Cy5 maleimide labelling for sensitive detection of free thiols in native protein extracts: identification of seed proteins targeted by barley thioredoxin h isoforms

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Barley thioredoxin h isoforms HvTrxh1 and HvTrxh2 differ in temporal and spatial distribution and in kinetic properties. Target proteins of HvTrxh1 and HvTrxh2 were identified in mature seeds and in seeds after 72 h of germination. Improvement of the established method for identification of thioredoxin-targeted proteins based on two-dimensional electrophoresis and fluorescence labelling of thiol groups was achieved by application of a highly sensitive Cy5 maleimide dye and large-format two-dimensional gels, resulting in a 10-fold increase in the observed number of labelled protein spots. The technique also provided information about accessible thiol groups in the proteins identified in the barley seed proteome. In total, 16 different putative target proteins were identified from 26 spots using tryptic in-gel digestion, matrix-assisted laser-desorption ionization–time-of-flight MS and database search. HvTrxh1 and HvTrxh2 were shown to

have similar target specificity. Barley α -amylase/subtilisin inhibitor, previously demonstrated to be reduced by both HvTrxh1 and HvTrxh2, was among the identified target proteins, confirming the suitability of the method. Several α -amylase/trypsin inhibitors, some of which are already known as target proteins of thioredoxin h, and cyclophilin known as a target protein of m-type thioredoxin were also identified. Lipid transfer protein, embryo-specific protein, three chitinase isoenzymes, a single-domain glyoxalase-like protein and superoxide dismutase were novel identifications of putative target proteins, suggesting new physiological roles of thioredoxin h in barley seeds.

Key words: barley seed proteome, Cy5 maleimide labelling, disulphide reduction, monobromobimane, target proteins, thioredoxin h.

INTRODUCTION

Thioredoxins are small (12 kDa), ubiquitous proteins with protein disulphide reductase activity [1]. The cysteine residues in the consensus active-site sequence WC(G/P)PC of thioredoxins form a disulphide bond. Electron donors, via thioredoxin reductases, reduce the disulphide bond in thioredoxins to the catalytically active dithiol form. Thioredoxins in the reduced form reduce disulphide bonds in target proteins and can thereby modulate the activity of proteins involved in a variety of cellular processes.

Uniquely, plants have various types of thioredoxins with distinct subcellular localizations. Thioredoxins f and m, located in chloroplasts, are well-studied plant thioredoxins that regulate photosynthetic enzymes [2]. Chloroplastic thioredoxins are regenerated to the reduced form by ferredoxin as the electron donor via ferredoxin-dependent thioredoxin reductase. Another thioredoxin system was identified in mitochondria of arabidopsis (*Arabidopsis thaliana*) [3].

In the cytosol, plants have multiple isoforms of thioredoxin h. At least five thioredoxin h isoforms have been identified in arabidopsis [4]. Arabidopsis thioredoxin h isoforms are capable of differentially complementing thioredoxin-deficient phenotypes in yeast [5], suggesting that they have different roles. Reduced thioredoxin h is regenerated from the oxidized form by NADPH via NTR (NADP-dependent thioredoxin reductase) [6].

Several target proteins of thioredoxin h have been identified so far. The thiol-specific fluorescent probe, mBBBr (monobromo-

bimane), in combination with protein separation in one-dimensional SDS/PAGE has been applied to visualize reduction by thioredoxin of storage proteins, such as hordeins in barley (*Hordeum vulgare*) and glutenins and gliadins in wheat (*Triticum aestivum*) [7], some α -amylase/trypsin inhibitor proteins [8] and BASI (barley α -amylase/subtilisin inhibitor) [9]. A systematic identification of *Chlamydomonas reinhardtii* thioredoxin h target proteins in peanut seeds was approached by using mBBBr labelling in combination with two-dimensional gel electrophoresis and MS [10]. This strategy was also successfully applied for the identification of target proteins in the embryo of germinating barley seeds using the same thioredoxin [11] and most recently in endosperm from mature wheat seeds using *Escherichia coli* thioredoxin [12]. However, the molar absorption coefficient for mBBBr ($5000 \text{ M}^{-1} \cdot \text{cm}^{-1}$) is much lower than for fluorescence dyes normally used for general protein detection in two-dimensional gels, such as cyanine dyes which have extinction coefficients above $10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The low molar absorption coefficient and thus low sensitivity of mBBBr would limit the target survey to the major proteins. In the present study, mBBBr was replaced with a monoreactive maleimide derivative of a cyanine dye, Cy5m (Cy5 maleimide), which has an absorption coefficient of $250\,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Thiol-specific maleimide derivatives of cyanine dyes have been used for protein detection in two-dimensional gels in a saturation labelling technique to enhance sensitivity of fluorescence imaging by labelling proteins under reducing and denaturing conditions [13]. Amine-reactive cyanine dyes have also been used

Abbreviations used: BASI, barley α -amylase/subtilisin inhibitor; cCBB, colloidal Coomassie Brilliant Blue; Cy5m, Cy5 maleimide; ESP, embryo-specific protein; EST, expressed sequence tag; G3PD, glyceraldehyde 3-phosphate dehydrogenase; IPG, immobilized linear pH gradient; LTP1, lipid transfer protein 1; MALDI, matrix-assisted laser-desorption ionization; mBBBr, monobromobimane; NTR, NADP-dependent thioredoxin reductase; TC, tentative consensus.

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for fluorescence imaging in the two-dimensional-DIGE technique [14].

Transgenic barley seeds overexpressing wheat thioredoxin h showed an increased level of limit dextrinase activity [15] and earlier increase in α -amylase activity in germinated [16] than normal seeds. Results from previous studies suggest that thioredoxin h regulates activity of hydrolytic enzymes and their inhibitors during seed germination to mobilize carbon and nitrogen sources required for growth.

Using the techniques of two-dimensional electrophoresis and MS, two thioredoxin h isoforms, HvTrxh1 and HvTrxh2, were recently identified in the barley seed proteome. The proteins had 51 % sequence identity and showed different patterns of appearance in seed tissues and during germination [17]. Recombinant HvTrxh1 and HvTrxh2 were produced in *E. coli* and shown to differ in kinetic properties [17]. In the present study, endogenous thioredoxin h isoforms, HvTrxh1 and HvTrxh2, were used in a target protein survey in mature barley seeds and in seeds after 72 h germination, which have considerably different protein profiles [18,19]. The established method for target identification was modified by replacement of mBBR with Cy5m to achieve higher sensitivity of target detection.

EXPERIMENTAL

Materials

Purified *A. thaliana* NTR [20] was a gift from J.-P. Jacquot (INRA, Nancy, France). The pETTrxTa expression system [21] was kindly provided by M. Gautier (INRA, Montpellier, France). Barley thioredoxin h1 (HvTrxh1), barley thioredoxin h2 (HvTrxh2) and wheat thioredoxin h (TrxhTa) were produced as described previously [17]. Cy5m mono-Reactive Dye 5 pack was from Amersham Biosciences (Uppsala, Sweden). Monobromobimane and NADPH were from Fluka (Buchs, Switzerland).

Plant material and protein extraction

Spring barley (*Hordeum vulgare* cv. Barke) was field-grown in Fyn (Denmark) in the 2000 season. Mature seeds and seeds micro-malted according to standard procedures [18] for 72 h (germinating seeds) were frozen in liquid nitrogen and freeze-dried before milling and extraction. Proteins were extracted from 4 g of milled seeds in 20 ml extraction buffer (5 mM Tris/HCl, pH 7.5/1 mM CaCl_2) for 30 min at 4 °C, as described previously [22]. After centrifugation to remove debris, supernatants containing soluble proteins were transferred to clean tubes and stored at -80 °C until required.

Target protein survey using mBBR labelling

The labelling method was based on a previously published procedure [10]. Reaction mixtures containing 0.48 mM NADPH, 0.59 μM arabidopsis NTR, 1.8 μM wheat TrxTa (no thioredoxin in control) and 150 μl mature seed extract (approx. 150 μg of protein) in a total volume of 375 μl in 50 mM Tris/HCl (pH 7.5) were incubated at room temperature (22 °C) for 30 min. For mBBR labelling, 0.6 μmol of mBBR in acetonitrile was added to each sample and incubated at room temperature for 20 min. Proteins in mBBR-treated extracts were precipitated with 4 vol. of acetone at -20 °C for 24 h, resuspended in reswelling buffer [8 M urea/2 % (w/v) CHAPS/0.5 % (v/v) IPG (immobilized linear pH gradient) buffer 3–10 (Amersham Biosciences)/20 mM dithiothreitol and a trace of Bromophenol Blue] and loaded on to 7 cm IPG strips,

pI 3–10, according to the manufacturer's recommendations. Second dimension vertical SDS/PAGE gels (NuPAGE 4–12 % Bis-Tris Zoom gel, 7 cm $\times$ 8 cm; Invitrogen) were run on the Xcell SureLock Mini-Cell (Invitrogen) according to the manufacturer's recommendations. The labelled proteins were visualized by photographing under UV light. Total proteins were visualized by cCBB (colloidal Coomassie Brilliant Blue) staining [23].

Target protein survey using Cy5m labelling

Reaction mixtures containing 0.72 mM NADPH, 0.44 μM arabidopsis NTR, 3.0 μM HvTrxh1 or HvTrxh2 (no thioredoxin in control) and 100 μl mature seed extract or germinating seed extract (approx. 100 and 160 μg of protein respectively) in a total volume of 250 μl in 50 mM Tris/HCl (pH 7.5) were incubated at room temperature (22 °C) for 1 h. Cy5m (10 μg) in 2 μl acetonitrile was added to each reaction mixture and incubated overnight at 4 °C in the dark. To avoid oxidation of cysteine residues, vials containing reaction mixtures were flushed with nitrogen.

Proteins in labelled extracts were desalted using a G25 desalting column (NAP10 column, Amersham Biosciences) to remove excess Cy5m and to improve focusing of high pI proteins [19]. Desalted proteins were acetone-precipitated as above and resuspended in reswelling buffer. Duplicate gels were run for each experiment and unnecessary exposure to light was avoided. Isoelectric focusing was performed using 18 cm IPG strips, pI 3–10, run on a Pharmacia Multiphor II (Amersham Biosciences) as described previously [22]. Second dimension SDS/PAGE gels (12–14 %, 18 cm $\times$ 24 cm; Amersham Biosciences) were run on a Pharmacia Multiphor II according to the manufacturer's recommendations. Gels were scanned by Storm 860 (Amersham Biosciences) in red fluorescence detection mode (excitation at 635 ± 5 nm; detection of emissions above 650 nm), and then stained with cCBB.

In-gel digestion and MALDI (matrix-assisted laser-desorption ionization)-time-of-flight MS

Spots were excised from cCBB-stained gels and subjected to in-gel trypsin digestion [24]. Tryptic peptides were desalted and concentrated on a home-made 5 mm nano-column [25] as described previously [22]. Peptides were eluted with 0.8 μl matrix (20 mg/ml α -cyano 4-hydroxycinnamic acid in 70 % acetonitrile/0.1 % trifluoroacetate) and deposited directly on to the MALDI target. A Bruker REFLEX IV MALDI-time-of-flight MS (Bruker-Daltonics, Bremen, Germany) in positive-ion reflector mode was used to analyse tryptic peptides. The *m/z* software (Proteometrics, New York, NY, U.S.A.) was used to analyse the spectra. Spectra were calibrated using trypsin autolysis products (*m/z* 842.51 and 2211.1) as internal standards. To identify proteins, the SwissProt and NCBI non-redundant sequence databases and the NCBI EST (expressed sequence tag) databases were searched with peptide masses using the Mascot (<http://www.matrixscience.com>) server. The following parameters were used for database searches: monoisotopic mass accuracy 80 p.p.m., missed cleavage 2, allowed modifications carbamidomethylation of cysteine (complete) and oxidation of methionine (partial). For a positive identification, more than 15 % sequence coverage was required, including at least three matched peptides with mass derivation of < 50 p.p.m. from the theoretical value. TC (tentative consensus) sequences corresponding to identified EST sequences were obtained by searching the Institute for Genomic Research (TIGR) sequence database (www.tigr.org/tldb/tgi/hvgi).

Figure 1 Target proteins of thioredoxin h in mature barley seed extracts visualized by mBBR labelling

Approx. 150 μ g of mature seed proteins were incubated in the absence (**A**, **B**) or presence (**C**) of TrxTa for disulphide reduction. Free thiol groups were labelled with mBBR and proteins were separated by two-dimensional gel electrophoresis in the pI range 3–10. (**A**) cBBB-stained image; (**B**, **C**) fluorescence images. Molecular-mass standards are indicated in (**A**). White arrows indicate spots considered to contain proteins reduced by TrxTa (Table 1).

RESULTS

Target protein survey in barley seeds using mBBR

To initiate identification of proteins targeted by thioredoxin h in barley seeds, an established method [10] was used. For reduction of disulphide bonds in possible target proteins, a protein extract from mature seeds was incubated with wheat TrxTa [21]. To detect proteins reduced by thioredoxins, accessible thiol groups were labelled with mBBR and the proteins were subsequently separated on two-dimensional gels (7 cm $\times$ 8 cm, pI 3–10). The gels were photographed under UV light (Figures 1B and 1C) to detect labelled proteins and stained with cBBB (Figure 1A) for general protein detection. Compared with the control (Figure 1B), incubation with TrxTa increased fluorescence intensities in nine

spots (Figure 1C, spots 1–9). Spots 1–5 were identified by peptide mass fingerprinting (Table 1) to contain two isoforms of G3PD (glyceraldehyde 3-phosphate dehydrogenase; spot 1), 1-Cys peroxiredoxin (spots 2–4) and a fragment of superoxide dismutase (spot 5). Spots 6 and 7 were estimated to contain α -amylase/trypsin inhibitors by spot-pattern comparison with identified proteins on two-dimensional gels of the same extracts [18,19]. Spot 9 contained TrxTa.

However, due to the small number of spots visualized by fluorescence detection (<30 in the control, Figure 1B) and cBBB staining (<120, Figure 1A) and observed spot overlapping (e.g. spot 1), the sensitivity of mBBR labelling and resolution of two-dimensional gels used here were estimated to be unsatisfactory for a comprehensive survey of thioredoxin h target proteins. Furthermore, the requirement of UV light for detection is incompatible with the use of the large two-dimensional gels supported with plastic sheets regularly used in our proteomics studies [18,19,22] due to background fluorescence. The small gels used here instead have lower resolution with more spot overlapping, which makes unambiguous identification of fluorescence-labelled proteins difficult. To improve sensitivity of detection and compatibility with our experimental system, mBBR was replaced with Cy5m, a highly sensitive thiol-specific fluorescent probe excited at longer wavelength.

Two thioredoxin h isoforms, HvTrxh1 and HvTrxh2 identified in barley seeds were cloned, expressed and purified [17] and used to identify target proteins in the present study. Protein extract from mature seeds or germinating seeds was incubated in the presence or absence of recombinant thioredoxin h isoforms. Accessible thiol groups in the extracted proteins were then labelled by adding a large amount of Cy5m to achieve high dye/protein ratio. Proteins were subsequently separated on large two-dimensional gels (18 cm $\times$ 24 cm) for a high resolution of spot patterns. Gels were fluorescence-scanned to detect Cy5m labelled proteins and cBBB-stained for general protein detection (Figures 2A–2F).

Analysis of sensitivity and labelling specificity of Cy5m

Spot patterns in the cBBB-stained image (Figure 2A) and the fluorescence image (Figure 2B) of the control gel of mature seed extract were analysed and compared. High-resolution protein separation was seen in both cBBB-stained and fluorescence images. In total, around 300 fluorescent spots were visualized in the control gel of mature seeds, representing a >10-fold increase compared with spots visualized using mBBR and smaller two-dimensional gels (Figure 1B). Treatment with Cy5m did not significantly alter the overall spot pattern, as untreated and Cy5m-treated samples gave rise to highly similar spot patterns (results not shown; Figures 3A and 3B). However, spot patterns were quite dissimilar between the cBBB-stained image and fluorescence image of the same gel (Figures 2A and 2B) as a result of the highly selective Cy5m labelling.

A section from the fluorescence (Figure 3C) and cBBB-stained images (Figure 3B) of the control mature seed gel (Figures 2A and 2B) and the corresponding section in a silver-stained two-dimensional gel of the same protein extract not treated with Cy5m (Figure 3A) [19] were analysed in detail (Figure 3). Several spots only faintly stained with cBBB (e.g. spots 176, 179, a and b; Figure 3) were detected with high sensitivity in the fluorescence image (Figure 3C). Some spots visible in the cBBB-stained image were seen in the fluorescence image whereas others were absent. Spots visible in the fluorescence image were considered to contain proteins with one or more accessible thiol groups, whereas those absent were considered to contain proteins without accessible

Table 1 Target proteins of TrxTa in mature barley seeds identified using mBBr labelling, two-dimensional electrophoresis and MS

Spot*	Function	Matched peptides	Sequence coverage (%)	Observed mass (kDa)	Observed pI	Accession no.†
1	G3PD (G3PC)	5	23	40	7.4	P08477
	G3PD (G3PX)	9	26			P26517
2	1-Cys peroxiredoxin	10	38	28	6.2	P52572
3	1-Cys peroxiredoxin	10	38	28	6.6	P52572
4	1-Cys peroxiredoxin	10	38	28	7.0	P52572
5	Superoxide dismutase (fragment)	2	23	18	6.1	gi 7433318
6‡	α -Amylase/subtilisin inhibitor CMe (#436)	—	—	13	7.2	CAA35188
7‡	α -Amylase inhibitor BDAI-I (#116)	—	—	9	5.3	gi 123970
8	Not determined	—	—	9	5.6	—

* Spot numbers refer to Figure 1.
† Accession nos. refer to SwissProt or NCBI databases.
‡ Identification inferred from position in two-dimensional gel pattern.

Figure 2 Target proteins of thioredoxin h in mature and germinating barley seed extracts visualized by Cy5m labelling

Approx. 100 μ g of proteins extracted from mature (A–C) or germinating (D–F) seeds were incubated in the presence (C, F) or absence (A, B, D, E) of HvTrxh1. Free thiol groups were labelled with Cy5m and proteins were separated by two-dimensional electrophoresis in the pI range 3–10. A region of the two-dimensional gels spanning approx. pI 4.6–8.7 and 8–75 kDa is shown. Molecular-mass standards are indicated in (A). (A, D) cBBB-stained images; (B, C, E, F) fluorescence images. A potential target protein of thioredoxin h is indicated (T13). Boxes 1–8 indicate sections of the two-dimensional gels shown in Figures 3 and 4.

Figure 3 Detailed analysis of the cCBB-stained and Cy5m-labelled proteins in mature seed extracts without reduction by thioredoxin h

Close-up view of a region of the two-dimensional gels spanning approx. pI 6.6–7.4 and 29–55 kDa (box 1, Figure 2). (A) The corresponding section of a silver-stained two-dimensional gel of the same protein extract not treated with Cy5m. (B) cCBB-stained image and (C) fluorescence image of Cy5m-labelled mature seed extracts. Identified protein spots are numbered and two unidentified spots labelled by Cy5m are indicated (a and b).

thiol groups. Spots 54, 55 and 411 that clearly appear in the silver-stained and Coomassie-stained gels were previously identified as grain peroxidase 1 (spots 54 and 55) and aldose reductase (spot 411) [19] and are absent in the fluorescence image. The absence of barley grain peroxidase 1 spots in the fluorescence image was in agreement with the three-dimensional structure (PDB accession no. 1BGP), which showed all eight cysteine residues in disulphide form [26]. No structural information is available for barley aldose reductase.

Spots 27 and 175, and spots 57, 176 and 179 (Figure 3A) contain two isoforms of G3PD, each appearing with multiple pI values [19]. No three-dimensional structures are available for barley G3PDs, however work with G3PD from other organisms has shown that alkylating reagents react with a conserved catalytically active cysteine residue in the active-site pocket [27]. Spots 175, 176 and 179 were visible in the fluorescence image, as expected,

in this observation, whereas spots 27 and 57 were absent, suggesting that they represent forms of the protein in which this cysteine is inaccessible or modified. HvTrxh1 (Figures 4A and 4B) and HvTrxh2 (results not shown), which have reactive cysteine residues, were also visible in the fluorescence image.

Selected identified spots from the mature barley seed proteome [18,19] (O. Østergaard, C. Finnie, S. Laugesen, P. Roepstorff and B. Svensson, unpublished work) are listed in Table 2, grouped by their presence or absence in the fluorescence image of the control gel. Several identified spots were unlabelled and most of them contained proteins either with no cysteine residues or with all cysteine residues in disulphide form (Table 2), according to three-dimensional structural data or by homology to other proteins with known structure, further supporting the labelling specificity of Cy5m towards thiol groups. Thus this technique can provide information about the state of cysteine residues in identified proteins.

Detection and identification of proteins reduced by HvTrxh1 and HvTrxh2

Spots in mature and germinating seed gels were grouped into three categories based on the influence of thioredoxins on their patterns of appearance: (1) spots visible in the cCBB-stained control gel whose fluorescence intensities did not increase after incubation with thioredoxin h; (2) spots visible in the cCBB-stained control gel whose fluorescence intensities increased after incubation with thioredoxin h; and (3) spots not visible in the cCBB-stained control gel, but appearing both in the fluorescence image and in the cCBB-stained image after incubation with thioredoxins, presumably due to a pI shift caused by labelling with Cy5m.

Spots in category 1 were considered to contain proteins not reduced by thioredoxins. In the mature seed gels, 26 spots were in category 2 or 3 and considered to contain proteins reduced by HvTrxh1 (Figures 2A–2C). Proteins in 23 of these spots were successfully identified by peptide mass fingerprinting (Table 3). Spot patterns of germinating seed gels were significantly different from mature seed patterns both in the fluorescence- and cCBB-stained images (Figures 2D–2F). In germinating seed gels, 23 spots were considered to contain proteins reduced by HvTrxh1, of which 15 were successfully identified. When HvTrxh2 was used, the same target spots were observed both in mature and germinating seed extracts (results not shown). In total, 16 different proteins were identified in 26 spots (Table 3), of which four proteins were identified as targets only in mature seeds and two proteins only in germinating seeds.

Identified putative target proteins of thioredoxin h

α -Amylase/trypsin inhibitors

Barley α -amylase/trypsin inhibitors have ten conserved cysteine residues in their primary structures. Formation of five disulphide bonds is expected from their homology to α -amylase/trypsin inhibitors with known three-dimensional structures of proteins from other sources such as ragi seeds [28]. Both in mature and germinating seed gels α -amylase/trypsin inhibitors appeared as large cCBB-stained and non-fluorescent spots in the control (Figure 2, box 2; Figure 4A, control, spots 1/118, 111, 112, 113a, 115b, 116, 117, 495 and T2).

In mature seed gels, category 2 spots were identified that contained α -amylase inhibitor BMAI-I (spots 111 and 112), α -amylase inhibitor BDAI-I (spot 495), α -amylase/trypsin inhibitor CMb (spot 117), α -amylase/trypsin inhibitor CMd (spot 115b),

Figure 4 Identification of possible target proteins of thioredoxin h in mature and germinating barley seed extracts

Close-up views of boxes 2–8 (Figure 2) from mature (**A**, **C–H**) and germinating (**B**) seed gels, spanning approx. pI 4.6–6.6 and 11–21 kDa (box 2; **A** and **B**), pI 7.6–8.7 and 11–15 kDa (box 3; **C**), pI 6.7–7.5 and 20–25 kDa (box 4; **D**), pI 5.0–5.8 and 25–31 kDa (box 5; **E**), pI 7.5–8.7 and 26–40 kDa (box 6; **F**), pI 5.9–6.7 and 32–44 kDa (box 7; **G**) and pI 8.3–8.7 and 19–30 kDa (box 8; **H**). The cCBB-stained images (upper) and fluorescence images (lower) are shown for extracts treated in the absence (control) or presence (HvTrxh1) of thioredoxin h. Identified proteins are numbered and listed in Table 3. Arrowheads indicate possible target proteins of thioredoxin h (Table 3). (**H**) Comparison of silver- and cCBB-stained images of extracts untreated (silver) or treated (cCBB and HvTrxh1) with Cy5m.

α -amylase/trypsin inhibitor pUP13 (spot 113a) and trypsin inhibitor CMe (spot T2). The category 3 spots T3 and T4 contained CMe. Spot 1/118 also increased in fluorescence intensity. However, it was not possible to identify the target protein unambiguously because this spot contained multiple proteins: α -amylase/trypsin inhibitors CMA, CMd and at least one additional protein.

In germinating seed gels (Figure 4B), BMAI-I, BDAI-I, CMb (additionally in spot T1), CMd and pUP13 were again identified as targets. Compared with mature seeds, the amounts of these proteins were decreased and reduction by thioredoxins was less significantly visualized. An exception was pUP13 (spot 113a) which increased in abundance and was reduced more intensively. Reduction of CMe was not observed in any spots in germinating seed gels. CMA (spot 21) was additionally identified as a target.

The reduction of different α -amylase/trypsin inhibitors were visualized with very different intensity by Cy5m labelling, despite their homology and the expected common disulphide structure. The fluorescence intensity was especially strong in BMAI-I in spot 112 and in CMe (spots T2 and T4), whereas it was very weak in BDAI-I (spot 495) (Figure 4A).

Single-domain glyoxalase-like protein

Previously, this protein was identified in spots 20 and 312 (Figure 4A) [22] (O. Østergaard, C. Finnie, S. Laugesen, P. Roepstorff and B. Svensson, unpublished work). In control gels of mature seeds, spot 312 was fluorescent whereas spot 20 was non-fluorescent. Incubation with thioredoxins made spot 20 strongly fluorescent (Figure 4A). Although the major component of spot 20 was single-domain glyoxalase-like protein, the MS spectrum obtained from this spot contained minor peaks matching peptides derived from CMe and CMA, making the target identification uncertain. Spots 20 and 312 were absent in germinating seed gels.

Superoxide dismutase

The category 2 spot 302 observed in mature seed gels contains superoxide dismutase (O. Østergaard, C. Finnie, S. Laugesen, P. Roepstorff and B. Svensson, unpublished work) (Figure 4A). In germinating seed gels spot 302 was faint and reduction by thioredoxins was not observed.

LTP1 (lipid transfer protein 1)

LTP1 has approx. 20% sequence identity to α -amylase/trypsin inhibitors and was previously identified in spots 25 and 425 [19] (Figure 2, box 3; Figure 4C). LTP1 has eight cysteine residues that form four disulphides [29]. In the present study, LTP1 spots appeared in mature and germinating seed gels as large non-fluorescent spots as expected. After incubation with thioredoxins, fluorescence intensities of spots 25 and 425 did not increase, but a new fluorescent spot emerged (spot T5, Figure 4C) with slightly higher molecular mass and lower pI than spot 425 and was found to contain LTP1.

BASI

The three-dimensional structure of BASI shows participation of all of its four cysteine residues in the formation of two disulphide bonds [30]. In control gels of mature (Figure 2, box 4; Figure 4D) and germinated seeds (results not shown), spots 56 and 62 containing BASI [19] were non-fluorescent. Fluorescence intensities of spots 56 and 62 did not increase after incubation with thioredoxins, but in mature seed gels, fluorescent spots T6, T7 and T8 emerged and were identified to contain BASI (Figure 4D). Only spot T6 appeared after incubation with thioredoxins in germinating seed gels (results not shown).

Table 2 Cy5m-labelling status of identified spots in cCBB-stained mature seed control gels

Id. no.*	Function	Accession no.†	No. of cysteine residues‡	No. of disulphides§
(A) Spots not visible in the fluorescence image				
Proteins without cysteine residues				
219	Nucleoside diphosphate kinase 1	gi 400404	0	0
211	Subtilisin–chymotrypsin inhibitor CI-1B	gi 124127	0	0
212	Subtilisin–chymotrypsin inhibitor CI-1A	gi 124125	0	0
103	Superoxide dismutase (Mn)	gi 7433345	0	0
Proteins with known or expected disulphide formation				
116, 495	α -Amylase inhibitor BDAI-I	gi 123970	10	5
111, 112	α -Amylase inhibitor BMAI-I	gi 2506771	10	5
117	α -Amylase/trypsin inhibitor CMb	gi 585290	11	5
115b	α -Amylase/trypsin inhibitor CMd	gi 82418	10	5
113a	Trypsin/ α -amylase inhibitor pUP13	gi 225102	10	5
28, 436	Trypsin inhibitor CMe	CAA35188	10	5
56, 62	BASI	gi 4699833	4	2
54, 55	Barley grain peroxidase 1	gi 2624498	8	4
68	Chitinase-two-rowed barley	JC5918	7	3
414	26 kDa endochitinase 1	P11955	17	7
25, 425	Non-specific lipid transfer proteins	P07597	8	4
58, 432	Perm1 protein	gi 7442172	16	8
415	Peroxidase (by similarity to X58396, barley)	TC29818	9	4
302	Superoxide dismutase (Cu-Zn) 2 (chloroplast)	gi 7433318	2	1
417	Thaumatococcus-like protein TLP8	AAK55326	16	8
423	Barley wound-induced protein (Barwin)	P28814	6	3
Proteins with unknown disulphide formation				
473	Allyl alcohol dehydrogenase	TC56569	3	
411, 418	Aldose reductase	P23901	5	
472	Auxin-induced protein	TC45978	6	
8	ESP	TC45910	5	
471	Epoxide hydrolase	TC47183	1	
66, 67	β -Glucosidase	A57512	5	
102	Glutathione-S-transferase	gi 6683765	2	
27	G3PD (cytosolic)	P26517	2	
57	Homologue to G3PD, cytosolic 3 (Q43247, maize)	TC26911	5	
416	Homologue to hypothetical proteins (BAB56043)	TC30606	3	
149	Glyoxalase I (fragment)	TC45305	5	
468	Glyoxalase I	TC45305	5	
11	Phosphoglycerate kinase (cytosolic)	gi 129916	1	
20	Single-domain glyoxalase-like protein	TC57273	2	
(B) Spots visible in the fluorescence image				
454	Aconitate hydratase (cytoplasmic)	TC56216	10	
455	Aconitate hydratase (cytoplasmic)	TC56432	9	
100	Aldose reductase	gi 113595	5	
406	ESP	TC45910	5	
273	Enolase 1	gi 119355	4	
193	Enolase 2	gi 1169528	5	
420	Weak similarity to epoxide hydrolase homologue	TC31145	1	
175	G3PD (cytosolic)	P26517	2	
176, 179	G3PD (cytosolic)	P08477	4	
312	Single-domain glyoxalase-like protein	TC57273	2	
77	Malate dehydrogenase (cytoplasmic)	TC65946	6	
13	Malate dehydrogenase (mitochondrial)	TC45040	4	
202, 269	Protein Z (Z4) serpin	gi 1310677	2	
154	Protein Z (Z7) serpin	gi 7438218	1	
122, 138	Triosephosphate isomerase (cytosolic)	gi 2507469	4	

* Id. numbers for previously identified spots [17,18] (O. Østergaard, C. Finnie, S. Laugesen, P. Roepstorff, B. Svensson, unpublished work).

† Accession nos. refer to SwissProt, NCBI or TIGR databases.

‡ The number of cysteine residues in protein sequences or the coding region of translated TC sequences, excluding known signal sequences.

§ Number of disulphide bonds in the three-dimensional structure of the listed protein (wherever available).

|| Number of disulphide bonds in the three-dimensional structure of plant protein homologous with listed protein.

Embryo-specific protein (ESP)

ESP was identified in spots 8 and 406 in developing and mature seeds [22] (O. Østergaard, C. Finnie, S. Laugesen, P. Roepstorff and B. Svensson, unpublished work). In the present study, spot 406 and an additional spot containing ESP (spot T14) were fluorescent in control gels of mature seeds (Figure 2, box 5; Fig-

ure 4E), but spot 8 was non-fluorescent. After incubation with thioredoxins, two new fluorescent spots containing ESP (spots T15 and T16) emerged with lower pI than spots 406 and T14, and the fluorescence intensities of spots 406 and T14 also increased. cCBB-stained images showed a corresponding strong decrease in the size of spot 8, suggesting a pI shift of reduced and labelled ESP. Spots containing ESP were absent from germinating seeds.

Table 3 Target proteins of barley thioredoxin h identified in mature and germinating barley seeds using Cy5m labelling, two-dimensional electrophoresis and MS

Spot no.*	Function	Location†	Matched peptides	Sequence coverage (%)	Observed mass (kDa)	Observed pI	Accession no.‡
Defence proteins and inhibitors							
495	α -Amylase inhibitor BDAI-I	M + G	10	74	13.7	5.0	P13691
111	α -Amylase inhibitor BMAI-1	M + G	9	66	16.1	5.4	P16968
112	α -Amylase inhibitor BMAI-1	M + G	9	66	14.2	5.4	P16968
21	α -Amylase/trypsin inhibitor CMa	G	7	65	15.1	6.1	TC66096
117	α -Amylase/trypsin inhibitor CMb	M + G	10	46	15.1	5.4	P32936
T1	α -Amylase/trypsin inhibitor CMb	G	10	46	14.5	6.2	P32936
115b	α -Amylase/trypsin inhibitor CMd	M + G	9	78	16.8	5.2	P11643
113a	Trypsin/ α -amylase inhibitor pUP13	M + G	5	42	15.1	4.9	Gi 225102
T2	Trypsin inhibitor CMe	M	4	37	15.8	6.3	P01086
T3	Trypsin inhibitor CMe	M	4	37	16.1	6.1	P01086
T4	Trypsin inhibitor CMe	M	4	37	16.8	6.0	P01086
T5	Non-specific lipid transfer protein	M + G	6	85	12.6	7.8	P07597
T6	BASI	M + G	14	71	22.7	7.1	P07596
T7	BASI	M	12	63	23.2	6.9	P07596
T8	BASI	M	11	63	23.7	6.8	P07596
T9	26 kDa endochitinase 2	M + G	8	38	26.2	7.8	P23951
T10	Homologue of wheat chitinase 3	M + G	9	33	36.9	6.3	TC78715
T11	26 kDa endochitinase 1	M + G	5	27	35.9	8.0	P11955
T12	26 kDa endochitinase 1	M + G	5	27	38.0	8.2	P11955
Proteins with other functions							
T13	Cyclophilin	G	6	27	20.1	7.6	TC76659
302	Cu,Zn superoxide dismutase (chloroplast)	M	3	27	17.8	5.1	TC65532
Proteins with unknown functions							
20	Single-domain glyoxalase-like protein	M	14	72	15.1	6.1	TC67562
406	ESP	M	13	67	27.9	5.4	TC66773
T14	ESP	M	13	67	26.8	5.4	TC66773
T15	ESP	M	12	62	27.3	5.3	TC66773
T16	ESP	M	12	62	27.9	5.1	TC66773

* Spot numbers refer to Figures 2 and 4.

† The proteins were identified as target proteins of barley thioredoxin h isoforms in mature seeds (M), in germinating seeds (G) or in both (M + G).

‡ Accession nos. refer to SwissProt, NCBI or TIGR databases.

Chitinase isoenzymes

Spots 414 and 68 containing endochitinases 1 and 2 (Chitinase-two-rowed barley) respectively [19] remained non-fluorescent after incubation with thioredoxins (Figure 2, box 6; Figure 4F). Several category 3 spots, some of which were identified as endochitinase 1 (T11 and T12) and endochitinase 2 (spot T9), were observed at lower pI (Figure 4F). The cCBB-stained spot 414 was no longer visible in thioredoxin-treated extracts, whereas the more intense spot 68 remained (Figure 4F).

An additional fluorescent spot also emerged at lower pI (spot T10, Figure 2, box 7; Figure 4G). Peptide mass data from this spot initially matched barley chitinase 2 with five peptides and 17% sequence coverage, but a subsequent search in EST databases resulted in a match with six peptides to a wheat EST sequence (gi|22211906). Using homology search in the TIGR barley gene index using the EST sequence, a TC sequence encoding a protein different from endochitinases 1 and 2 but 97% identical with wheat chitinase 3 was obtained. A theoretical trypsin digest of the translated TC sequence was compared with the peptide mass data from spot T10 and resulted in nine matched peptides and 33% sequence coverage. This particular chitinase has not been identified previously in barley seeds. Endochitinase 1 (26 kDa) and wheat chitinase 3 belong to chitinase class I, consisting of two domains both rich in disulphides: an N-terminal wheatgerm agglutinin lectin domain important for chitin binding and a C-terminal chitinase domain catalysing hydrolysis of chitin [31]. Endochitinase 2 belongs to chitinase class II, which lacks the wheatgerm agglutinin lectin domain. Reduction of all three

chitinase isoenzymes was also observed in the germinating seed gels, although fluorescence intensity of chitinase 3 spot was rather faint (results not shown).

Cyclophilin

A category 2 spot present only in germinating seed extract was identified as cyclophilin (spot T13, Figures 2D–2F). Another cyclophilin isoenzyme in spot 227 [19] was present in both mature seed and germinating seed gels and was 75% identical with the cyclophilin in spot T13. In control gels, the relative intensity of cCBB-stained spot 227 was much less than that in extracts untreated with Cy5m. When incubated with thioredoxins, the intensity of the cCBB-stained spot 227 increased (Figure 2, box 8 and Figure 4H). This behaviour was opposite to that displayed by expected thioredoxin target proteins and is discussed later.

DISCUSSION

Modification and evaluation of the method for target identification

The applicability of mBBBr labelling in combination with two-dimensional gel electrophoresis for identification of proteins targeted by thioredoxins has been demonstrated recently in various studies [10–12]. In the present study, the method was successfully used to identify proteins reduced by thioredoxin h in the barley seed proteome.

Cy5m has been used for general protein detection in two-dimensional gels by labelling in reducing and denaturing conditions

[13]. In the present study, labelling with Cy5m was performed under native conditions in the absence of reducing chemicals to achieve selective protein detection. The higher sensitivity of detection of Cy5m in comparison with mBBR and enhanced protein separation by large format gels made it possible to include an increased number of seed proteins in the current target survey, and increased confidence in target protein identification, since each spot most probably contained only a single protein. Furthermore, compatibility with the experimental system used for previous and ongoing studies of the barley seed proteome [18,19,22] (O. Østergaard, C. Finnie, S. Laugesen, P. Roepstorff and B. Svensson, unpublished work) enabled prior knowledge of two-dimensional spot patterns of barley seed proteins to be exploited.

Labelling with Cy5m caused occasional shifts of labelled proteins in high pI spots to lower pI, presumably due to a negative charge in Cy5m. Altered spot patterns were also observed in the saturation labelling technique using Cy5m [13]. The pI shift actually increased the certainty of identification of some target proteins, since identification of multiple spots containing the same protein minimized the risk of wrong identification caused by spot overlapping. Reduction in intensity of the original unlabelled cCBB-stained spots also supported target identifications in some cases (e.g. ESP and endochitinase 1).

Although some proteins (CMe and BDAI-I) were identified both by mBBR and Cy5m labelling, the two techniques are complementary, as they led to identification of different target proteins (Tables 1 and 3). The enhanced sensitivity achieved by Cy5m labelling made it possible to visualize target proteins present in small amounts or weakly reduced, like chitinase isoenzymes. Nevertheless, reduction of 1-Cys peroxiredoxin and G3PD (Table 1) was not observed using Cy5m. Both of these proteins have previously been identified as possible target proteins using mBBR labelling [11,12] but their target status has not been confirmed by other methods. G3PD spots were present in control gels, whereas 1-Cys peroxiredoxin spots 59, 60 and 99 [19] (O. Østergaard, C. Finnie, S. Laugesen, P. Roepstorff and B. Svensson, unpublished work) were absent from cCBB-stained gels possibly due to pI shift. For some proteins, pI shift due to Cy5m labelling may result in co-migration with other fluorescent spots, preventing their identification as target proteins. Alternatively, mBBR and Cy5m may have differential capability to access, e.g. partially buried thiol groups.

The list of possible target proteins identified here only partially corresponds to those identified in a recent study, in which 23 putative targets of *E. coli* thioredoxin were identified in the wheat endosperm proteome using mBBR labelling [12]. Several of the proteins reported in the above study have been located in the barley seed proteome (Table 2) [18,19,22] (O. Østergaard, C. Finnie, S. Laugesen, P. Roepstorff and B. Svensson, unpublished work), although their reduction by thioredoxin was not observed here. The variation may be explained by the modified method and the different protein extracts used here and/or the dissimilar specificities of *E. coli* thioredoxin and barley thioredoxin h. Although enhanced protein reduction with thioredoxins was observed at higher temperatures (results not shown), in the present study, 22 °C was chosen rather than 37 °C [12] to reduce the risk of non-specific reactions.

Roles of HvTrxh1 and HvTrxh2

Since seed proteins were visualized in two-dimensional gels by cCBB staining regardless of their interactions with thioredoxins, the selectivity of thioredoxin reduction could be observed. Thioredoxins preferentially reduced a number of seed proteins, whereas

several abundant proteins containing one or more disulphide bonds, such as barley grain peroxidase (Figure 3), Barwin, perm-1 and thaumatin (Table 2), were not observed to be reduced and additional spots due to potential pI shifts did not appear nearby.

HvTrxh1 and HvTrxh2 differ both in temporal and spatial distribution in the seeds and in kinetic properties, suggesting that they may have different physiological roles [17]. The present study has indicated high similarity in their specificities, as all the identified targets were reduced by both the isoforms. Surface charges around active-site cysteine residues on thioredoxins influence recognition of chloroplastic target proteins [32–34]. The conservation of residues around the active site in modelled three-dimensional structures of HvTrxh1 and HvTrxh2 [17] may explain the similarities in target specificity. However, their *in vivo* target proteins and physiological roles may differ, because HvTrxh1 and HvTrxh2 have different patterns of temporal and tissue distribution and thus have the possibility to interact with different sets of proteins. Analysis of catalytic properties of HvTrxh1 and HvTrxh2 towards identified target proteins is required to study potential differences in affinity.

The barley seed proteome undergoes great changes during germination [18,19]. Several proteins were identified as possible thioredoxin h target proteins in both mature and germinating seeds and some target proteins were identified only in mature or germinating seed extracts. Even for the targets identified in both, the abundances were in many cases changed during germination.

BASI, α -amylase/trypsin inhibitors, chitinase isoenzymes and LTP1, identified as target proteins, are related to seed defence. Chitinase isoenzymes and LTP1 have been identified as possible target proteins for the first time here, whereas reduction of BASI by wheat thioredoxin h [9], HvTrxh1 and HvTrxh2 [17], and reduction of some α -amylase/trypsin inhibitors by wheat thioredoxin h [8] have been observed previously. However, BASI and a broad range of barley α -amylase/trypsin inhibitors are shown here to be selectively reduced by HvTrxh1 and HvTrxh2 in a complex mixture with other seed proteins. BASI inhibits endogenous barley α -amylase 2 and serine proteases of pathogens and pests [35,36]. Some barley α -amylase/trypsin inhibitors are known to inhibit insect α -amylase or bovine trypsin, and their inhibitory activity towards endogenous serine endoprotease is also reported [37]. Chitinases catalyse hydrolysis of chitin [38], a major component of cell walls in many fungi, and are reported to contribute to resistance against fungal attack [39]. The physiological role of LTP is still unknown, but association with defence against pathogens is suggested [40]. Moreover, redox regulation of the activities of some of these proteins is reported. Reduction of BASI is shown to render it more susceptible to proteolytic cleavage and to lower its inhibitory activity towards α -amylase 2 [9]. Thus it may aid the germination process in barley seeds by mobilizing starch reserves. Chemical reduction and alkylation of disulphide bonds in the chitin-binding domain in class I chitinases lowered their hydrolytic activity towards insoluble chitin [41]. The present identifications suggest that redox regulation of specific defence proteins is an important role for thioredoxin h in barley seeds.

The functions of embryo-specific protein and single-domain glyoxalase-like proteins are still unknown. Both proteins are abundant in mature seeds but their amounts decrease rapidly during germination. Thus reduction of these proteins is probably a particular role for thioredoxins in mature seeds and in seeds at the early stage of germination.

Conversely, the cyclophilin isoform in spot T13 is identified as a target in germinating seeds only. Cyclophilin catalyses *cis*–*trans* isomerization of proline residues to accelerate protein folding. Spinach chloroplast cyclophilin was identified as a possible target

of m-type thioredoxin, and reduction of two disulphide bonds in recombinant chloroplast cyclophilin from *Arabidopsis* is shown to improve its catalytic activity [42,43]. The cyclophilin isoform identified in spot T13 is closely similar to other cytosolic cyclophilins and lacks a signal sequence, and hence it is assumed to be a cytosolic protein. Three out of four cysteine residues present in *Arabidopsis* chloroplast cyclophilin are conserved and one additional cysteine residue is present in the barley cyclophilin isoform identified in spot T13. Hence at least one disulphide bond specific for redox activation may be present. The cyclophilin isoform in spot 227 behaved uniquely in the present study. All four cysteine residues in *Arabidopsis* chloroplast cyclophilin are conserved and two additional cysteine residues are present in this protein. The disappearance of spot 227 from the cCBB-stained image of control gels after treatment with Cy5m (Figure 4H) indicates that free thiol group(s) may become labelled, resulting in a pI shift of the protein. Reappearance of spot 227 after incubation with thioredoxins could be explained by novel disulphide formations after thioredoxin reduction of original disulphide bond(s) as reported for chloroplastic malate dehydrogenase [44,45]. Further work will be required to investigate this possibility.

Superoxide dismutase catalyses reduction of O_2^- to H_2O_2 , and is important for defence against oxidative stress. Plants have several superoxide dismutase isoforms with distinct subcellular locations [46]. The protein identified here is probably chloroplastic since it has an N-terminal extension characteristic of transit peptides [47], and has high homology to chloroplast superoxide dismutase from other plants. Thioredoxin h is cytosolic, thus interaction with this superoxide dismutase is unlikely to have physiological importance. However, superoxide dismutase could be targeted by chloroplastic thioredoxins.

Generally, biochemical properties of identified possible target proteins in oxidized and in reduced forms must be characterized to determine the regulatory importance of HvTrxh1 and HvTrxh2. Furthermore, many identified target proteins contain several disulphide bonds, thus identification of the specific disulphides targeted by thioredoxins is required to understand fully the mechanisms involved in their regulation.

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