Thioredoxin links redox to the regulation of fundamental processes of plant mitochondria

Yves Balmer*, William H. Vensel†, Charlene K. Tanaka†, William J. Hurkman†, Eric Gelhaye‡, Nicolas Rouhier‡, Jean-Pierre Jacquot‡, Wanda Manieri§, Peter Schürmann§, Michel Droux¶, and Bob B. Buchanan*‖

*Department of Plant and Microbial Biology, University of California, 111 Koshland Hall, Berkeley, CA 94720; †Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Albany, CA 94710; ‡Interaction Arbres Microorganismes, Institut National de la Recherche Agronomique, Unité Mixte de Recherche 1136, Université de Nancy I, 54506 Vandoeuvre Cedex, France; §Laboratoire de Biochimie Végétale, Université de Neuchâtel, 2007 Neuchâtel, Switzerland; and ¶Laboratoire Mixte Centre National de la Recherche Scientifique/Bayer CropScience, 14-20 Rue Pierre Baizet, 69263 Lyon Cedex 9, France

Contributed by Bob B. Buchanan, December 22, 2003

Mitochondria contain thioredoxin (Trx), a regulatory disulfide protein, and an associated flavoenzyme, NADP/Trx reductase, which provide a link to NADPH in the organelle. Unlike animal and yeast counterparts, the function of Trx in plant mitochondria is largely unknown. Accordingly, we have applied recently devised proteomic approaches to identify soluble Trx-linked proteins in mitochondria isolated from photosynthetic (pea and spinach leaves) and heterotrophic (potato tubers) sources. Application of the mitochondrial extracts to mutant Trx affinity columns in conjunction with proteomics led to the identification of 50 potential Trx-linked proteins functional in 12 processes: photorespiration, citric acid cycle and associated reactions, lipid metabolism, electron transport, ATP synthesis/transformation, membrane transport, translation, protein assembly/folding, nitrogen metabolism, sulfur metabolism, hormone synthesis, and stress-related reactions. Almost all of these targets were also identified by a fluorescent gel electrophoresis procedure in which reduction by Trx can be observed directly. In some cases, the processes targeted by Trx depended on the source of the mitochondria. The results support the view that Trx acts as a sensor and enables mitochondria to adjust key reactions in accord with prevailing redox state. These and earlier findings further suggest that, by sensing redox in chloroplasts and mitochondria, Trx enables the two organelles of photosynthetic tissues to communicate by means of a network of transportable metabolites such as dihydroxyacetone phosphate, malate, and glycolate. In this way, light absorbed and processed by means of chlorophyll can be perceived and function in regulating fundamental mitochondrial processes akin to its mode of action in chloroplasts.

Thioredoxins (Trxs) are small, widely distributed proteins with a redox-active disulfide group. Two types of Trx systems have been described in plants based on the source of reducing power: the ferredoxin/Trx system located in chloroplasts [in which electrons from ferredoxin reduce Trx by means of the iron–sulfur enzyme ferredoxin/Trx reductase (1–4)] and the extraplastidic NADP/Trx system [whereby NADPH reduces Trx by means of the flavoenzyme NADP/Trx reductase (NTR) (3, 4)].

Trx has two main functions by nature of its ability to reduce specific S-S groups: (*i*) as a substrate for enzymes that catalyze reactions such as the reduction of ribonucleotides, methionine sulfoxide, and hydrogen peroxide; and (*ii*) as a regulator that alters the activity or other functional properties of interacting target proteins (1–4). The regulatory role of Trx is prominent in plants where the first redox-controlled enzyme was identified in chloroplasts >25 years ago (1, 2). Since that time, the number of Trx-regulated enzymes has steadily increased (2, 4). Recently developed proteomics-based approaches have accelerated progress in making possible the systematic identification of Trx-linked proteins in complex extracts. With the addition of previously unrecognized potential Trx targets, our knowledge of the function of Trx is relatively extensive for chloroplasts and cytosol (5–11).

In contrast, we have scant understanding of the role of Trx in plant mitochondria. It has been proposed that Trx is involved in the regulation of cyanide-resistant respiration and, by analogy to the animal and yeast organelles, in antioxidant defense (3, 4, 12). However, direct evidence for linking Trx to these or other mitochondrial processes is quite limited.

To help fill this gap, we have applied recently devised approaches to identify soluble Trx-linked proteins of mitochondria from both photosynthetic (leaf) and heterotrophic (tuber) sources. Preparations of the isolated organelles were analyzed by using proteomics in combination with (*i*) affinity chromatography in which a mutated Trx (5) traps targets in applied extracts by forming a stable heterodisulfide (6–8) and (*ii*) fluorescence gel electrophoresis in which target proteins are identified visually after reduction of the preparation by Trx (9). These experiments have led to the identification of 50 potential Trx-linked proteins functional in 12 processes that, in some cases, depend on the source of mitochondria. The successful use of proteomics in combination with both modes of target isolation complements recent studies in which these procedures were applied separately to seed preparations (gel procedure) and chloroplasts, algae, and cyanobacteria (column procedure). The results raise the possibility that, by sensing the redox state of parent organelles, Trx functions in an interorganelle network that enables communication between chloroplasts and mitochondria. Communication is achieved by means of transportable metabolites such as dihydroxyacetone phosphate, malate, and glycolate, whose concentration changes as a result of illumination and energy status.

Materials and Methods

Materials. Materials were as described in ref. 8.

Recombinant Mutant Trx Proteins. Recombinant mutant spinach Trx *m* C40A and poplar Trx *h1* C42S were overexpressed in *Escherichia coli* and purified from cell extracts following procedures described for the WT enzymes (6, 13). Recombinant poplar Trx *h2* and *Arabidopsis* NTR used in gel experiments were prepared and purified as described (14, 15).

Isolation of Matrix from Mitochondria. Matrices were obtained from intact mitochondria prepared from spinach and pea leaves and from potato tubers (16).

Preparation of Trx-Sepharose Resin and Separation of Mitochondrial Extracts. The preparation of the affinity resin and the separation of the protein extracts on the affinity column were as described (8) except that a chloroplast mutant Trx *m* was used as bait because of availability and relatedness to mitochondrial Trx *o* (3, 12). Additionally, Trx target isolation was achieved with a monocysteinic Trx *h*. The two affinity columns yielded similar protein patterns with minor differences in spot intensities. The apparent loss of specificity

Abbreviations: Trx, thioredoxin; NTR, NADP/Trx reductase; HSP, heat shock protein.

‖To whom correspondence should be addressed. E-mail: view@nature.berkeley.edu.

www.pnas.org/cgi/doi/10.1073/pnas.0308583101

of mutant Trxs toward their respective targets was previously observed in chloroplasts (6, 8).

Fluorescent Labeling of the Potential Trx Target Proteins. Mitochondria extracts were applied to mutant Trx columns, and the retained fraction was eluted with DTT. Excess DTT was removed by extensive (48-h) dialysis against 50 mM Tris·Cl, pH 7.5, with two buffer changes. Subsequently, the protein sample was treated with 1 mM *N*-ethylmaleimide (NEM) to block remaining free cysteines (17). Excess NEM was removed by addition of 1 mM 2-mercaptoethanol. Finally, half of the fraction was reduced by using the *E. coli* Trx system, and the remaining half was incubated in parallel with water (control) as described (9, 10). The control and reduced samples were treated with 2 mM monobromobimane, a fluorescent thiol-specific probe, to label the newly exposed SH groups (9). In a separate experiment, reduction was carried out with poplar Trx *h2* and *Arabidopsis* NTR, and the gels were analyzed following the same protocol.

2D Gel Separation. Separation of the column eluate by 2D isoelectric focusing SDS/PAGE was as described (8) using the Protean Isoelectric Focusing (IEF) Cell and Criterion Precast System (Bio-Rad).

Protein Spot Excision, Digestion, and Identification. Spots were manually excised, digested with trypsin, and analyzed by electrospray ionization tandem mass spectrometry using a QSTAR Pulsar *i* quadrupole time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex, Toronto); data were analyzed as described in ref. 10. Amino acid sequence alignments for each polypeptide detected were generated by using BLASTP (18) and CLUSTALW (19) to identify conserved cysteines that could form disulfide bonds.

Results

The major spots of soluble mitochondrial proteins isolated by mutant Trx *m* affinity chromatography were identified by mass spectrometry. Only proteins detected with at least three different tryptic peptides and an expectation score $<1 \times 10^{-3}$ were selected as potential targets. Mitochondria extracts from three sources (pea and spinach leaves and potato tubers) were analyzed to compare the organelles from photosynthetic and heterotrophic tissues. These sources were selected because of proven success in yielding mitochondrial preparations of high purity. The pea and tuber extracts were also subjected to the affinity procedure using a mutant poplar Trx *h*. Aside from differences in intensity, the spots were the same as those found for the procedure with Trx *m* (data not shown) and reflect of the high degree of interchangeability of mutated Trxs in the affinity column procedure (6–8).

The proteomic approach led to the identification of 55 proteins, of which 50 represent potential mitochondrial Trx targets. The remaining five were contaminants, four of which were of chloroplast origin present only in leaf preparations (Rubisco large and small subunits, Rubisco activase, and carbonic anhydrase), plus the mutant Trx *m* or *h* used to prepare the matrices. Forty-seven of the 50 mitochondrial proteins detected in the affinity column eluates can be grouped into 38 enzymes or protein complexes functional in 12 biological processes: photorespiration; citric acid cycle (and associated enzymes); lipid, nitrogen, and sulfur metabolism; electron and membrane transport; ATP synthesis and transformation; translation; protein assembly and folding; hormone synthesis; and stress-related reactions (Table 1). The remaining three mitochondrial proteins are hypothetical proteins without assigned function. The approach was designed to detect soluble proteins rather than targets located in the mitochondrial membranes, an exciting area that awaits exploration.

To confirm their role as potential targets, the eluates from the affinity column were reduced by Trx and the change in redox state was measured by fluorescence coupled with gel electrophoresis. To this end, the eluted proteins were first treated with *N*-ethylmaleimide to block free SH groups and then incubated with the NADP/Trx system from *E. coli* to reduce disulfide bonds. The newly formed SH groups were labeled with monobromobimane and separated by 2D electrophoresis. The Trx-reduced samples showed, relative to untreated controls, an increase in fluorescence of most protein spots (Fig. 1). A comparison of the gels revealed that, aside from the proteins lacking conserved cysteines (see *Results*), each of the candidate targets eluted from the affinity columns became fluorescent, i.e., was reduced, after treatment with the Trx system. Similar results were obtained when *E. coli* Trx and NTR were replaced, respectively, by a form of Trx that appears to be associated with mitochondria (poplar Trx *h2*) (E.G., N.R., and J.-P.J., unpublished results) and *Arabidopsis* NTR (data not shown). Based on this finding, it appears that, as with seed proteins studied earlier (10), the mitochondrial targets can recognize bacterial in addition to indigenous plant Trxs.

An examination of results obtained with the three preparations (pea and spinach leaves and potato tubers) shows that half of the proteins were identified in mitochondria from at least two sources and that many polypeptides analyzed were found only in the potato organelle. Moreover, the relative amount of the different proteins recovered from the affinity column varied greatly in extracts from photosynthetic and heterotrophic sources, reflecting specific mitochondrial function. Thus, consistent with the work of others (20), photorespiratory enzymes represented a large proportion of the proteins of leaf mitochondria, whereas they were minor components with tuber extracts. An extension of this analysis revealed that photorespiration was the only major process clearly paramount in photosynthetic tissue, whereas lipid metabolism, electron transport, membrane transport, nitrogen metabolism, and hormone synthesis were prominent in heterotrophic preparations. Enzymes participating in the citric acid cycle, ATP synthesis, translation, protein assembly and folding, sulfur metabolism, and stress response were uniformly distributed between mitochondria from the two types of tissues (Table 1).

Potential Trx Target Proteins in Mitochondria

Photorespiration. Photorespiration, a process initiated in chloroplasts and continued, sequentially, in peroxisomes and mitochondria, is a primary function of photosynthetic tissue that recovers energy and carbon from the phosphoglycolate produced by the oxygenase activity of Rubisco (21). The two mitochondrial enzymes functional in photorespiration (glycine cleavage complex and serine hydroxymethyl transferase) were both identified as Trx-linked proteins. The targets included three of the four subunits of the glycine cleavage complex, H, P, and T. The fourth subunit (L), a dihydrolipoamide dehydrogenase, was also identified. However, because this enzyme is part of both the glycine cleavage system and the pyruvate dehydrogenase complex (22), this protein was listed under the citric acid cycle in Table 1.

Until now, there was no evidence of a link between Trx and either the glycine cleavage or serine hydroxymethyl transferase enzymes. Due to the presence of lipoamide bound to subunit H, it is possible that a nonspecific disulfide could be formed between the mutant Trx and this subunit of glycine decarboxylase, thus trapping the complex on the column. However, based on knowledge that glycine cleavage activity depends on a nonphysiological substitute for Trx, DTT, it seems likely that the complex is an authentic target (23). Such a link to redox complements the earlier view that regulation of the mitochondrial photorespiratory enzymes is due mainly to feedback inhibition by the final reaction products, NADH and serine (21). Recent evidence showing that an enzyme synthesizing NO is a variant of the P protein is consistent with an involvement of Trx in NO signaling (24).

Citric Acid Cycle-Associated Enzymes. Seven members of the citric acid cycle (aconitase, malic enzyme, succinyl-CoA ligase, and

Table 1. Potential thioredoxin target proteins of plant mitochondria identified by affinity chromatography

Protein	Spinach	Pea	Potato	Cys*
Photorespiration				
Glycine cleavage system H protein	++	++	++	5
Glycine cleavage system P protein	++	++	++	14
Glycine cleavage system T protein	++	++	++	5
Serine hydroxymethyl transferase	++	++	++	2
Citric acid cycle-associated reactions				
Aconitase	++		++	8 (4)
Dihydrolipoamide acetyltransferase		++	++	5 (1)
Dihydrolipoamide dehydrogenase		++	++	5 (4)
Isocitrate dehydrogenase			+	6 (2)
Malate dehydrogenase		++	++	3 (2)
Malic enzyme			+	2 (2)
Pyruvate dehydrogenase E1, alpha su			+	5 (2)
Pyruvate dehydrogenase E1, beta su	++	++	++	3 (0)
Succinate dehydrogenase (flavoprotein su)		++	++	9 (5)
Succinyl-CoA ligase, alpha su		+		6 (6)
Succinyl-CoA ligase, beta su	++	++	++	5 (3)
Lipid metabolism				
CoA-thioester hydrolase			+	4
Electron transport				
Cytochrome c oxidase su 5b			+	3 (3)
Cytochrome c oxidase su 6b			+	4 (4)
NADH-ubiquinone oxidoreductase 75-kDa su			+	14 (13)
Ubiquinol-cytochrome *c* reductase su II			+	1 (0)
ATP synthesis/transformation				
Adenylate kinase			+	4 (3)
ATP synthase, alpha su	++	++		5 (3)
ATP synthase, beta su	++	++	++	1 (0)
ATP synthase, delta su		++	++	0
Nucleoside diphosphate kinase	++	++		1 (0)
Membrane transport				
Porin (VDAC)			+	1
Translation				
Elongation factor Tu		++	++	2
Protein assembly/folding				
Chaperonin HSP 60	++	++	++	4
Dna-K molecular chaperone HSP 70	++	++	++	2
Nitrogen metabolism				
Alanine aminotransferase		++	++	8
Aspartate aminotransferase			+	4
Branched-chain keto acid decarboxylase E1, beta su			+	8
Glutamate dehydrogenase			+	4
Isovaleryl-CoA dehydrogenase			+	10
Leucyl aminopeptidase		+		3
Methylmalonate-semialdehyde dehydrogenase			+	7
Sulfur metabolism				
Cysteine synthase	++	++	++	0
Mercaptopyruvate sulfurtransferase		++	++	3
Hormone synthesis				
Allene oxide cyclase			+	1
Stress-related reactions				
Alcohol dehydrogenase CPRD12		+		2
Aldehyde dehydrogenase	++	++	++	5
Catalase			+	7
Formate dehydrogenase	++	++	++	3
Glutaredoxin-like protein		+		1
Peroxiredoxin	++	++	++	2
Phospholipid hydroperoxide GSH reductase			+	3
Superoxide dismutase Mn	++	++	++	0
Miscellaneous				
Hypothetical protein (29.8 kDa)		+		2
Hypothetical protein (36.2 kDa)		+		2
Putative protein At5g10860.1	++	++	++	1

++, Identification of target proteins in more than one mitochondrial preparation; +, the protein was detected in a single preparation. The SwissProt ID numbers for each protein is from the isoform with the best match. The ID number of the enzymes from top to bottom are P16048, P26969, P49364, P34899, Q8L784, Q8RWN9, P31023, Q7XK22, O48904, P37225, P52902, P52904, Q9ZPX5, P53586, Q8LAV0, Q9LKJ1, Q9LW15, Q9SXV0, Q43644, P29677, O82514, P05493, P17614, Q9SP13, P42056, Q9ZT91, Q05046, P37900, Q8GRN4, P46643, 082450, P93541, Q9FS87, O65557, Q9SI43, Q43153, O64530, Q9LEG5, P93697, P93344, P55312, Q9S7E4, Q8LBK6, Q9XGP1, O48646, O81233, Q94GV5, Q9SUK9, and Q9LEV3.

*Number of conserved cysteines in plant proteins. Numbers in parentheses represent conserved cysteines common to plant and animal enzymes. Members of each group have their own invariant cysteines.

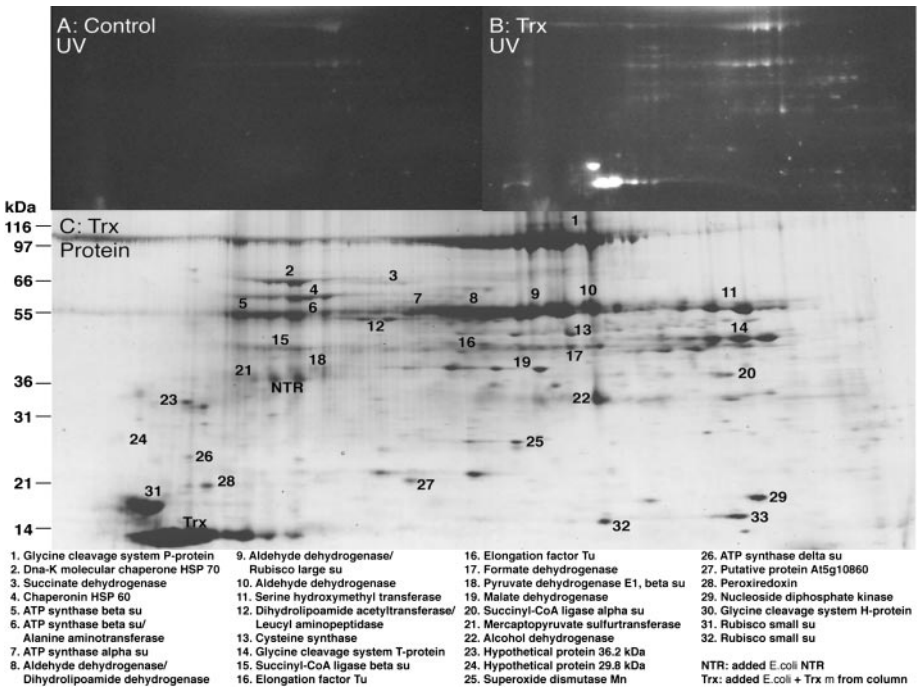

Fig. 1. Reduction of proteins of pea leaf mitochondria by Trx after treatment with *N*-ethylmaleimide to remove background SH groups. The mutant affinity column eluate was either not treated (*A*) or reduced by Trx (*B*) using *E. coli* Trx, NADPH, and NTR. After reduction, the proteins were derivatized with monobromobimane and subjected to 2D electrophoresis. (*C*) As in *B*, but stained for protein.

isocitrate, malate, pyruvate, and succinate dehydrogenases) were covalently trapped by mutant Trx columns. A link between Trx and citric acid cycle enzymes has not been described for plant mitochondria. However, Trx was linked to this process in animal organelles (25).

In plants, aconitase, an iron–sulfur enzyme, is readily inactivated by H_2O_2 (26). Thus, Trx could be involved in both the protection and reactivation of this enzyme. The mitochondrial pyruvate dehydrogenase complex is inactivated *in vivo* by light, possibly through a signal originating in the chloroplast (27). It will be interesting to know whether Trx is part of this signaling mechanism (see *Discussion*). Finally, NAD-dependent malate dehydrogenase of the cytosol (10, 11) and mitochondria (11) has recently been identified as a Trx target in seeds, and NADP-dependent malate dehydrogenase was one of the first Trx-linked enzymes discovered in chloroplasts (1–4).

Lipid Metabolism. The β-oxidation of lipids was believed for years to occur only in peroxisomes in plants. However, recent evidence suggests that this process also takes place in mitochondria in plants as with animals (28). Identification of CoA-thioester hydrolase as a possible Trx target in potato tubers strengthens this dual localization and adds insight as to its mode of regulation.

Electron Transport. NADH-ubiquinone oxidoreductase (subunit 75 kDa), ubiquinol–cytochrome *c* reductase (subunit II), and cytochrome *c* oxidase (subunits 5b and 6b) were identified in the eluate of the affinity column with potato tuber extracts. Evidence of regulation of these three enzymes by means of redox is lacking in plants. However, it has been demonstrated with animal preparations that DTT reverses the inhibition of complex I (NADH-ubiquinone oxidoreductase) and cytochrome *c* oxidase by reactive oxygen species (29). Additionally, the ubiquinol–cytochrome *c* reductase (also known as a processing peptidase) is a bifunctional enzyme involved in electron transport as well as protein import, a process known to be inhibited by the modification of thiol groups in plant preparations (30).

ATP Synthesis and Transformation. Three enzymes functional in the synthesis or transformation of ATP were identified: adenylate kinase, which transfers Pi from ATP to AMP to yield two equivalents of ADP; nucleoside diphosphate kinase catalyzing the transfer of a Pi from ATP to a nucleoside diphosphate; and three subunits of ATP synthase (α, β, and δ) functional in the synthesis of ATP. A link among these three enzymes and Trx could achieve a general control of ATP utilization and production, thereby resembling the ATP synthase of chloroplasts, which is linked to Trx (2, 4).

Membrane Transport. One of the proteins identified in Table 1, porin, forms a voltage-dependent anion channel (VDAC) across the outer mitochondrial membrane that allows the transport of low-molecular weight metabolites such as ATP and ADP. In addition, VDAC interacts with other proteins to form a complex (the permeability transition pore complex) that, upon stimulation, triggers apoptosis and cell death (31). In animal mitochondria, the permeability transition appears to be under redox control (32).

Translation. Another candidate, elongation factor Tu, promotes the GTP-dependent binding of aminoacyl-tRNA to the ribosome during the elongation of newly synthesized protein. Chloroplast elongation factor Tu was identified as a potential Trx target with chloroplasts (8).

Protein Assembly and Folding. Two chaperones, heat shock protein 70 kDa (HSP 70) and DnaK molecular chaperone 60 kDa, were identified in the affinity column eluate. Chloroplast and yeast counterparts were previously found to be potential plant Trx targets (8, 33). Significantly, the HSP 70 seems to function not only in protein assembly and folding, but also in protein import (34). This process has been reported to be redox-regulated and possibly light-regulated in a noncircadian manner (30, 35).

Nitrogen Metabolism. Seven enzymes of nitrogen metabolism were identified as potential Trx targets, primarily in preparations from potato tubers. Six of these enzymes (alanine and aspartate aminotransferase, branched-chain keto acid decarboxylase, glutamate, isovaleryl-CoA, and methylmalonate-semialdehyde dehydrogenase) function in amino acid metabolism, whereas leucyl aminopeptidase is likely involved in protein turnover. Only alanine aminotransferase was previously seen as a cytosolic Trx target (in wheat starchy endosperm) (10).

Sulfur Metabolism. Like its chloroplast counterpart (8), mitochondrial cysteine synthase appears to be a Trx target. A second enzyme functional in sulfur metabolism, mercaptopyruvate sulfurtransferase, trapped by the affinity column is activated by thiols, including Trx (36).

Hormone synthesis. Allene oxide cyclase catalyzes the third reaction of the lipoxygenase pathway in the synthesis of jasmonic acid. The biosynthesis of jasmonate is generally viewed as a chloroplast process. However, the recent identification of four genes coding for allene oxide cyclase in *Arabidopsis thaliana* raises the possibility of dual localization in mitochondria (37).

Stress-related proteins. Eight proteins linked to stress were found to be potential Trx targets of plant mitochondria. Peroxiredoxin is unique among the proteins identified in Table 1 in using reduced Trx specifically as a substrate (4). According to a recent study, another of the candidate enzymes, phospholipid hydroperoxide, uses either glutathione or Trx in reducing organic peroxides (38). The remaining stress-related proteins (alcohol dehydrogenase, aldehyde and formate dehydrogenases, catalase, glutaredoxin-like protein, and Mn superoxide dismutase) were not previously known to be linked to Trx. However, it is known that aldehyde dehydrogenase of rat liver forms disulfide bonds that can be reduced by DTT concordant with some restoration of activity (39). Catalase stands out as the only recognizable peroxisome contaminant identified in the present study. If confirmed, this finding raises the question of the presence and role of Trx in peroxisomes.

Unknowns. Three hypothetical proteins were identified as potential Trx targets in mitochondria, and several spots of 10–30 kDa (data not shown) failed to give a significant match to known plant proteins.

Conserved Cysteines. One of the features highlighted in the present study concerns the number of conserved cysteines in proteins targeted by Trx. Most of the 50 proteins identified contained two or more conserved cysteines in keeping with their function as classical Trx targets in which intramolecular disulfide bonds are reduced (Table 1). Moreover, most of the proteins involved in the major relevant mitochondrial processes, i.e., citric acid cycle, electron transport, and ATP synthesis, showed additional conserved cysteines when compared with animal counterparts. However, seven candidates appeared to contain a single conserved cysteine, implying other modes of reduction: ubiquinol-cytochrome *c* reductase, ATPase β-subunit, nucleoside diphosphate kinase, porin, allene oxidase cyclase, glutaredoxin-like protein, and putative protein AT510860.1. Such mechanisms could entail reduction of either an intermolecular disulfide bond as occurs with 1-Cys peroxiredoxin, a mixed disulfide with glutathione or, based on recent evidence, of a sulfenic acid residue (40, 41). Finally, three putative targets (cysteine synthase, Mn superoxide dismutase, and ATP synthase δ-subunit) appeared not to have conserved cysteines. It is possible that these proteins were trapped by virtue of their noncovalent interaction either with Trx (42) or putative targets. Further work will be required to resolve this point and also to determine whether the apparent presence of a single or no conserved cysteines is real or whether it is due to the lack of reliable information on isoforms rendering sequence alignment analysis difficult.

Discussion

Before this study, little was known of the function of Trx in plant mitochondria. The present findings suggest that Trx participates in the regulation of 12 mitochondrial processes, ranging from reactions of energetics and metabolism to protein synthesis and stress. In fulfilling its function, Trx appears to act not only to regulate biochemical processes under optimal cellular conditions but also, as proposed for chloroplasts (8), to restore the function of a number of activities after oxidative stress. Thus, although historically linked to the reduction of substrates and the regulation of enzymes under typical physiological conditions, Trx is now recognized to act in other capacities, some of which are highlighted below.

Regulation of Metabolism. Trx is well known to modulate the activity of target enzymes through thiol-disulfide exchange. This type of regulation is prominent in chloroplasts, where the Trx redox status is directly linked to photosynthetic electron flow through ferredoxin and ferredoxin/Trx reductase. In the light, Trx achieves the activation (or deactivation) of selected chloroplast enzymes by reduction of specific disulfide bonds. Although possibly less pronounced, a similar mechanism could apply to mitochondria, as proposed for the activation of the membrane-bound alternative oxidase (3, 4).

Protein Synthesis. In plants, the synthesis, assembly, and transport of proteins are inhibited by SH oxidants and are stimulated by DTT (30). Trx (or a Trx-like protein) could participate in one or more of these steps as suggested by the number of identified targets that function in translation and protein assembly and folding. For example, in cooperation with other proteins, the mitochondrial HSP 70 produces the driving force for the import of precursor proteins (30). The cleavage of the targeting sequence is achieved by a mitochondrial processing peptidase, an enzyme integrated in the ubiquinol-cytochrome *c* reductase of the electron transport chain that, as seen above, appears to be linked to Trx. The proper folding of the newly imported protein is assisted by two potential Trx targets (chaperonin HSP 60 and DnaK molecular chaperone HSP 70) and possibly by Trx itself (43). Moreover, the assembly of cytochrome *c* in plant mitochondria seems similar to the pathway in proteobacteria (system I), which involves a battery of Trx-like proteins (44).

Stress. Stress-related proteins were among the major targets found to be linked to Trx in plant mitochondria, a major site for the generation of reactive oxygen species. The candidates ranged from alcohol and aldehyde dehydrogenases, enzymes believed to function under anaerobic stress, to counterparts such as catalase, superoxide dismutase, and peroxiredoxin, which act under opposing, oxidative conditions. Mitochondria are thus similar to other systems investigated with respect to Trx, stress, and redox conditions (8, 10).

Additionally, through its ability to catalyze the reversible reduction of disulfides, Trx could assist in the adaptation of plants to temperature. Enzymes of psychrophilic organisms show high flexibility and low thermal stability, whereas thermophilic enzymes show the reverse, i.e., low flexibility and high thermal stability (45). When plants cope with pronounced temperature shifts, the Trx system could modulate the catalytic properties of enzymes to adapt to a new environment. The formation of disulfides would increase stability at high temperature, whereas their subsequent reduction would increase flexibility at low temperature and enhance catalytic activity.

Regulation of Energetics. As the main depot of oxygen-dependent reactions, the proteins involved in the generation of ATP are likely subject to redox regulation, especially during transient oxidative stress (46, 47). The identification of subunits of the major electron transfer chain complexes as potential Trx targets [complex I, NADH-ubiquinone oxidoreductase (75 kDa subunit); complex II, succinate dehydrogenase (flavoprotein subunit); complex III, ubiquinol-cytochrome *c* reductase (subunit II); and complex IV, cytochrome *c* oxidase (subunit 5b and 6b)] raises the possibility that oxidative regulation can directly control the process that produces reactive oxygen species. Such a route of regulation is reminiscent of feedback controls known to be linked through metabolites to biosynthetic pathways throughout biology.

Associated Processes. ***Programmed cell death.*** The release of proteins from the mitochondrial inter-membrane space is a factor initiating programmed cell death. Trx participates in this mechanism in animals by acting as a signal for initiating apoptosis (47, 48). The

identification of a porin (voltage-dependent anion channel) as a potential target raises the possibility that Trx could trigger apoptosis through the regulation of cytochrome *c* release (31, 47, 48). Porins form an anion channel in the outer membrane (31) that could be kept in a reduced state by Trx or a Trx-like protein. Under oxidative stress, the reactive oxygen species scavenging system could lead to the formation of an intermolecular disulfide between the unique conserved cysteine of the porin and another subunit of either the porin or another protein. This oxidation could trigger the subsequent formation of the permeability transition pore that releases cytochrome *c* and initiates apoptosis.

Male sterility. Cytoplasmic male sterility, a maternally inherited condition characterized by a plant's inability to generate functional pollen, can be linked to mitochondria. In maize, sterility is mediated by a polypeptide (URF13) that forms a mitochondrial inner membrane pore (49). Fertility can be restored by nuclear genes, thus making this, the T-cytoplasm system, useful for the production of hybrids of commercial interest. One of the genes responsible for the restoration of maize sterility (*Rf2* restorer) is a mitochondrial aldehyde dehydrogenase (49). Although Trx has been reported to be essential for pollen incompatibility in *Brassica* (50), the identification of aldehyde dehydrogenase as a potential target is the first indication of a link to male sterility.

Interorganelle Communication. It has long been known that light influences the regulation of leaf mitochondrial processes such as the citric acid cycle, which becomes modified to function with partial reactions (51). However, the basis for such effects has remained elusive. The present findings suggest that Trx could act in this capacity and coordinate processes in the two organelles by means of redox status. As seen in Fig. 2, the light-dependent Trx signal of chloroplasts could be transmitted to mitochondria through major metabolites exported to the cytosol, i.e., dihydroxyacetone phosphate, malate, and glycolate. The malate, NAD(P)H or photorespiratory glycine (derived from glycolate) imported from the cytosol would serve to reduce Trx by means of indigenous NTR, thereby completing transmission of the light signal. In this way, light absorbed and processed in plastids could be recognized and used to adjust operation of the mitochondrial biochemical machinery.

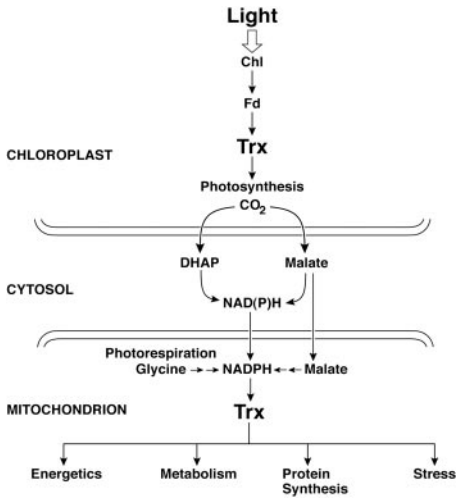

Fig. 2. Role of Trx as a regulatory link between chloroplasts and mitochondria. Reactions linking Trx to reproduction and apoptosis are not included. The mitochondrial pools of NADH and NADPH would be equilibrated by transhydrogenase activities (45). Chl, chlorophyll; Fd, ferredoxin.

Concluding Remarks

In uncovering the potential role of Trx, the present results emphasize the importance of mitochondria as independent units and as a partner with other organelles. It appears that Trx enables mitochondria to communicate with chloroplasts and possibly other organelles in a range of basic cellular reactions (energetics, metabolism, and protein synthesis) and in response to change in environment (adaptation to stress). Trx also appears to be linked to related responses affecting the whole plant, e.g., apoptosis and reproduction. In short, it seems that the role of Trx in plant mitochondria will be as varied and as rich as that deduced for chloroplasts in the past three decades.

We thank Nick Cai for assistance with the illustrations. This work was supported by funds from Syngenta, Inc., and the California Agricultural Experiment Station.

1. Buchanan, B. B. (1980) *Annu. Rev. Plant Physiol.* **31,** 341–374.
2. Buchanan, B. B., Schürmann, P., Wolosiuk, R. A. & Jacquot J.-P. (2002) *Photosynth. Res.* **73,** 215–222.
3. Balmer, Y. & Buchanan, B. B. (2002) *Trends Plant Sci.* **7,** 191–193.
4. Schürmann, P. & Jacquot J.-P. (2000) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **51,** 371–400.
5. Verdoucq, L., Vignols, F., Jacquot, J.-P., Chartier, Y. & Meyer, Y. (1999) *J. Biol. Chem.* **274,** 19714–19722.
6. Motohashi, K., Kondoh, A., Stumpp, M. T. & Hisabori, T. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 11224–11229.
7. Goyer, A., Haslekas, C., Miginiac-Maslow, M., Klein, U., Le Maréchal, P., Jacquot, J.-P. & Decottignies, P. (2002) *Eur. J. Biochem.* **269,** 272–282.
8. Balmer, Y., Koller, A., del Val, G., Manieri, W., Schürmann, P. & Buchanan, B. B. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 370–375.
9. Yano, H., Wong, J. H., Lee, Y. M., Cho, M. J. & Buchanan, B. B. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 4794–4799.
10. Wong, J. H., Balmer, Y., Cai, N., Tanaka, C. K., Vensel, W. H., Hurkman, W. J. & Buchanan, B. B. (2003) *FEBS Lett.* **547,** 151–156.
11. Maeda, K., Finnie, C. & Svensson, B. (2004) *Biochem. J.*, in press.
12. Laloi, C., Rayapuram, N., Chartier, Y., Grienenberger, J.-M., Bonnard, G. & Meyer, Y. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 14144–14149.
13. Behm, M. & Jacquot, J.-P. (2000) *Plant Physiol. Biochem.* **38,** 363–369.
14. Gelhaye, E., Rouhier, N., Laurent, P., Sautière, P. E., Martin, F. & Jacquot, J.-P. (2002) *Physiol. Plant* **114,** 164–171.
15. Jacquot, J.-P., Rivera-Madrid, R., Marinho, P., Kollarova, M., Le Maréchal, P., Miginiac-Maslow, M. & Meyer, Y. (1994) *J. Mol. Biol.* **235,** 1357–1463.
16. Douce, R., Bourguignon, J., Brouquisse, R. & Neuburger, M. (1987) in *Methods in Enzymology*, eds. Packer, L. & Douce, R. (Academic, San Diego), pp. 403–415.
17. Yano, H. (2003) *Anal. Chem.* **75,** 4682–4685.
18. Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997) *Nucleic Acids Res.* **25,** 3389–3402.
19. Thompson, J. D., Higgins, D. G. & Gibson T. J. (1994) *Nucleic Acids Res.* **22,** 4673–4680.
20. Bardel, J., Louwagie, M., Jaquinod, M., Luche, S., Rabilloud, T., Macherel, D., Garin, J. & Bourguignon, J. (2002) *Proteomics* **2,** 880–898.
21. Douce, R., Bourguignon, J., Neuburger, M. & Rébeillé, F. (2001) *Trends Plant Sci.* **6,** 167–176.
22. Bourguignon, J., Merand, V., Rawsthorne, S., Forest, E. & Douce, R. (1996) *Biochem. J.* **313,** 229–234.
23. Walker, J. L. & Oliver, D. J. (1985) *J. Biol. Chem.* **261,** 2214–2221.
24. Chandok, M. R., Ytterberg, A. J., van Wijk, K. J. & Klessig, D. F. (2003) *Cell* **113,** 469–482.
25. Bunik, V. I. (2003) *Eur. J. Biochem.* **270,** 1036–1042.
26. Verniquet, F., Gaillard, J., Neuburger, M. & Douce, R. (1991) *Biochem. J.* **276,** 643–648.
27. Budde, R. J. A. & Randall, D. D. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 673–676.
28. Masterson, C. & Wood, C. (2001) *Proc. R. Soc. London* **268,** 1949–1953.
29. Borutaite, V., Budriunaite, A. & Brown, G. C. (2000) *Biochim. Biophys. Acta* **1459,** 405–412.
30. von Stedingk, E. M., Pavlov, P. F., Grinkevich, V. A. & Glaser, E. (1997) *Plant Mol. Biol.* **35,** 809–820.
31. Godbole, A., Vaghese, J., Sarin, A. & Mathew, M. K. (2003) *Biochim. Biophys. Acta* **1642,** 87–96.
32. Petronilli, V., Costantini, P., Scorrano, L., Colonna, R. & Bernardi, P. (1994) *J. Biol. Chem.* **269,** 16638–16642.
33. Vignols, F., Mouaheb, N., Thomas, D. & Meyer, Y. (2003) *J. Biol. Chem.* **278,** 4516–4523.
34. Glaser, E., Sjöling, S., Tanudji, M. & Whelan, J. (1998) *Plant Mol. Biol.* **38,** 311–338.
35. Dessi, P. & Whelan, J. (1997) *FEBS Lett.* **415,** 173–178.
36. Papenbrock, J. & Schmidt, A. (2000) *Eur. J. Biochem.* **267,** 5571–5579.
37. Stenzel, I., Hause, B., Miersh, O., Kurz, T., Maucher, H., Weichert, H., Ziegler, J., Feussner, I. & Wasternack, C. (2003) *Plant Mol. Biol.* **51,** 895–911.
38. Herbette, S., Lenne, C., Leblanc, N., Julien, J. L., Drevet, J. R. & Roeckel-Drevet, P. (2002) *Eur. J. Biochem.* **269,** 2414–2420.
39. Shen, M. L., Lipsky, J. J. & Naylor, S. (2000) *Biochem. Pharmacol.* **60,** 947–953.
40. Kumar, R. A., Koc, A., Cerny, R. L. & Gladyshev, V. N. (2002) *J. Biol. Chem.* **277,** 37527–37535.
41. Rouhier, N., Gelhaye, E. & Jacquot, J.-P. (2002) *J. Biol. Chem.* **277,** 13609–13614.
42. Balmer, Y., Koller, A., del Val, G., Schürmann, P. & Buchanan, B. B. (2004) *Photosynth. Res.*, in press.
43. Li, W. & Churchich, J. E. (1997) *Eur. J. Biochem.* **246,** 127–132.
44. Kranz, R., Lill, R., Goldman, B., Bonnard, G. & Merchant, S. (1998) *Mol. Microbiol.* **29,** 383–396.
45. Georlette, D., Damien, B., Blaise, V., Depiereux, E., Uversky, V. N., Gerday, C. & Feller, G. (2003) *J. Biol. Chem.* **278,** 37015–37023.
46. Møller, I. M. (2001) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52,** 561–591.
47. Sweetlove, L. J., Heazlewood, J. L., Herald, V., Holtzapffel, R., Day, D. A., Leaver, C. J. & Millar, A. H. (2002) *Plant J.* **32,** 891–904.
48. Ueda, S., Masutani, H., Nakamura, H., Tanaka, T., Ueno, M. & Yodoi, J. (2002) *Antioxid. Redox. Signal.* **4,** 405–414.
49. Schnable, P. S. & Wise, R. P. (1998) *Trends Plant Sci.* **3,** 175–180.
50. Cabrillac, D., Cock, J. M., Dumas, C. & Gaude, T. (2001) *Nature* **410,** 220–223.
51. van Lis, R. & Atteia, A. (2004) *Photosynth. Res.,* in press.