A complete ferredoxin/thioredoxin system regulates fundamental processes in amyloplasts

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A growing number of processes throughout biology are regulated by redox via thiol–disulfide exchange. This mechanism is particularly widespread in plants, where almost 200 proteins have been linked to thioredoxin (Trx), a widely distributed small regulatory disulfide protein. The current study extends regulation by Trx to amyloplasts, organelles prevalent in heterotrophic plant tissues that, among other biosynthetic activities, catalyze the synthesis and storage of copious amounts of starch. Using proteomics and immunological methods, we identified the components of the ferredoxin/Trx system (ferredoxin, ferredoxin–Trx reductase, and Trx), originally described for chloroplasts, in amyloplasts isolated from wheat starchy endosperm. Ferredoxin is reduced not by light, as in chloroplasts, but by metabolically generated NADPH via ferredoxin–NADP reductase. However, once reduced, ferredoxin appears to act as established for chloroplasts, i.e., via ferredoxin–Trx reductase and a Trx (*m*-type). A proteomics approach in combination with affinity chromatography and a fluorescent thiol probe led to the identification of 42 potential Trx target proteins, 13 not previously recognized, including a major membrane transporter (Brittle-1 or ADP-glucose transporter). The proteins function in a range of processes in addition to starch metabolism: biosynthesis of lipids, amino acids, and nucleotides; protein folding; and several miscellaneous reactions. The results suggest a mechanism whereby light is initially recognized as a thiol signal in chloroplasts, then as a sugar during transit to the sink, where it is converted again to a thiol signal. In this way, amyloplast reactions in the grain can be coordinated with photosynthesis taking place in leaves.

redox regulation | target proteins | ferredoxin–thioredoxin reductase

Our understanding of the function of the regulatory disulfide protein thioredoxin (Trx) has increased dramatically in the past few years, with the advent of new methodologies. Recent approaches make it possible to identify proteins linked to Trx by combining proteomics with affinity chromatography (1) or thiol probes (2–4). These capabilities have defined an extended role of Trx in chloroplasts (1, 5) and helped elucidate its function in other plant systems: mitochondria (6), seeds (2, 3, 7, 8), and seedlings (4, 9). Currently almost 200 proteins appear to be linked to Trx in plants (10). One major organelle that has been neglected, however, is the amyloplast, a plastid of heterotrophic tissues that performs a wide range of biosynthetic reactions, including the synthesis and storage of abundant quantities of starch.

To help fill this gap, we have applied proteomic and immunological approaches to investigate the occurrence and function of Trx in amyloplasts. We now report evidence that amyloplasts isolated from wheat starchy endosperm resemble chloroplasts in containing a complete ferredoxin/Trx system composed of ferredoxin, ferredoxin–Trx reductase (FTR), and Trx (*m*-type). Application of affinity chromatography and fluorescent probe procedures led to the identification of 42 multifunctional Trx-linked amyloplast proteins, one-third of which were previously unrecognized. The results add a new dimension to our understanding of Trx and its role in regulating heterotrophic processes in plants.

Table 1. Identification of proteins associated with the ferredoxin/Trx system in isolated amyloplasts

Protein	Identification
System members	
Ferredoxin III	Proteomics
FTR	Proteomics
Trx *m*	Western blot
Support enzymes	
Glucose-6-phosphate dehydrogenase	Proteomics
6-phosphogluconate dehydrogenase	Proteomics
FNR	Proteomics

Evidence for Trx *m* is presented in this article. For proteomics, proteins from isolated amyloplasts were separated by 2D gel electrophoresis and identified by mass spectrometry.

Results and Discussion

Background. Initially believed to be restricted to chloroplasts, ferredoxin and the enzyme catalyzing its reduction with NADPH [ferredoxin–NADP reductase (FNR)] were later purified and found to be isoforms specific to nonphotosynthetic tissues (11–13). In addition to FNR, amyloplasts were found to contain enzymes capable of generating the NADPH needed for reduction of ferredoxin via FNR, namely glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (14–17).

Aside from the presence of the enzymes needed to reduce ferredoxin, two lines of evidence suggest a role for Trx in amyloplasts. First, enzymes shown to be regulated by Trx reside in the organelle, namely, ADP-glucose pyrophosphorylase (18), NADP-glucose 6-phosphate dehydrogenase (19, 20), and α-glucan, water dikinase (21). Second, analyses of the constituent proteins (proteome) of amyloplasts have revealed the presence of a number of other enzymes that, in chloroplasts, are linked to Trx (refs. 16 and 68). However, despite these indications, evidence for the presence of a complete Trx system in amyloplasts is lacking. This problem has been addressed in a recent proteomic analysis (68) and in the experiments below.

Identification of Members of the Ferredoxin/Trx System and Support Enzymes in Isolated Amyloplasts. By applying 2D gel electrophoresis and MS analysis, we obtained proteomic evidence for the presence of FNR, ferredoxin, and FTR among the 284 proteins identified in amyloplasts isolated from wheat endosperm (68). As summarized in Table 1, we also confirmed the presence of the

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Abbreviations: Trx, thioredoxin; FTR, ferredoxin–Trx reductase; FNR, ferredoxin–NADP reductase; G6PD, glucose-6-phosphate dehydrogenase; 2-DE, 2-dimensional gel electrophoresis.

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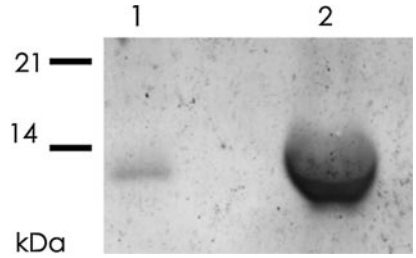

Fig. 1. Trx *m* was detected in amyloplasts isolated from wheat starchy endosperm by using antibodies against spinach chloroplast Trx *m*. Lane 1, amyloplast extract, wheat endosperm; lane 2, chloroplast extract, spinach leaves. Immunoblot was prepared as before by using 20 μg of protein per lane (33).

two above-mentioned enzymes of the oxidative pentose phosphate pathway that generate the NADPH needed to reduce ferredoxin: glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (13). However, our proteomic approach failed to identify a central component of the ferredoxin/Trx system so far not described in amyloplasts, namely, Trx.

To pursue this problem, polyclonal antibodies raised against spinach chloroplast Trxs *f* and *m*, known not to crossreact, were used to analyze the soluble protein fraction from isolated amyloplasts. Whereas our efforts to detect the *f*-type protein were unsuccessful (data not shown), Trx *m* was observed, although seemingly at a level less than in chloroplasts (Fig. 1). It is not clear whether the relatively weak signal seen with amyloplasts was due to low abundance of the protein, stage of development, or epitope differences between the native wheat amyloplast Trx *m* and the spinach chloroplast counterpart used to generate the antibody. Future work should resolve this issue and also whether Trx *f* may have been missed for one of these reasons. It is noted that others have found Trx *f* in root amyloplasts (Juan de Dios Barajas, A. Chueca, and M. Sahrawy, personal communication).

A related question concerns the nature of amyloplast Trx *m*, i.e., whether the protein expressed is the same as that in photosynthetic tissue. Because multiple genes encode Trx *m* (four in *Arabidopsis*), there is opportunity for tissue-dependent expression (22, 23). Finally, it will be of interest to determine whether the Trxs more recently identified in chloroplasts (types *x* and *y*) also occur in amyloplasts (22, 24, 25).

The finding of a complete ferredoxin/Trx system opens the door to a role for redox in reversibly regulating enzymes of amyloplasts as established for chloroplasts. However, in contrast to chloroplasts, where reducing power is derived directly from photosynthetic electron transport, the reduction of Trx in amyloplasts would be accomplished with NADPH generated enzymatically from entering glucose-6-phosphate (26) by glucose-6-phosphate and 6-phosphogluconate dehydrogenases. Furthermore, the amyloplast isoforms of ferredoxin (type III with a more oxidizing redox potential) and FNR (with high affinity for type III ferredoxin) favor the transfer of electrons from NADPH to ferredoxin, thereby enabling ferredoxin-dependent reactions to proceed (27). In the current case, FTR would divert a portion of these electrons to Trx which, when reduced, would regulate target enzymes by cleaving specific disulfide bonds as in other systems (Fig. 2). In the future, it will be of interest to determine the properties of the FTR in amyloplasts because in *Arabidopsis* there appear to be two genes encoding the variable subunit of the enzyme, but only one for the catalytic subunit (28). It is possible that, as with the resident ferredoxin, amyloplast FTR may have a more oxidizing redox potential than its chloroplast counterpart.

Identification of Trx Target Proteins in Isolated Amyloplasts. The finding of a complete ferredoxin/Trx system prompted us to look for Trx-regulated proteins in amyloplasts. To this end, we applied recently devised proteomic approaches based on fluorescent labeling (2, 8) and affinity chromatography (1, 5). As observed previously (8), the two techniques were complementary. Of the 42 proteins identified, approximately one-third were detected individually by either fluorescent labeling or affinity chromatography, and one-half were recognized by both procedures (Table 2). With the exception of chloroplast inorganic pyrophosphatase, each of the candidates contains conserved cysteine(s), consistent with the presence of a regulatory disulfide bond(s). It seems likely that the pyrophosphatase enzyme is not related to Trx and was identified because of colocalization in 2D gels with a true Trx target that escaped detection by MS.

Thirteen of the proteins identified represent previously unrecognized Trx targets that function in the biosynthesis of starch, lipids, nucleotides, and amino acids as well as protein assembly/folding (Table 2). The remaining 29 proteins function in metabolic processes previously found in studies on chloroplasts and other systems (10). The function of these different targets is discussed below.

Previously Unrecognized Targets Identified. ***Starch metabolism.*** None of the three candidate proteins participating in the metabolism of starch was previously recognized as a Trx target. One, α-1,4-glucan phosphorylase, releases glucose 1-phosphate from the starch chain, possibly as part of a stress response (29, 30). Trx seems ideal to participate in such a response by way of the oxidative regulatory mechanism described for certain chloroplast enzymes, e.g., transketolase (5).

Another potential target relevant to starch metabolism, Brittle-1, was shown to be an ADP-glucose transporter after its identification in mutant maize enriched in sugar and deficient in starch (31). The contribution of Brittle-1 gained prominence in subsequent studies showing that the cytosol, rather than the plastid, is the major site of ADP-glucose formation in wheat starchy endosperm (17, 32). A link to Trx thus provides evidence for a role for redox in regulating transport of the bulk of the sugar used for starch synthesis in wheat amyloplasts.

The final previously unrecognized starch-related target iden-

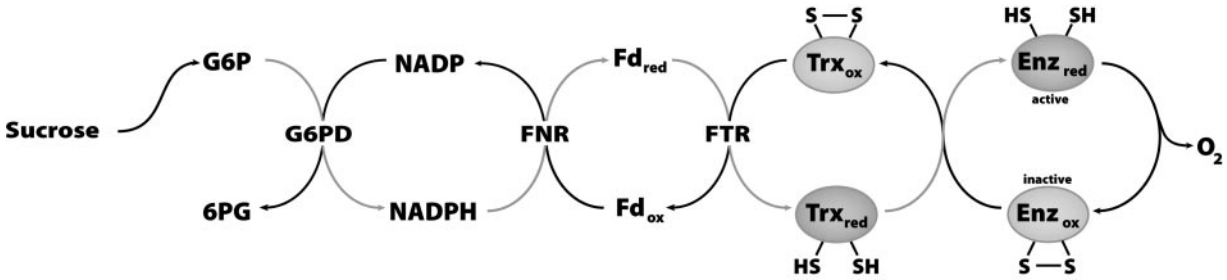

Fig. 2. Regulation of amyloplast enzymes by the ferredoxin/Trx system.

Table 2. Potential Trx target proteins identified in isolated amyloplasts by proteomics

Target	Protein	Cys	Monobromobimane	Affinity
Previously unrecognized targets	Starch metabolism			
	α1,4-glucan phosphorylase	9	●	●
	Brittle-1 protein (plastidial ADP-glucose transporter)	4	●	
	Starch branching enzyme IIa	3		●
	Lipid biosynthesis			
	Acyl-[acyl-carrier protein] thioesterase	3	●	
	Amino acid biosynthesis			
	Arginine			
	Acetylornithine aminotransferase	5	●	
	Ornithine carbamoyltransferase, putative	3	●	
	Argininosuccinate lyase	5		●
	Histidine			
	Imidazole glycerol phosphate synthase hisHF	4	●	
	Tryptophan			
	Tryptophan synthase β-chain 1	4	●	
	Valine/isoleucine			
	Dihydroxy-acid dehydratase	9	●	
	Amino acid-derived molecule			
	Aminotransferase AGD2, putative	5	●	●
	Nucleotide biosynthesis			
	Adenylosuccinate synthetase	5	●	●
	Protein assembling/folding			
	Endoplasmin homolog	1		●
Previously reported targets	Lipid biosynthesis			
	Acetyl-coenzyme A carboxylase	11		●
	Carbohydrate metabolism			
	Aldolase	2	●	
	Enolase, putative	2		●
	Phosphoglycerate dehydrogenase, putative	3	●	
	Phosphoglycerate kinase	1	●	
	Pyruvate dehydrogenase E1 α-subunit	6	●	●
	Transketolase	5	●	●
	Lipoamide dehydrogenase, putative	5	●	
	ADP-glucose pyrophosphorylase LS	7	●	●
	ADP-glucose pyrophosphorylase SS	3	●	
	Glucose-6-phosphate isomerase	4	●	
	Amino acid metabolism			
	3-isopropylmalate dehydratase small subunit	3	●	
	Ketol-acid reductoisomerase, putative	4	●	●
	Leucine aminopeptidase, putative	2	●	
	Threonine synthase	8	●	
	ATP-dependent clp protease ATP-binding subunit clpA	2	●	●
	Fd-GOGAT	19		●
	Protein assembly/folding			
	Cyclophilin-like protein	3	●	
	HSP 70 kDa	2	●	●
	HSP 82 kDa	4		●
	HSP ClpB, putative	3		●
	Protein disulfide isomerase	4	●	●
	Rubisco SU binding-protein α-subunit	1	●	
	Rubisco SU binding protein β-subunit	4	●	●
	Miscellaneous			
	Chloroplast inorganic pyrophosphatase	0	●	
	Peroxiredoxin BAS1 (2-cys)	2		●
	Ribulose bisphosphate carboxylase LS	7	●	
	Serine hydroxymethyltransferase	4	●	●
	Thiamine biosynthesis protein ThiC	9		●
	Thiosulfate sulfurtransferase	3	●	

The SwissProt ID numbers of the proteins (from top to bottom) are Q6UZD6, Q6E5A5, Q9ATB5, Q8L6B1, O04866, Q6YVI0, Q9LEU8, Q9SZ30, P43283, Q6YZH8, Q6VMN8, O24396, P36183, O48959, Q7X9K7, Q9C9C4, Q7XMP6, P12782, Q7XTJ3, Q9FPB6, Q94CN9, P12299, P55238, Q76E42, Q6H6I1, Q8RZF3, Q6K669, Q9S7B5, P31542, O04186, Q6XPZ6, Q9SEW1, Q43638, Q75I57, Q93XQ8, Q7X9A7, Q43831, Q9LXC9, Q96468, Q93XQ8, O23254, Q9AXS1, and Q9ZPK0.

tified in our analysis, starch branching enzyme, catalyzes the formation of α-1–6 linkages, a critical step distinguishing branched starch (amylopectin) from its linear counterpart (amylose). This finding is of interest in view of earlier work with germinating transgenic barley demonstrating that overexpressed Trx increased the activity of starch debranching enzyme (pul-

lulanase or limit dextrinase), which catalyzes the hydrolytic cleavage of α-1–6 bonds (33). These findings raise the question of whether these opposing enzymes respond to the oxidized or reduced form of Trx. It is noted that starch branching enzyme is generally prepared and purified in the presence of DTT or 2-mercaptoethanol (34).

Lipid biosynthesis. The identification of acyl-[acyl-carrier-protein] thioesterase as a potential target protein indicates that Trx has broader control of *de novo* fatty acid synthesis than was previously thought. Accordingly, with addition of thiolesterase, Trx appears to control the terminal step of the pathway in addition to the previously established first step catalyzed by acetyl-CoA carboxylase (35).

Amino acid biosynthesis. Seven of the 13 previously unrecognized targets function in the synthesis of amino acids, a major process in amyloplasts based on proteomic analysis (refs. 36 and 68). Three (acetylornithine aminotransferase, ornithine carbamoyltransferase, and argininosuccinate lyase) are implicated in the synthesis of arginine, catalyzing the fourth, sixth, and eighth (last) step of the pathway, respectively (37). Interestingly, possibly reflecting activation by redox, sulfhydryl compounds were reported to increase the activity of ornithine carbamoyltransferase (38).

Other potential Trx targets function in the biosynthesis of valine and isoleucine (dihydroxy acid dehydratase), histidine (imidazole glycerol phosphate synthase), and tryptophan (tryptophan synthase β-subunit). Dihydroxy acid dehydratase purified from spinach leaves resembles aconitase in containing an iron–sulfur cluster (39) and, as seen in Table 2, in being a target of Trx (10). Although the iron–sulfur cluster is expected to participate in catalysis and possibly in regulation (40), the disulfide site could enable the enzyme to respond to alternate redox signals. For example, the iron–sulfur cluster could respond to reactive oxygen species and the disulfide could respond to thiol status. The enzyme of histidine biosynthesis, imidazole glycerol phosphate synthase, apparently has not been obtained in pure form from plants. Furthermore, despite being pointed out early on as a deficiency (41), a mechanism for the posttranslational regulation of this enzyme had, until now, been a mystery. The β-subunit of tryptophan synthase catalyzes the last step in tryptophan biosynthesis. Regulation by Trx would, therefore, provide a means for redox to control output from the pathway.

Finally, a novel aminotransferase known as AGD2 seems to be involved in the synthesis of an essential amino acid-derived molecule affecting development and defense (42). If confirmed by future work, the role of redox, currently linked to disease resistance by Cf-9 (via CITRX) (43) and NPR1 (via an unknown mediator) (44), would be extended to pathogenesis and development by AGD2 (via Trx).

Nucleotide biosynthesis. One of the proteins identified, adenylosuccinate synthetase, participates in a two-step reaction that results in the formation of AMP from IMP. As the first committed step of AMP biosynthesis, a link of this reaction to Trx introduces a role for redox in the biosynthesis of a key coenzyme. In a study with adenylosuccinate synthetase from etiolated seedlings, DTT, a thiol reagent known to mimic Trx, was present in all preparation and assay solutions (45).

Protein assembly/folding. Endoplasmins are members of a large group of proteins known as reticuloplasmins that reside in the lumen of the endoplasmic reticulum (46). The identification of a member of this family as a Trx target is consistent with evidence for the occurrence of Trx *h* in that compartment (47).

Previously Reported Targets. The identification of known Trx targets builds on the recent description of the amyloplast proteome (refs. 16 and 68) and evinces a role for Trx in regulating a number of processes analogous to chloroplasts. Included are targets functional in lipid biosynthesis, carbohydrate metabolism, amino acid metabolism, and protein folding in addition to those active in miscellaneous processes (thiamin biosynthesis, oxidative stress, C-1, and sulfur metabolism).

We were able to detect both subunits of ADP-glucose pyrophosphorylase, an enzyme known to be regulated by Trx in amyloplasts as well as chloroplasts, (18, 48). Taking into consideration the previously unrecognized targets of starch metabolism listed in Table 2, it appears that Trx controls this process at several levels in plastids, including transport, breakdown, and synthesis.

In addition to ADP-glucose pyrophosphorylase, five of the previously reported targets in Table 2 have been biochemically linked to Trx: acetyl-CoA carboxylase (35), cyclophilin (49), peroxiredoxin BAS1 (50), protein disulfide isomerase (51, 52), and thiosulfate sulfurtransferase (53). Two previously reported amyloplast targets, glucose-6-phosphate dehydrogenase (19) and α-glucan, water dikinase (21), were not confirmed in our work. These enzymes, originally characterized in potato tuber, could have been missed because of low abundance or lack of sequence correspondence between the wheat proteins and counterparts from potato or *Arabidopsis*. The finding of ribulose bisphosphate carboxylase in a heterotrophic organelle could be due to imprecise control of expression, contamination from small amounts of photosynthetic tissue in endosperm, or a possible unknown function.

Comparison of the 29 previously reported Trx target proteins identified in isolated amyloplasts (Table 2) with recent proteomic studies reveals that 10 were identified in the related chloroplast organelle (1, 5), and 3 were identified in the parent endosperm fraction (3, 7, 8). The remaining 16 were identified in other plant systems (10). This comparison shows the importance of organelle isolation to enrich proteins of interest for MS analysis.

Trx as a Regulatory Link Between Photosynthesis and Metabolic Processes in Amyloplasts. The amount of photosynthate produced and exported by leaves has long been known to be greatly enhanced by light (54). In addition to providing energy and essential building blocks, metabolites transported in the phloem, such as sucrose and hexoses, have more recently been shown to be part of a signaling network whereby source and sink tissues communicate (55, 56). The current study provides new insight into how these metabolites could regulate processes of heterotrophic plastids.

The finding that amyloplasts resemble chloroplasts in having the potential to use redox for the control of a spectrum of enzymes raises the possibility that Trx could coordinate biochemical activities between the two organelles. The organelles would be linked by Trx in a manner fundamentally the same as that recently proposed for chloroplasts and mitochondria in leaves (6). As seen in Fig. 3, the Trx-linked buildup of sucrose in illuminated leaves would increase the NADPH/NADP ratio after its transport to sink tissues, such as seeds, thereby increasing the extent of Trx reduction and, in effect, informing amyloplasts that the plant is illuminated and that biochemical processes should be adjusted accordingly (carbohydrate, lipid and nitrogen metabolism, and protein structure). Light would be initially recognized as a thiol signal in chloroplasts, then as a sugar during transit to the seed, and finally, again, as an NADPH-generated thiol signal in amyloplasts. In this way, metabolic processes in amyloplasts could be adjusted in accord with photosynthetic activity. It is possible that Trx *h*, a major constituent of phloem, contributes to long-distance thiol signaling in a manner not yet apparent (57, 58).

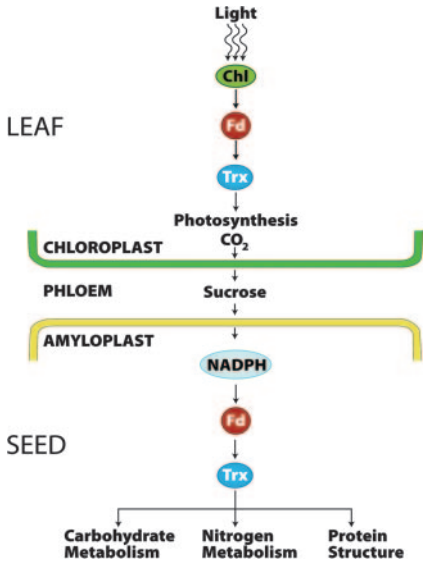

Fig. 3. Trx as a regulatory link between photosynthesis and metabolic processes in amyloplasts.

Concluding Remarks

The occurrence of a complete ferredoxin-linked Trx system in amyloplasts might be predicted from earlier reports on the presence of Trx target proteins and enzymes promoting the reduction of ferredoxin. However, to our knowledge, this is the first evidence for all components needed to provide reduced Trx in these organelles. If future experiments confirm the current work, the function of Trx will be extended in new directions and include major biochemical pathways of amyloplasts. As with other systems, Trx could influence target proteins at several levels, including classical modulation of activity, oxidative regulation, and assembly or folding (10). Additionally, analogous to its role as a regulatory link between chloroplasts and mitochondria (6), Trx could provide a mechanism that enables sink tissues to sense light processed via the reactions of photosynthesis. In view of the presence of FNR and a specific ferredoxin, it seems possible that chromoplasts, highly colored nonphotosynthetic organelles widely distributed in flowers and fruits, may also have the capability to sense light in this manner (59).

Materials and Methods

Materials. NuPage Zoom gels (4–12%) and IPG (immobilized pH gradient) strips were purchased from Invitrogen. Wheat (*Triticum aestivum* L. cv. Butte) was grown in a climate-controlled greenhouse under a 16-h day (supplemented with 100-W sodium lamps) and 8-h night regimen. Maximum daytime and nighttime temperatures were 24°C and 17°C, respectively. Water and fertilizer (Plantex 20-20-20, 500 ml of 0.6 g/liter per pot per day) were applied by drip irrigation. Heads were harvested 8–10 days after anthesis and used within 2 h.

Amyloplast Preparation. Amyloplasts were isolated according to ref. 60. Embryos from 20 heads were excised from the kernels, and the endosperm was squeezed into ice-cold buffer (0.5 M sorbitol/50 mM Hepes, pH 7.5), transferred to plasmolysis buffer (0.8 M sorbitol/50 mM Hepes, pH 7.5/1 mM EDTA/1 mM KCl/2 mM $MgCl_2$), and incubated for 1 h on ice. Plasmolyzed endosperm was chopped with razor blades. The resulting homogenate was filtered through Miracloth and gently pipetted into a conical tube containing a cushion of Nycodenz (Nycomed) (plasmolysis buffer plus 2% Nycodenz) layered over a 2-ml pad of 1% agar. After centrifugation (30 × *g* for 10 min at 4°C), the supernatant was discarded, the pellet was gently suspended in plasmolysis buffer, and the Nycodenz separation was repeated.

Isolation of Amyloplast Proteins. The pellet containing intact amyloplasts was suspended in plasmolysis buffer without sorbitol but with protease inhibitors (Complete Mini, Roche). The suspension was freeze/thawed three times to rupture the amyloplasts. The lysate was centrifuged (10,000 × *g* for 20 min at 4°C), and the soluble amyloplast proteins were collected.

Isolation of Potential Trx Targets by Fluorescence. Isolation of potential Trx target proteins using fluorescence labeling was achieved with monobromobimane (6, 8). Soluble amyloplast proteins were incubated with a mixture of 10 mM iodoacetamide and 10 mM *N*-ethylmaleimide for 20 min at 25°C to block free thiol groups (6, 61). Then, equimolar 2-mercaptoethanol was added to quench excess blocking reagents, and the solution was dialyzed extensively vs. 50 mM Tris·HCl (pH 7.5) at 4°C. Subsequently, an aliquot of dialyzed protein was reduced with a combination of NADPH and *Escherichia coli* NADP–Trx reductase and Trx. After a 20-min incubation, 2 mM monobromobimane was added to the reduced sample and a nonreduced (control) sample. The fluorescence was recorded after a parallel separation of the two samples by 2D gel electrophoresis (8).

Isolation of Potential Trx Targets by Affinity Chromatography. Potential Trx target proteins were isolated by affinity Sepharose chromatography by using recombinant mutant spinach Trx *m* (5, 6, 8). The only modification was that the sample applied to the column had been pretreated with the iodoacetamide/*N*-ethylmaleimide mixture to block free thiol groups and avoid nonspecific interactions between the remaining active site cysteine of the Trx and free thiol groups of the applied proteins.

2D Gel Electrophoresis. Isoelectric focusing and SDS/PAGE were performed by using the Invitrogen system according to the manufacturer's instructions. Proteins were solubilized in the following isoelectric focusing buffer: 7 M urea, 2 M thiourea, 0.5% ampholytes, 2% β-dodecyl maltoside, and 10 mM DTT (62). Isoelectric focusing was carried out by using strips with a nonlinear pH range from 3 to 10. The second dimension was developed with a NuPage 4–12% Bis-Tris Zoom gel. Gels were stained with Coomassie brilliant blue G-250 (63).

Protein Spot Excision, Digestion, and Identification. Gels were scanned (Powerlook III, Umax), and spots were detected with PROGENESIS (Nonlinear Dynamics). Spots were excised by using an automated spot picker (Investigator, Genomics Solution), destained, reduced, alkylated with iodoacetamide, and digested with trypsin by using a DigestPro gel-spot-processing robot (Intavis, Langenfeld, Germany). LC/tandem MS of tryptic peptides of proteins was carried out by using a QSTAR *Pulsar i* quadrupole TOF mass spectrometer (Applied Biosystems/MDS Sciex, Toronto) equipped with a Proxeon Biosystems (Odense, Denmark) nanoelectrospray source to perform ESI-MS of the tryptic peptides as described (68). Matching of tandem MS spectra to known protein or nucleotide sequences was carried out by using a locally installed copy of the spectrum modeler X!TANDEM, an open source program that is a part of the Global Proteome Machine (64, 65). Version 2004.09.01.2 of X!TANDEM was used, with one missed tryptic cleavage and unanticipated cleavages allowed. Each search was against a flat file containing amino acid sequences of all plant proteins in HARVEST:WHEAT 1.04 (http://harvest.ucr.edu), the National Center for Biotechnology Information nonredundant green plant database, National Center for Biotechnology Information *T. aestivum* UniGene Build 37, and the wEST Database

(http://wheat.pw.usda.gov/wEST) (68). Further analysis and examination of the data were accomplished by using a locally installed copy of the Global Proteome Machine (www.thegpm.org). Reported mass spectrometer identifications from the plant protein database search had expectation values of $<1 \times 10^{-3}$. Amino acid sequence alignments for each of the potential Trx-target proteins were generated by using BLASTP (66) and CLUSTALW (67) to identify conserved cysteines that could form disulfide bonds.

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