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Short communication

Determination of *in vivo* disulfide-bonded proteins in *Arabidopsis*

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ABSTRACT

Protein thiol-disulfide oxidoreduction plays an important role in redox regulation of cellular processes. Here we present a proteomic approach to visualize and map *in vivo* disulfide-bonded proteins in plants. A proteomic map of the disulfide-bonded proteins was achieved using 2D gel electrophoresis of *Arabidopsis* protein extract. Along with novel proteins identified as potentially redox regulated, we have also shown the feasibility of mapping some of the cysteines involved in the formation of disulfide bonds. This study presents an important tool for characterizing redox-regulated proteins.

1. Introduction

Redox regulation of thiol groups is one of the many protein post-translational modifications that universally occur in myriad of cellular processes. For example, changing the redox state of protein thiol groups serves as regulatory switches of protein functions in carbon storage [1], photosynthesis [2] and leaf senescence [3]. This redox regulation is known to be mediated by several systems, including the ferredoxin–thioredoxin system and the glutathione–glutaredoxin system [2,4].

Proteomic approaches have been applied to identify protein targets of thioredoxin and glutaredoxin function [4,5]. There are two major techniques, affinity purification with mutant thioredoxin column and gel separation of proteins with thiol groups fluorescently labeled with monobromobimane (mBBr). In plants, the disulfide proteome was not well studied. A Korean group investigated the *Arabidopsis* disulfide proteome using a thiol-affinity purification procedure [6], which is different from the above two major techniques used for identifying thioredoxin and glutaredoxin targets. Although some interesting disulfide-bonded proteins were identified, the technique displayed limitations. Because affinity chromatography was used to isolate disulfide proteins, information about the original protein characteristics such as molecular weight (Mw) and isoelectric point (p*I*) were missed. In addition, it was not possible to determine the dynamics of protein redox state changes and to discriminate protein redox changes from overall expression changes.

Here we report a 2D gel proteomic approach that eliminates the above limitations in analyzing *in vivo* disulfide proteins. We utilized mBBr as a fluorescent dye to label the thiol groups of proteins obtained after alkylation of free thiol groups and reduction of disulfide bonds. The labeled proteins were separated on 2D gels. After visualization of disulfide-bonded proteins, total proteins were stained with SyproRuby to compare the levels of protein expression with the levels of mBBr signal. We have established a proteomic map of potential redox-regulated proteins from *Arabidopsis*, identified new disulfide-bonded proteins, and demonstrated the utility in mapping the cysteines involved in disulfide bond formation.

2. Experimental

2.1. Seedling growth

Seeds from *Arabidopsis thaliana* ecotype Col-0 were sterilized using 50% bleach for 10 min, followed by washing four to five times with sterilized water. Seeds were germinated on a half strength Murashige–Skoog agar medium containing 1% sucrose and transferred to a growth chamber under a photosynthetic flux of 140 μmol photons m^{-2} s^{-1} with a photoperiod of 16 h at 24 °C and 18 °C at night for 10 days. Shoots were dissected, weighed and immediately frozen in liquid nitrogen.

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2.2. Protein TCA precipitation and quantification

Shoot samples (approximately 100 mg fresh weight, three replicates) were ground to a fine powder in liquid nitrogen. One millilitre of 10% TCA in acetone was added, followed by incubation on ice for 30 min. After centrifugation at 14,000 rpm for 10 min at 4 °C, the supernatant was removed and 1 mL of cold acetone was added. The samples were precipitated for 45 min at −20 °C. The acetone precipitation step was repeated. The pellets were resuspended in 5 M urea, 2 M thiourea, 2% CHAPS, 2% SB 3-10, 40 mM Tris, 0.2% Bio-Lyte 3/10 ampholyte (Biorad, USA). Protein amounts were determined using a CB-X Protein Assay kit (GenoTech, USA).

2.3. mBBr labeling of protein thiols involved in disulfide bond formation

Two hundred microlitres of alkylation buffer (100 mM Tris–HCl pH 7.5, 200 mM iodoacetamide) were added to 250 μg protein sample and incubated for 1 h in the dark. Proteins were then precipitated by adding 1 mL 80% cold acetone at −20 °C for 1 h. The pellets were resuspended in 200 μL of reduction buffer (100 mM Tris–HCl pH 7.5, 10 mM DTT) and incubated for 1 h at room temperature. Labeling of the proteins was performed by adding 20 μL of mBBr solution (1 μg/100 μL) to each sample. The samples were then incubated for 30 min at room temperature in the dark. The labeling was terminated by adding 10 μL of 10% SDS. Proteins were then precipitated in 1 mL 80% acetone at −20 °C overnight.

2.4. One dimensional (1D) and two dimensional (2D) gel electrophoresis

Protein pellets were resuspended in Laemmli loading buffer (BioRad, USA) and run on 12% Precise gels (Pierce, USA). The mBBr-labeled proteins were visualized and imaged under 365 nm UV light. The gels were then placed in SyproRuby solution and stained according to manufacturer's instructions (Molecular Probes, USA). The total proteins were visualized using a Typhoon 9410 laser scanner (GE Healthcare, USA).

For 2D gel electrophoresis, the samples were resuspended in a destreak rehydration solution (GE Healthcare, USA). One hundred and fifty micrograms of proteins were loaded onto 11 cm immobilized pH gradient gel strips (pH 3–10 NL). Isoelectric focusing and gel electrophoresis were performed as previously described [7].

2.5. Protein identification using liquid chromatography–tandem MS (LC–MS/MS)

Spots from gels were excised and digested by trypsin as previously described [8,9]. Ten microliters of protein digests were injected onto a capillary trap column (PepMap, Dionex, USA) and desalted for 5 min before being loaded onto a C18 PepMap nanoflow column. The elution gradient started at 3% solvent A (0.1%, v/v acetic acid, 3%, v/v ACN and 96.9%, v/v H_2O), 97% solvent B (0.1%, v/v acetic acid, 96.9%, v/v ACN and 3%, v/v H_2O) and finished at 60% solvent A, 40% solvent B for 20 min. MS analysis was carried out on a quadrupole-time of flight mass spectrometer (QSTAR XL, Applied Biosystems, USA). The focusing potential and ion spray voltage were set to 275 and 2600 V, respectively. The information-dependent data acquisition (IDA) was employed in which a survey scan from m/z 400–1500 was acquired followed by collision-induced dissociation (CID) of three most intense ions. Survey scan and each MS/MS spectrum were accumulated for 1 and 3 s, respectively. The spectra were searched against NCBInr database (version 22 June 2007) using Mascot software (http://www.matrixscience.com). Unambiguous identification was judged by the number of peptide sequences, sequence coverage, Mascot score, and the quality of tandem MS spectra [9].

3. Results

3.1. Validation of the mBBr labeling procedure

The method used to identify the disulfide-bonded proteins consists of alkylation of all free thiol groups with iodoacetamide, and reduction of all the disulfide bonds with DTT. The resulting thiol groups are then labeled with mBBr. After separation of the labeled proteins on gels, the disulfide-bonded proteins can be visualized under 365 nm UV light. In order to confirm that the alkylation of free thiol groups and the reduction of disulfide bonds were complete before mBBr labeling, shoot protein extracts were aliquoted and subjected to alkylation, reduction, or alkylation and reduction steps before mBBr labeling (Fig. 1A). Fig. 1B shows that after alkylation followed by labeling, few proteins were labeled by mBBr. In contrast, when the sample was totally reduced, most of the proteins reacted with mBBr. The intensities of the gel bands from the samples that were not alkylated were higher than those from the alkylated and reduced samples. The SyproRuby stained image showed that not all the proteins were labeled with mBBr (Fig. 1B).

3.2. Identification of disulfide-bonded proteins in Arabidopsis shoots

Proteins extracted from *Arabidopsis* shoots were labeled with mBBr and resolved on 2D gels. In order to discriminate the differences between protein mBBr labeling and the overall expression levels, the gels were stained with SyproRuby fluorescent stain. Fig. 2 shows an example of 2D gel separation of proteins labeled by mBBr, and later stained with SyproRuby. A total of 165 protein spots were detected after mBBr labeling out of 290 spots revealed by SyproRuby staining. Forty spots labeled with mBBr were not detected by SyproRuby staining, indicating these were low abundance proteins. A total of 125 spots did not show signal after mBBr labeling, indicating that the proteins are highly reduced and do not contain disulfides.

Forty spots on the 2D gel with decent reproducible mBBr signals were excised for protein identification using LC–MS/MS. A total of 63 proteins were identified out of 36 spots picked (Supplemental Table 1). This is because multiple proteins were identified from a single gel spot (spots # 2196, 2213, 2278, 2413, 2604, 2668, 2785, 3053, 3058, 3063, 3081, 3110 and 3128). With the increased sensitivity and resolution of modern mass spectrometers, it becomes common to identify multiple proteins in one 2D gel spot [8,9]. The sequences of the proteins identified were analyzed for the presence of cysteine residues. The number of cysteines in each protein was listed in Supplemental Table 1. As expected, 50 proteins identified did contain cysteines. However, 13 proteins did not have cysteines. Eight of them were identified as labeled by mBBr because each was separated at the same pI and Mw as a disulfide-bonded protein. Five of them were identified without being associated to mBBr labeled proteins. The probable explanation is that the mBBr-labeled protein comigrating with the protein was of lower abundance and was not identified. Most of the proteins identified were previously reported as thiol/disulfide containing proteins [2,4,10–12]. We identified five proteins with disulfide bonds that had never been reported before as potential targets of redox regulation. Among them, we identified one protein involved in programmed cell death, mosaic death 1 (MOD1), one protein involved in photorespiration, glutamate:glyoxylate aminotransferase 1 (GGT1), one universal stress protein and two uncharacterized proteins.

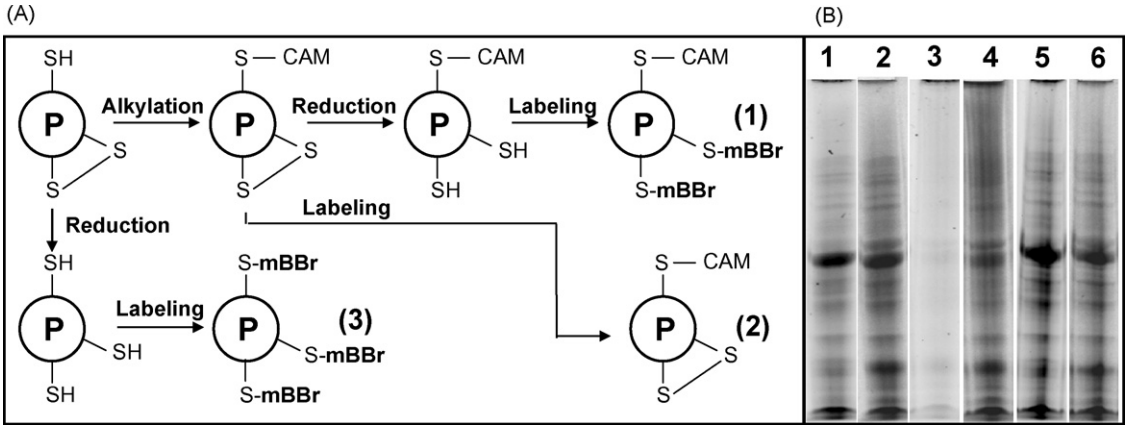

Fig. 1. Test of mBBr labeling procedure using proteins extracted from *Arabidopsis*. (A) Outline of mBBr labeling procedure; (B) proteins labeled with mBBr (lanes 1, 3 and 5) and counterstained with SyproRuby (lanes 2, 4 and 6). Lanes 1 and 2, the complete labeling procedure was followed. Lanes 3 and 4, the proteins were alkylated and labeled. Lane 5 and 6, the proteins were reduced and labeled.

3.3. Characterization of cysteines involved in disulfide bonds

Labeling with mBBr allows for convenient visualization of disulfide-bonded proteins on 2D gels and efficient identification of the proteins. Meanwhile, from the MS/MS spectra acquired for protein identification, the cysteine residues involved in the disulfide bond formation could be mapped. In this study, two cysteine residues were confidently identified in two different proteins. The C_{192} in peptide AVYE**C**LR derived from a ribulose bisphosphate carboxylase and C_{41} in peptide GPQSPSGYS**C**K from a germin-like protein were modified by mBBr (Fig. 3). Using a disulfide bond prediction software GDAP (Genomic Disulfide Analysis Program) [13], the two cysteines found modified by mBBr were predicted to be involved in the formation of a disulfide bond C_{172}–C_{192} in ribulose bisphosphate carboxylase and a disulfide bond C_{26}–C_{41} in the germin-like protein.

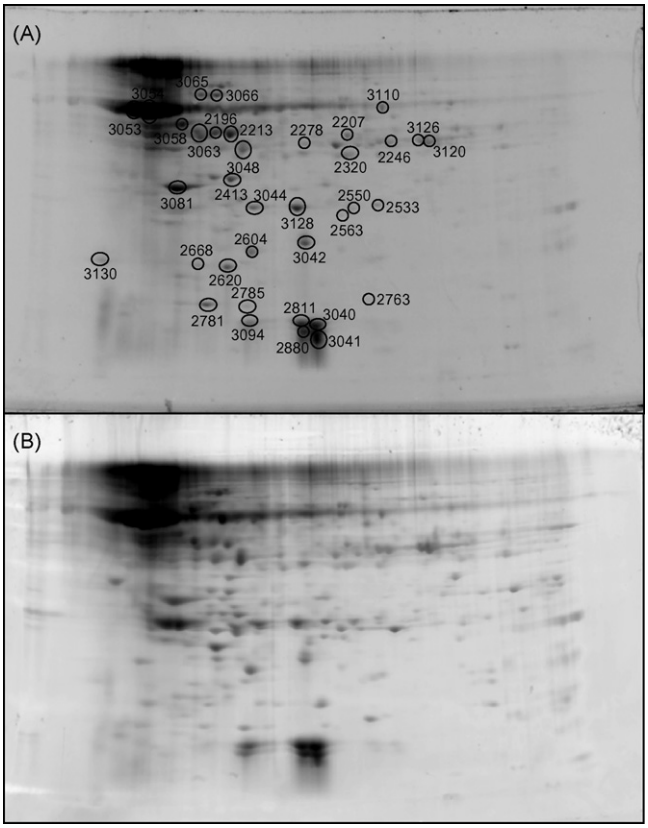

Fig. 2. 2D gel map of disulfide-bonded proteins and total proteins from *Arabidopsis*. (A) Gel pattern of disulfide-bonded proteins. The spot numbers indicate positive protein identification and correspond to those reported in Supplemental Table 1; (B) gel pattern of total proteins.

4. Discussion

By applying mBBr labeling and MS to the study of *Arabidopsis* shoot proteins, we have demonstrated the utility of this method in identifying potential redox proteins and mapping the cysteine residues involved. Out of the 50 proteins that we identified potentially regulated by reduction–oxidation, most were previously characterized as the targets of thioredoxins or glutaredoxins [2,4]. Another mechanism of redox regulation is S-glutathionylation of proteins, a process also known as thiolation [14]. In this study, we identified three protein targets of thiolation modification. The first one is the dehydroascorbate reductase (DHAR). This enzyme involved in the reduction of dehydroascorbate (DHA) into ascorbate is known to be modified by thioltransferases [10]. DHAR was S-glutathionylated as an intermediate form in the course of the reduction of DHA into ascorbate [15]. The two other proteins are enzymes involved in the Calvin cycle: glyceraldehyde-3-phosphate dehydrogenase (GDPH) and fructose bisphosphate aldolase (FBA). Thiolation of both enzymes led to the inactivation of the enzymes [16,17]. Our method could be easily adapted to study protein S-glutathionylation using glutathione ester or radiolabeled glutathione.

Five new thiol/disulfide containing proteins were identified in this study. Little information about their regulation is known. Among them, we found an enzyme involved in photorespiration, *i.e.*, GGT1. The fact that this enzyme is only located in the peroxisomes [18] where reactive oxygen species are highly produced suggests that GGT1 is likely to be regulated by redox. In addition, we identified another protein MOD1, an enoyl-acyl carrier protein reductase involved in fatty acid biosynthesis. Mutation of MOD1 led to premature cell death and dramatic alterations in plant growth and development [19]. The regulation of this enzyme has not been studied before. Here we present evidence that this protein is potentially redox regulated. Moreover, a member of the universal stress protein family was also identified. Nothing is known about the function and regulation of this type of proteins in plants. The protein was originally named after the universal stress protein A (USPA) in *Escherichia coli*. Based on an evolutionary study of conserved struc-

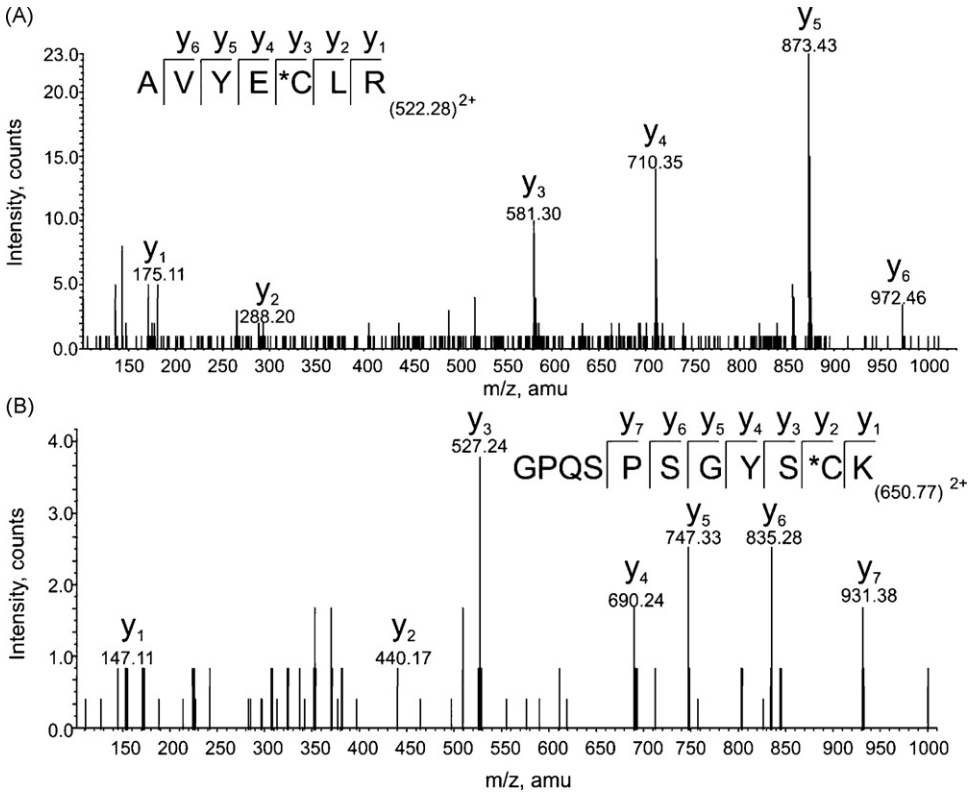

Fig. 3. CID-MS/MS identification of cysteines involved in disulfide bond formation. (A) CID-MS/MS sequencing of a tryptic peptide derived from ribulose bisphosphate carboxylase mapped the cysteine residue modified by mBBr as indicated by the asterisks; (B) CID-MS/MS sequencing of a tryptic peptide derived from germin-like protein mapped the cysteine residue modified by mBBr as indicated by the asterisks.

tural domains, it was suggested that the ancestral function of USPA might be nucleotide binding and signal transduction [20,21].

In summary, the proteomic approach involving mBBr labeling, 2D gel separation and MS analysis is powerful to identify disulfide proteins and/or redox-regulated proteins. Using this approach, we have identified many proteins, including five new proteins that are potentially regulated by redox. It allows the analysis of the dynamic features of protein redox modifications because of the possibility to differentiate the changes specific to protein redox status from those of overall protein expression. In addition, the approach enables mapping of the cysteines involved in disulfide bond formation and redox regulation. This proteomic approach has many potential applications, *e.g.*, to characterize the effect of environmental stresses on protein redox status and the role of redox regulation in plant signaling and metabolism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2008.11.027.

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