

Introduction of the Disulfide Proteome: Application of a Technique for the Analysis of Plant Storage Proteins as Well as Allergens[†]

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Redox regulation plays an important role across a broad spectrum of biology. Accumulating evidence suggests that thioredoxin, a widely distributed redox enzyme, participates in the redox control of numerous target proteins, thus, playing a key role as a signaling intermediate that senses the metabolic state or environmental changes, and transmits information to induce appropriate reactions for survival. The recent development of proteomic strategies has facilitated the identification of thioredoxin targets to allow clarification of new thioredoxin-dependent redox mechanisms. This review examines the usefulness of a disulfide proteome technique for the analysis of thioredoxin-dependent redox regulation as well as for use in allergen studies.

Keywords: Allergen • disulfide proteome • redox regulation • thioredoxin

Introduction

Intracellular redox status is an important parameter through which eukaryotes balance information from their metabolism and the environment.¹ Thioredoxins (Trxs) are small, widely distributed redox proteins involved in the regulation of numerous target proteins via thiol/disulfide exchanges, and they therefore play a key role in the maintenance of cellular redox homeostasis.^{2,3} Historically, the detection of disulfide bonds in proteins targeted by Trx was achieved by biochemical experiments. The first identification of a regulatory disulfide stemmed from the observation that ferredoxin (Fdx) increased the activity of fructose-1,6-bisphosphatase in chloroplast extracts, whereas catalytic activity *per se* did not require a reductant.^{4,5} The discovery led to the identification (1) of thioredoxin as the protein reducing the fructose bisphosphatase, and (2) of ferredoxin-thioredoxin reductase (FTR) as the enzyme transferring reducing equivalents (electrons and H⁺) from Fdx to Trx.⁶ Since this fortuitous development introduced a fairly new concept of Trx functions, research has focused on uncovering new enzymes regulated by thiol/disulfide exchange. These searches were initially based on specific activation effects observed with a nonphysiological substitute for Trx, dithiothreitol (DTT), or with dark-adapted chloroplast enzymes following exposure to light (Comprehensive Review: refs. 3, 6). Other than regulation of enzyme activities, subsequent biochemical studies clarified Trx's func-

tions in activation/deactivation of protease inhibitors,⁷ venom neurotoxins,⁸ and changing susceptibilities of proteins against proteases.^{8,9}

As evidence are accumulating to suggest that Trx plays critical roles in a broad spectrum of biology, comprehensive analyses of Trx targets are expected to lead to the clarification of redox regulation. In 2001, two major proteomic approaches based on discrete principles were developed, and they have since contributed significantly to the impressive list of the putative Trx targets.¹⁰ The affinity column strategy¹¹ allows the concentration of the targets on a column, thus, facilitating the recovery of quantitatively minor targets, while the disulfide proteome technique^{12,13} is applicable to both *in vitro* and *in vivo* analyses. After a brief introduction of these two methods, the present short review focuses on the disulfide proteome technique, specifically its applications in the analysis of the redox regulation of cereal proteins as well as its use in allergen studies.

Proteomic Approaches to the Identification of Trx Targets

Affinity Column Procedure. The affinity column procedure takes advantage of the two-step reaction mechanism¹⁴ by which Trx reduces the disulfide bonds of its targets. In the first step, the N-terminal Cys residue in the active site (-CGPC-) attacks the disulfide bond of the target, forming an unstable intermolecular disulfide bond between the Trx and the target. The second step involves an attack by the second Cys residue (-CGPC-) on the mixed disulfide intermediate, releasing the reduced target and the oxidized Trx.¹⁴ Thus, a Trx mutated in the second Cys should form a stable intermolecular disulfide complex with its target. Kouwen et al.¹⁵ report that both active-site Cys residues can form mixed disulfides with the target proteins when the other active-site Cys is absent, but only the N-terminal active-site Cys forms stable interactions. Verdoucq et al.¹⁶ substituted the second Cys residue in the active site of

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Arabidopsis Trx for Ser, followed by expression of the monocysteine Trx mutant in yeast, and successfully stabilized the Trx-target intermediate. N-terminal polyhistidine extension of the mutant helped purify the intermediate, and peroxiredoxin was identified as a new target. In another study, Motohashi et al.¹¹ immobilized the monocysteine Trx *m* on a cyanogen bromide-activated resin. The application of chloroplast extract allowed adsorption of the targets on the column, and they were recovered by the subsequent DTT treatment. Most of the identified potential targets have been found to function in 10 chloroplast processes not previously linked to Trx^{11,17} (isoprenoid, tetrapyrrole and vitamin biosynthesis, protein assembly/folding, protein and starch degradation, glycolysis, HCO₃⁻/CO₂ equilibration, plastid division, and DNA replication/transcription), as well as in the processes already known to be regulated by Trx (the Calvin-Benson cycle, nitrogen metabolism, translation, and the oxidative pentose phosphate cycle).³ The crystal structure of the monocysteine Trx–target complex¹⁸ suggests that the recognition of features around protein disulfides plays a major role in the specificity. Trx shares a mechanism with glutaredoxin (Grx) and glutathione transferase for correctly positioning substrate cysteine residues at the catalytic groups but possesses a unique structural element that allows recognition of protein disulfides.¹⁸ So the immobilized monocysteine Trx should allow selective concentration of the targets on a column.

Fluorescent Gel Approach. In parallel with the mutant affinity column procedure, Buchanan and co-workers¹² developed a fluorescent gel approach to identify potential Trx targets. They used a specific fluorescent probe, monobromobimane (mBBr),¹⁹ to label free sulfhydryl groups of cellular proteins followed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis. Proteins whose disulfide status changes are detected as an increase or decrease in the fluorescence of the respective spots. The application of this technique in the analysis of Trx targets and in *in vivo* redox regulation is discussed below.

Comparison of the Two Strategies. Wong et al.²⁰ discuss the advantages and disadvantages of the two methods. The affinity column traps interacting proteins irrespective of their abundance, allowing concentration of the targets and, thus, facilitating the detection and recovery of target proteins with low abundance, while the mutant Trx, which normally forms a mixed disulfide with an authentic target on a column, could potentially also bind a glutathionylated or oxidized cysteine residue,^{21,22} or nontarget protein with a free, exposed cysteine. Furthermore, dilution of the extract, which is required to achieve a salt concentration suitable for the affinity protocol, may lead to precipitation and the loss of hydrophobic targets. On the other hand, under the mBBr fluorescence procedure, the comparison of control and Trx-reduced samples labeled with mBBr confirms that Trx-dependent reduction has taken place. Thus, the increase in fluorescence observed in the treated sample is due, in most cases, to the reduction of a disulfide linking two intra- or, in some cases, intermolecular cysteines. However, a single spot on the 2D gel may contain more than one protein, due to insufficient separation, and a nontarget may thus be misrecognized as a target. Wong et al.²⁰ conclude that a combination of the two approaches is best in order to obtain a comprehensive overview of potential targets. The complementarity of the two approaches has also been confirmed in subsequent studies.^{23,24}

Application of the Disulfide Proteome To Clarify Plant Redox Regulation

***In Vitro* Target Analysis of Trx in Peanut Seeds.** The first method used in disulfide proteomic research was diagonal 2D-PAGE.²⁵ In its unique separation system, protein extracts are consecutively separated in two dimensions first under oxidizing conditions and then under reducing conditions. Proteins that have no disulfide bond migrate along the diagonal line, while those with inter- and intramolecular disulfide bonds migrate to the areas below and above the diagonal line, respectively. Yano et al.¹² subjected peanut extract to diagonal 2D-PAGE (Figure 1). Most peanut proteins migrated to the areas above or below the diagonal, indicating that they have intra- or intermolecular disulfide bonds (Figure 1A). When the extract was treated with DTT, all proteins migrated along the diagonal line and were detected as fluorescent spots (Figure 1B). When the extract was treated with Trx *h*, only its targets, in this case intramolecular disulfide proteins, were reduced and migrated to the diagonal line, appearing as fluorescent spots (Figures 1C,D). Thus, the diagonal 2D system shows whether the targets have intra- or intermolecular disulfide bonds. Some proteins increased their fluorescence but migrated to the area above the diagonal line, indicating that they are partly reduced targets with intramolecular disulfide bonds (Figure 1C). On the other hand, when the same samples were separated using the isoelectric focusing/sodium dodecyl sulfate (IEF-SDS) 2D gel system, the higher resolution allowed the detection of 20 targets as spots with increased fluorescence (Figure 2). Five were identified: three allergens (Ara h2, Ara h3 and Ara h6), a desiccation-related protein and a seed maturation protein.¹² The desiccation-related protein is of particular interest since it is one of 5 proteins induced upon the desiccation of *Cratogeomys plantagineum* (a resurrection plant).^{26,27} Under desiccation conditions, the protein is reported to be synthesized *de novo* in the leaves of this desiccation-resistant plant and to disappear upon rehydration. The reduction of its intramolecular disulfide bond by Trx may be linked to the degradation of the protein during rehydration. The seed maturation protein shows both sequence homology to and activity characteristic of glucose and ribitol dehydrogenase.²⁸ The present results raise the question of whether reduction by Trx *h* alters activity and thereby serves to regulate the enzyme. The finding that 3 peanut allergens (Ara h2, Ara h3 and Ara h6) are reduced by Trx opens the possibility that their allergenicity may be mitigated by Trx.^{9,29}

***In Vivo* Analyses of Rice Seed Germination.** The fluorescent gel system is also applicable to *in vivo* analyses. Yano et al.³⁰ followed the degradation of rice glutelin in seed germination (Figure 3). Rice glutelin is composed of acidic (red arrows) and basic chains (blue arrows) which are interconnected by a single disulfide bond. The acidic chain also has an intramolecular disulfide bond. The basic chain contains a free sulfhydryl group, so it is detectable as a fluorescent spot. Because the full complement of responsible proteases in germination is not expressed without an embryo,³¹ a comparison was made between the 2D profiles of imbibed complete and de-embryonated seeds in an attempt to trap intermediates in the degradation process.³⁰ Monitoring the glutelin degradation in seed germination made it possible to visualize the processes with respect to the disulfide bonding (Figure 3). Until 5 days after imbibition, rice glutelin underwent truncation of the acidic chain, while the basic chain and both the intra- and intermo-

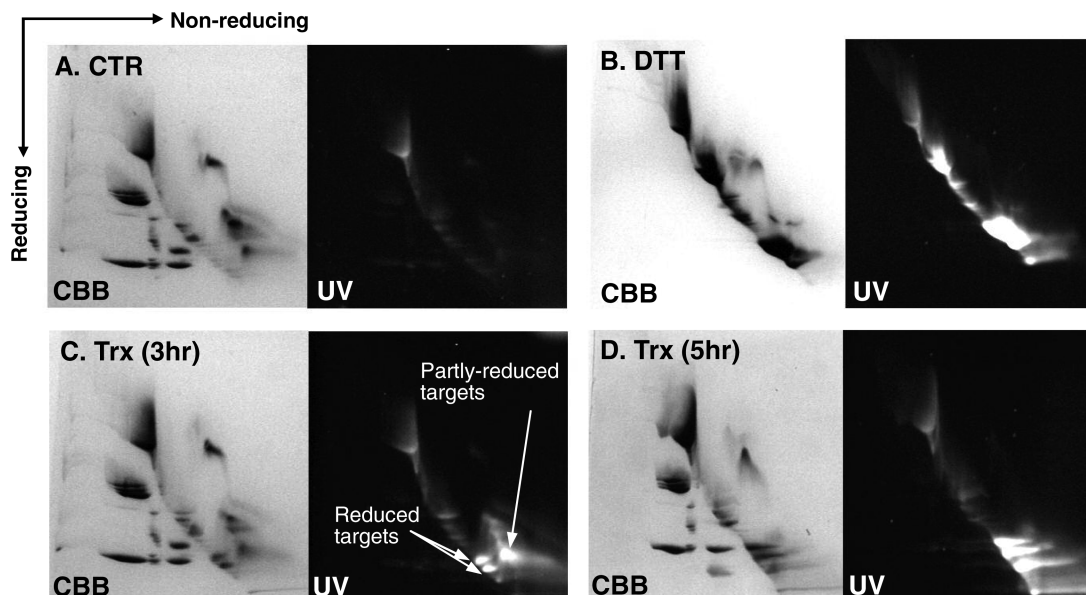


Figure 1. Analysis of peanut seed proteins following reduction by the NADP/thioredoxin system or DTT and labeling with mBBr using nonreducing/reducing 2D-PAGE. Peanut protein was incubated in the following conditions. (A) Control, no-addition; (B) DTT, the sample was heated in boiling water after addition of DTT; (C) Trx (3 h), incubation for 3 h in the presence of NADPH, NTR and Trx; (D) Trx (5 h), incubation under the same conditions as C, except for 5 h. Reprinted with permission from ref 12, with modification. Copyright 2001 National Academy of Sciences, U.S.A.

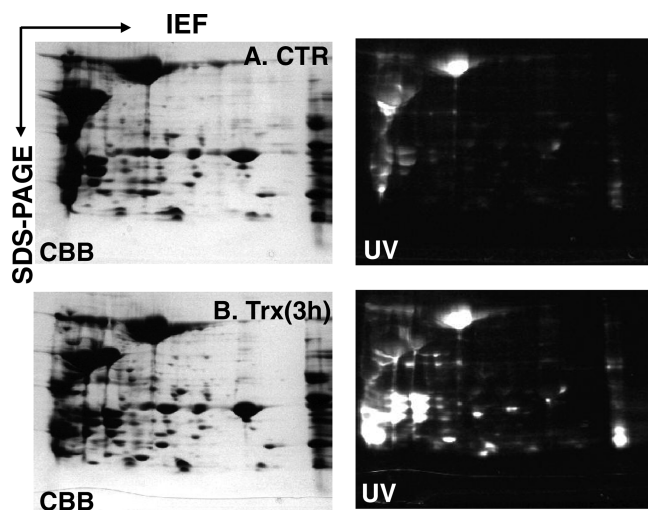


Figure 2. Analysis of peanut seed proteins following reduction by the NADP/thioredoxin system and labeling with mBBr using IEF-SDS 2D-PAGE. Peanut protein was incubated in the following conditions. (A) Control, no-addition; (B) Trx (3 h), incubation for 3 h in the presence of NADPH, NTR and Trx. Reprinted with permission from ref 12, with modification. Copyright 2001 National Academy of Sciences, U.S.A.

lecular disulfide bonds remained intact (Figure 3A). After truncation, at approximately 7 days after imbibition, both the intra- and intermolecular disulfide bonds were reduced, and the products of truncation were degraded to smaller pieces (green arrows). These observations suggest that, like proteolysis, reduction of glutelin occurs in an ordered fashion during germination (Figure 3B). First, limited proteolysis truncates the individual glutelin molecules, thereby disrupting the original ordered macromolecular conformation³² and making the protein more soluble and the disulfide bonds of its fragments accessible to reduction. Next, the intermediates are reduced and become susceptible to complete proteolysis. This mech-

anism should allow both compact packaging in the protein body and ready mobilization. In this way, the disulfide proteome monitors the behavior of proteins *in vivo* with respect to its disulfide status.

Degradation of Aleurone Proteins in Seed Germination.

Focused observations of bran proteins in seed germination have allowed the elucidation of Trx-linked exquisite mobilization mechanisms. Yano and Kuroda³³ examined Trx-dependent reactions in the rice bran, as it is rich in aleurone layer, one of the key organs for seed germination. They obtained buffer-soluble extract from rice bran, and endogenous Trx in the extract was activated by adding nicotinamide adenine dinucleotide phosphate (NADPH) and NADPH-dependent Trx-reductase (NTR) (Figure 4). The effect of activation was monitored by the disulfide proteome technique. The embryo-specific protein (ESP), one of the major soluble proteins in bran (Figure 4A, spots 1–3), disappeared upon activation of endogenous Trx in the extract, while globulins (spots 4, 5), the other major proteins, remained intact (Figures 4A,B). When Trx activation was conducted in the presence of leupeptin, a Cys-protease inhibitor, the ESP spots did not disappear but appeared as fluorescent spots, while they were nonfluorescent in the control sample (Figures 4A,C). These observations suggest that Trx unfolded the ESP, a specific substrate of the Cys-protease, by reducing its disulfide bond(s). Yet, the question remains of whether Trx activates the Cys-protease that digests the ESP. It is possible that the protease activity is Trx-independent, but that the folded structure, due to disulfide bonding, rendered the ESP resistant to the protease. To investigate whether the cysteine protease is activated by Trx, unfolded and mBBr-labeled ESPs (i.e., “locked” in its unfolded state) were subjected to the bran extract in the absence or presence of active Trx. The fluorescent ESP spots disappeared when the endogenous Trx was activated by the addition of NADPH and NTR, suggesting that the protease activity is Trx-dependent. Leupeptin impeded degradation, allowing us to confirm that Cys

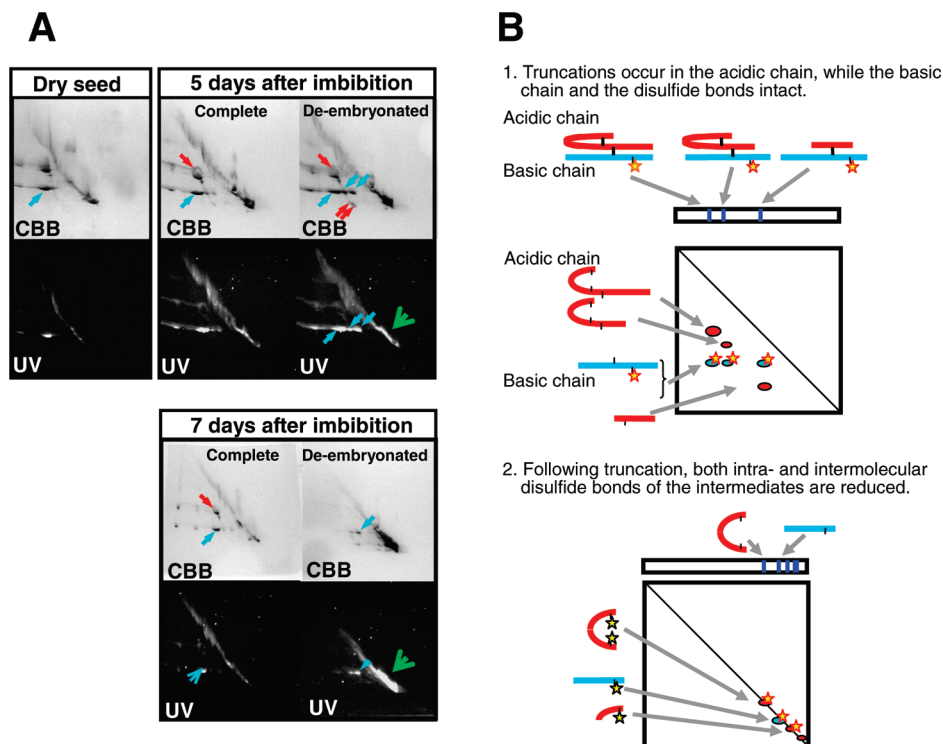


Figure 3. Monitoring glutelin degradation in seed germination using nonreducing/reducing 2D-PAGE. Buffer-soluble proteins were extracted from germinating complete rice seed or imbibed de-embryonated rice seed. (A) Free sulfhydryl groups of proteins were fluorescently modified with mBBR. (B) Explanatory figure of the protein profiles in A. Reprinted with permission from ref 30, with modification. Copyright 2001 Oxford University Press.

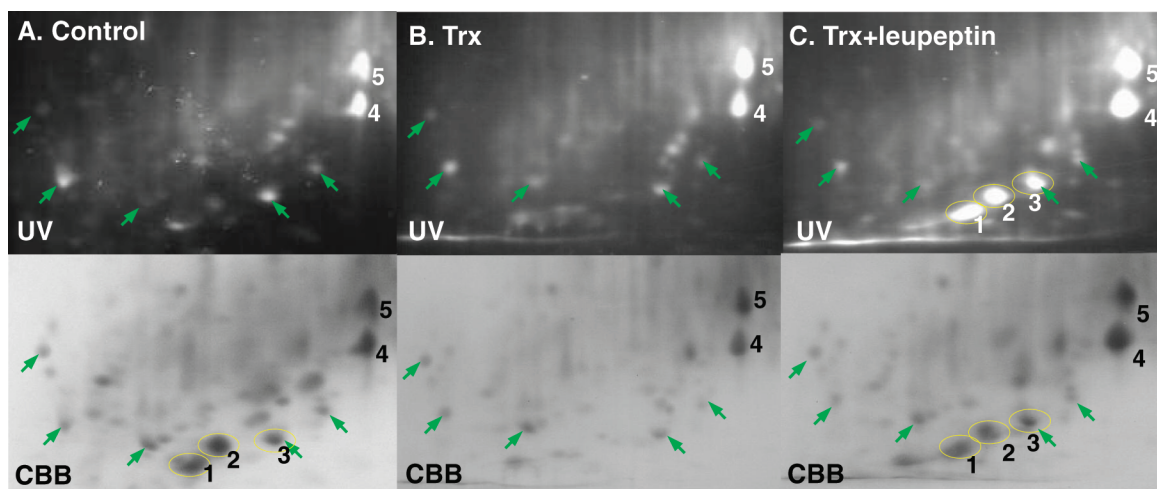


Figure 4. Analysis of rice bran proteins after reduction by the NADP/thioredoxin system and labeling with mBBR using IEF/SDS 2D-PAGE. Buffer soluble rice bran protein was incubated in the following conditions. (A) Control, no addition with 3 h incubation; (B) Trx, incubation for 3 h in the presence of NADPH and NTR; (C) Trx+leupeptin, incubation under the same condition as B, except in the presence of leupeptin. After electrophoresis, each gel was examined under the UV lamp (UV) or stained with CBB. Green arrows: landmarks. Reprinted with permission from ref 33, with modification. Copyright 2006 Wiley-VCH Verlag GmbH & Co. KGaA.

protease is responsible for degradation. Next, to investigate whether the same reaction occurs *in vivo*, isolated rice bran was imbibed in the presence of CaCl_2 and gibberellic acid. The ESP disappeared in 16 h incubation while globulins remained intact. When the incubation was carried out in the presence of leupeptin, the ESP remained as fluorescent spots. These *in vivo* and *in vitro* results correlate well suggesting that Trx controls enzyme reactions by acting on both the enzyme and its substrate. A question prompted by the study is whether it is appropriate to use the *in vitro* Trx reaction system to infer

in vivo reactions in seed germination. Available evidence supports that Trx plays important roles in seed germination. Trx exists in the aleurone cells of germinating wheat.³⁴ During germination of wheat, Trx is converted from the oxidized (inactive) to the reduced (active) form.³⁵ In germinating wheat, Trx activates thiocalcin, a serine protease specific for indigenous seed storage proteins.³⁶ Overexpression of Trx in barley endosperm enhances the activity of pullulanase³⁷ and α -amylases,³⁸ and also accelerates germination.³⁸ All these observations suggest that Trx plays critical roles in seed germination,

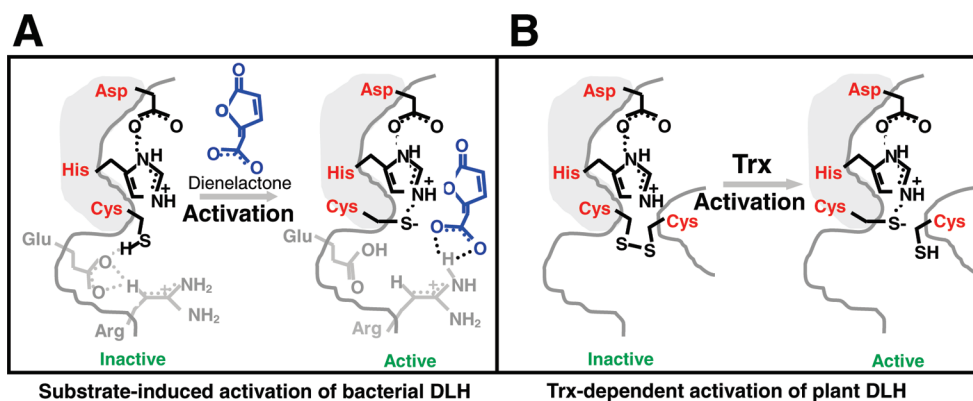


Figure 5. Comparative figure of plant and bacterial DLHs. Reprinted with permission from ref 77, with modification. Copyright 1995 Oxford University Press.

and justify correlating the Trx-linked *in vitro* reactions with seed germination. Besse et al.³⁶ report that Trx activates Ser-protease in barley seed germination to degrade globulins. The substrate specificity and regulation of protease activity suggest the presence of a Trx-dependent mobilization mechanism through which specific seed proteins are degraded in specific stages of germination. It is likely that Trx thus fine-tunes the degradation of storage proteins in seed germination.

Regulatory Mechanism of Rice Dienelactone Hydrolase. Disulfide proteome analysis suggests a new activation mechanism of dienelactone hydrolase (DLH) in higher plants.³³ In bacteria, the protein detoxifies dienelactone, an environmental toxin, to nontoxic maleylacetate. Bacterial DLH is reported to undergo a unique substrate-induced activation³⁹ (Figure 5A): In the absence of dienelactone, Glu and Arg near the active site interact with Cys in the active site to inactivate DLH, presumably to prevent auto-oxidation.^{40,41} DLH is activated upon the appearance of dienelactone. On the other hand, in the case of rice DLH, the Cys in the active site forms an S–S bond with another Cys near the active site (Figure 5B). During germination, Trx reduces the disulfide bond to activate the enzyme. The reactive property that makes Cys suitable for redox reactions and thiol-disulfide interchanges occasionally makes the amino acid extremely vulnerable to oxidation. Although the role of DLH in higher plants remains unclear, bacteria and plants seem to have obtained discrete mechanisms to protect DLH from auto-oxidation.

Improvement of the Disulfide Proteome. While the disulfide proteome has been utilized successfully in identifying putative targets in seeds,^{20,23,42,43} wheat amyloplasts,²⁴ the inner envelope membrane of chloroplasts,⁴⁴ the extracellular matrix of *Nicotiana glauca*,⁴⁵ and plant mitochondria,⁴⁶ and in investigating the mouse kidney,⁴⁷ improvement of the fluorescent gel methods is also in progress. To increase sensitivity, Maeda et al.⁴⁸ replaced mBBR (5000 M⁻¹ cm⁻¹) with high-absorption-coefficient Cy5 maleimide (250 000 M⁻¹ cm⁻¹). Marchand et al.⁴⁹ used ¹⁴C-labeled iodoacetamide to allow high sensitivity detection, while Chan et al.⁵⁰ applied 2D-difference gel electrophoresis (DIGE) technology to redox proteomics by synthesizing a pair of thiol-reactive iodoacetyl derivatives of Cy3 and Cy5. Leichert and Jakob⁵¹ adopted a differential thiol-trapping strategy.

Other Approaches to the Identification of Trx Targets

Affinity Chromatography To Utilize the Electrostatic Interaction between Trx and Targets. Mora-Garcia et al.⁵² have shown that the electrostatic interactions between Trx *f* and

some of its target proteins induce the formation of a noncovalent Trx–target complex, which is needed for the subsequent thiol/disulfide exchange. Immobilization of Trx *f* on agarose allowed Balmer et al.⁵³ to identify 27 previously unrecognized proteins functional in 11 chloroplast processes. These results provide evidence in favor of the hypothesis that an association with Trx allows the interacting target to achieve its optimal conformation to facilitate the transfer of reducing equivalents from the Fdx/FTR complex to a target protein to enable the channeling of metabolite substrates in some cases, and to function as a subunit in the formation of multienzyme complexes.⁵³

Yeast Two-Hybrid Systems. Classical yeast two-hybrid systems are not useful with most Trx's due to the presence of endogenous Trx in yeast that presumably competes with Trxs introduced as bait. Therefore, Vignols et al.⁵⁴ used a yeast strain in which cytosolic Trx genes were inactivated successfully isolating known Trx targets and putative ones such as peroxiredoxins AHP1 and TSA1, as well as phosphoadenosine-5-phosphosulfate (PAPS) reductase MET16, which were not detected using classical strains.

How To Confirm Specific Targets?

The application of proteomic strategies has increased the number of putative targets of Trx/Grx to approximately 300.^{2,3} These targets are implicated in almost all pathways, including the housekeeping metabolism, photosynthesis, abiotic and biotic stress defense and development. The data demonstrate an unexpected involvement of dithiol–disulfide regulation in almost all processes of plant life.² Nevertheless, proteomic methods are almost as specific but do not warrant interactions between a reductant and its putative targets *in vivo*.² Furthermore, knowledge lags behind on the individual roles played by Trx and NTR isoforms. In *Arabidopsis*, 40 genes encoding Trx and Trx-related proteins have been identified.⁵⁵ Compared to the extensive studies on the chloroplastic Trx system, genetic methods of identifying the functions of cytosolic Trxs are limited due to the existence of identical phenotypes among single mutants, presumably resulting from functional redundancies among Trx and even Grx members.^{55–57} To address this issue, several studies have been conducted. Using Trx *f* mutants in which surface charges near the active site were modified, Mora-Garcia et al.⁵² showed that the electrostatic interactions between Trx and some of its target proteins control the formation of the binary noncovalent complex needed for the subsequent thiol/disulfide exchange. The crystal structure of the Trx/barley alpha-amylase/subtilisin inhibitor (BASI) com-

plex reported by Maeda et al.¹⁸ suggests a conserved hydrophobic motif in Trx interacting with BASI through van der Waals contacts and backbone-backbone hydrogen bonds. Maeda et al.⁵⁸ also report that charge complementarity between specific amino acids in both Trx and the target near the interface plays a role in the specificity of Trx. These three reports demonstrate that the recognition of features around protein disulfides plays a major role in the specificity and protein disulfide reductase activity of Trx.

Yeast complementation by Trx demonstrates specific interactions between plant Trxs and their targets in yeast. Five Trx *h*'s were tested for their ability to complement phenotypes of a yeast mutant in which two endogenous cytosolic Trx genes were inactivated. Although all exogenous Trx *h*'s restored a normal cell cycle, only Trx *h* 2 restored sulfate assimilation (activation of PAPS reductase), and only Trx *h* 3 restored H₂O₂ tolerance (activation of peroxiredoxins).⁵⁹ These findings indicate that specific interactions between plant Trx's and their targets occur *in vivo*.

Other than molecular specificity, the expression pattern of each Trx gene is also expected to be an important factor in determining specific functions during the development or in response to environmental changes or stresses. The following observations suggest that Trx isoforms have a distinct spatiotemporal appearance, as well as distinct kinetic properties and regulatory systems. Gene expression analysis showed that Trx *y* 2 is expressed primarily in leaves and is induced by light, while Trx *y* 1 is expressed primarily in nonphotosynthetic organs, especially in seeds during the stage of major accumulation of storage lipids.⁶⁰ Maeda et al.⁶¹ report that barley seeds contain two distinct Trx *h* isoforms, *h* 1 and *h* 2, the former expressed mainly in endosperm, aleurones and embryos, and the latter in embryos. Their abundance profiles during germination and kinetic properties against NTR are also different. Shahpiri et al.⁶² report that, although the mRNA of both Trx *h* 1 and Trx *h* 2 isoforms is present in barley embryos and aleurone layers, the corresponding proteins differ in their spatiotemporal appearance. Additionally, the gene expression of HvNTR2, but not HvNTR1, is suggested to be regulated by gibberellic acid.

These studies not only provide evidence for the specificity between reductants and their targets, but also suggest ways to determine the authenticity of candidates (i.e., whether they have the reductant–target relation *in vivo*). Furthermore, in a recent study with germinating seeds of the legume *Medicago truncatula*, more than half of the 43 potential Trx targets identified by the mBBR-labeling procedure were reduced during germination.²³ These results suggest the validity of the disulfide proteome approach, since this technique is able to identify putative Trx targets as well as to confirm their reduction in *in vivo* reactions.

Application to Allergen Studies

As we have observed, cereal storage proteins, having a compact structure with multiple disulfide bonds, are degraded in a sophisticated way upon seed germination, like tangled yarn being unraveled before being cut into small pieces. On the other hand, when we eat cereals as food, it is like cutting the tangled yarn directly with scissors. This leads to the occasional production of protease-resistant fragments in the digestive system, fragments that may be adsorbed accidentally through the intestine and that may be allergens. Therefore, we extended our research field to include allergen studies.

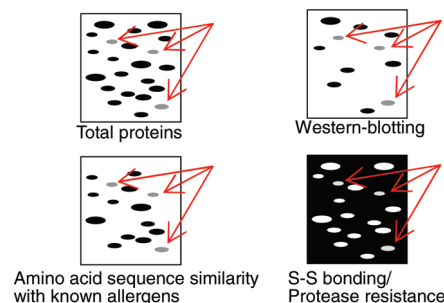


Figure 6. Strategy to identify allergens.

Screening of Allergens. Accumulating evidence suggests that disulfide bonds are responsible for the allergenicity of certain proteins.⁶³ Using a canine model, Buchanan, Frick and colleagues found that the reduction of the disulfide bonds of wheat²⁹ and milk,⁹ respectively, rendered several proteins less allergenic. Sen et al.⁶⁴ report that peptide fragments of Ara h2 that survived *in vitro* digestion by gastrointestinal proteases contained disulfide bonds and intact IgE-binding epitopes. They also report that the reduction of the disulfide bonds rendered Ara h2 susceptible to protease digestion. By disulfide proteome analysis, the allergenic fractions of peanut¹² and rice⁶⁵ were found to be composed primarily of disulfide proteins. All of these observations suggest a positive correlation among disulfide bonding, protease resistance and allergenicity. Thus, we conducted a feasibility study to see whether specific labeling of disulfide proteins can be applied to allergen screening. After alkylation of free SH groups by iodoacetamide, disulfide proteins in crude extracts of mite, pollen⁶⁶ and buckwheat⁶⁷ were reduced to expose free sulfhydryl groups which were selectively labeled with mBBR. Fluorescent proteins detected on the subsequent 2D gel were then subjected to internal amino acid sequence analysis. Most proteins were known allergens or proteins sharing high amino acid sequence similarities with known allergens. Additionally, all of the major protease-resistant fragments of the buckwheat allergen contained disulfide bonds.⁶⁷ These results demonstrate that the protocol is applicable as a preliminary screen for new allergens, although disulfide proteins are not necessarily allergenic.

Current studies designed to detect new allergens rely on the 2D analysis of allergenic fractions and immunoblotting using patient sera. Although this method has been used successfully to identify allergens,^{68–70} some may have been missed due to variations in reactivity between the sera of different patients or decreases in potency due to denaturation and blotting. Thus, disulfide proteome analysis may be useful as a supplemental tool to identify allergens that have escaped conventional methods.

To develop less allergenic cereals by cross-breeding, breeders must select a limited number of allergens to be removed, since it is technically difficult to remove large numbers of allergens. At our institution, we investigate allergens through several different approaches including Western blotting with patient sera, amino acid sequence similarity with known allergens, and disulfide-bonding/protease resistance (Figure 6). The proteins identified by each method are considered candidates. However, if a given proteins is detected by all of these methods (red arrows), it is highly likely that it is allergenic.

Reduction of allergen disulfide bonds by Trx has been applied successfully to alleviate their allergenicity.^{9,29} In these studies, using canine colonies, allergenicity was assessed

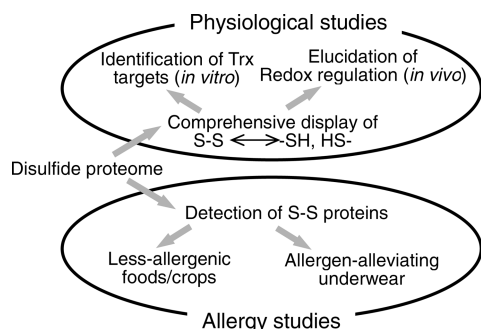


Figure 7. Schematic view of the disulfide proteome studies.

quantitatively by the hypersensitive skin test response (wheat) as well as by feeding challenges leading to vomiting and diarrhea (milk). Meanwhile, some recent studies based on IgE binding of sera from patients with baker's asthma suggest the allergenicity of Trx itself.^{71,72} Although the role of Trx in allergies is expected to be resolved in future studies,⁷³ industrial applications of Trx would have unlimited potential to alleviate allergens.

Further Practical Applications. Further studies are in progress. Phthalocyanine (Pc)-dyed fiber is reported to reduce atopic symptoms in some patients when they use underwear made of the fiber.^{74,75} In collaboration with Daiwabo Neu Co., Ltd. (Osaka, Japan), we have found that Pc fiber adsorbs house dust/pollen/food allergens with varied molecular weights and pI.⁷⁶ Disulfide proteome analyses have revealed that Pc fiber does not change the molecular weight or disulfide bonding of the adsorbed allergens.⁷⁶ These studies suggested that Pc fiber functions as a versatile “allergen trap”, a discovery that led to the commercialization of AllerCatcher AD, an allergen-alleviating underwear, which was released on the market in 2006.

Concluding Remarks

Disulfide bonds play a critical role in protein chemistry, sustaining not only protein conformations, but also dynamic redox regulation. The development of the disulfide proteome has added a new dimension to the analysis of protein chemistry, the clarification of redox regulation and allergen studies. A schematic view of disulfide proteome studies is shown in Figure 7.

Abbreviations: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; BASI, barley alpha-amylase/subtilisin inhibitor; DLH, diene lactone hydrolase; DTT, dithiothreitol; ESP, embryo-specific protein; Fdx, ferredoxin; FTR, ferredoxin-thioredoxin reductase; Grx, glutaredoxin; IEF-SDS, isoelectric focusing/sodium dodecyl sulfate; Mbb, monobromobimane; NADPH, nicotinamide adenine dinucleotide phosphate; NTR, NADPH dependent Trx-reductase; PAPS, phosphoadenosine-5-phosphosulfate; Pc, Phthalocyanine; Trx, thioredoxin.

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