YLR109 (C. Bréhélin and Y.M., unpublished results). One of these proteins presents an N-terminal extension that shows the characteristics of a mitochondrial targeting sequence. Therefore, as in yeast, the plant mitochondrial thioredoxin system could also contribute to the defense against oxidative stress as the electron donor of a mitochondrial plant TPx.

In addition, thioredoxin could be involved also in a plant-specific redox control of mitochondrial enzyme activities. Activation through the reduction of a disulfide bond has been proposed for higher plant citrate synthases in contrast to the redox-insensitive animal enzyme (42) and is now well documented for the plant alternative oxidase (AOX) (43). As for cytochrome *c* oxidase, AOX catalyzes the reduction of dioxygen to water. Plant AOX is a homodimeric protein localized in the inner mitochondrial membrane and possesses a single cysteine residue exposed to the mitochondrial matrix, which serves as a regulatory sulfhydryl/disulfide site (44). A function of AtTRX-o1, which we have shown to be a matrix protein, could be to reduce the oxidized AOX to the more active form and thereby limit reactive oxygen species production (41).

We thank Dr. Richard Cooke (Perpignan) for reading the manuscript. N.R. was supported by CEFIPRA Grant 2003-4.

1. Laurent, T. C., Moore, E. C. & Reichard, P. (1964) *J. Biol. Chem.* **239,** 3436–3444.
2. Arner, E. S. & Holmgren, A. (2000) *Eur. J. Biochem.* **267,** 6102–6109.
3. Meyer, Y., Vignols, F. & Reichheld, J.-P. (2001) *Methods Enzymol.*, in press.
4. Pedrajas, J. R., Kosmidou, E., Miranda-Vizuete, A., Gustafsson, J. A., Wright, A. P. & Spyrou, G. (1999) *J. Biol. Chem.* **274,** 6366–6373.
5. Spyrou, G., Enmark, E., Miranda-Vizuete, A. & Gustafsson, J. (1997) *J. Biol. Chem.* **272,** 2936–2941.
6. Miranda-Vizuete, A., Damdimopoulos, A. E., Pedrajas, J. R., Gustafsson, J. A. & Spyrou, G. (1999) *Eur. J. Biochem.* **261,** 405–412.
7. Konrad, A., Banze, M. & Follmann, H. (1996) *J. Plant Physiol.* **149,** 317–321.
8. Banze, M. & Follmann, H. (2000) *J. Plant Physiol.* **156,** 126–129.
9. Jacquot, J. P., Rivera-Madrid, R., Marinho, P., Kollarova, M., Le Marechal, P., Miginiac-Maslow, M. & Meyer, Y. (1994) *J. Mol. Biol.* **235,** 1357–1363.
10. Verdoucq, L., Vignols, F., Jacquot, J. P., Chartier, Y. & Meyer, Y. (1999) *J. Biol. Chem.* **274,** 19714–19722.
11. Sakamoto, W., Spielewoy, N., Bonnard, G., Murata, M. & Wintz, H. (2000) *Plant Cell Physiol.* **41,** 1157–1163.
12. Spielewoy, N., Schulz, H., Grienenberger, J. M., Thöny-Meyer, L. & Bonnard, G. (2001) *J. Biol. Chem.* **276,** 5491–5497.
13. Bowler, C., Alliotte, T., De Loose, M., Van Montagu, M. & Inze, D. (1989) *EMBO J.* **8,** 31–38.
14. Lamattina, L., Gonzalez, D., Gualberto, J. & Grienenberger, J. M. (1993) *Eur. J. Biochem.* **217,** 831–838.
15. Fey, J., Vermel, M., Grienenberger, J., Marechal-Drouard, L. & Gualberto, J. M. (1999) *FEBS Lett.* **458,** 124–128.
16. Wischmann, C. & Schuster, W. (1995) *FEBS Lett.* **374,** 152–156.
17. Li, X., Nield, J., Hayman, D. & Langridge, P. (1996) *Plant J.* **10,** 505–513.
18. Sahrawy, M., Hecht, V., Lopez-Jaramillo, J., Chueca, A., Chartier, Y. & Meyer, Y. (1996) *J. Mol. Evol.* **42,** 422–431.
19. Blanc, G., Barakat, A., Guyot, R., Cooke, R. & Delseny, M. (2000) *Plant Cell* **12,** 1093–1102.
20. Gonnet, G. H., Cohen, M. A. & Benner, S. A. (1992) *Science* **256,** 1443–1445.
21. Glaser, E., Sjoling, S., Tanudji, M. & Whelan, J. (1998) *Plant Mol. Biol.* **38,** 311–338.
22. Schneider, G., Sjoling, S., Wallin, E., Wrede, P., Glaser, E. & von Heijne, G. (1998) *Proteins* **30,** 49–60.
23. Nakai, K. & Horton, P. (1999) *Trends Biochem. Sci.* **24,** 34–36.
24. Emanuelsson, O., Nielsen, H., Brunak, S. & von Heijne, G. (2000) *J. Mol. Biol.* **300,** 1005–1016.
25. Marcus, F., Chamberlain, S. H., Chu, C., Masiarz, F. R., Shin, S., Yee, B. C. & Buchanan, B. B. (1991) *Arch. Biochem. Biophys.* **287,** 195–198.
26. Douce, R., Bourguignon, J., Brouquisse, R. & Neuburger, M. (1987) *Methods Enzymol.* **148,** 403–415.
27. Nito, K., Yamaguchi, K., Kondo, M., Hayashi, M. & Nishimura, M. (2001) *Plant. Cell. Physiol.* **42,** 20–27.
28. Filppula, S. A., Yagi, A. I., Kilpelainen, S. H., Novikov, D., FitzPatrick, D. R., Vihinen, M., Valle, D. & Hiltunen, J. K. (1998) *J. Biol. Chem.* **273,** 349–355.
29. Kotti, T. J., Savolainen, K., Helander, H. M., Yagi, A., Novikov, D. K., Kalkkinen, N., Conzelmann, E., Hiltunen, J. K. & Schmitz, W. (2000) *J. Biol. Chem.* **275,** 20887–20895.
30. Oda, T., Mizuno, T., Ito, K., Funai, T., Ichiyama, A. & Miura, S. (2000) *Cell Biochem. Biophys.* **32,** 277–281.
31. Sun, Q. A., Zappacosta, F., Factor, V. M., Wirth, P. J., Hatfield, D. L. & Gladyshev, V. N. (2001) *J. Biol. Chem.* **276,** 3106–3114.
32. Souciet, G., Menand, B., Ovesna, J., Cosset, A., Dietrich, A. & Wintz, H. (1999) *Eur. J. Biochem.* **266,** 848–854.
33. Duchene, A. M., Peeters, N., Dietrich, A., Cosset, A., Small, I. D. & Wintz, H. (2001) *J. Biol. Chem.* **276,** 15275–15283.
34. Mireau, H., Lancelin, D. & Small, I. D. (1996) *Plant Cell* **8,** 1027–1039.
35. Vision, T. J., Brown, D. G. & Tanksley, S. D. (2000) *Science* **290,** 2114–2117.
36. Mestres-Ortega, D. & Meyer, Y. (1999) *Gene* **240,** 307–316.
37. Danpure, C. J. (1995) *Trends Cell Biol.* **5,** 230–238.
38. Small, I. D., Wintz, H., Akashi, K. & Mireau, H. (1998) *Plant. Mol. Biol.* **38,** 265–277.
39. Diekert, K., Kispal, G., Guiard, B. & Lill, R. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 11752–11757.
40. Kranz, R., Lill, R., Goldman, B., Bonnard, G. & Merchant, S. (1998) *Mol. Microbiol.* **29,** 383–396.
41. Fabianek, R. A., Hennecke, H. & Thöny-Meyer, L. (2000) *FEMS Microbiol. Rev.* **24,** 303–316.
42. Stevens, F. J., Dong Li, A., Salman Lateef, S. & Anderson, L. E. (1997) *Photosynth. Res.* **54,** 185–197.
43. Umbach, A. L. & Siedow, J. N. (1993) *Plant Physiol.* **103,** 845–854.
44. Siedow, J. N. & Umbach, A. L. (2000) *Biochim. Biophys. Acta* **1459,** 432–439.