

Unraveling thioredoxin-linked metabolic processes of cereal starchy endosperm using proteomics

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Abstract Application of a thiol-specific probe, monobromobimane, with proteomics and enzyme assays led to the identification of 23 thioredoxin targets in the starchy endosperm of mature wheat seeds (*Triticum aestivum* cv. Butte), almost all containing at least two conserved cysteines. The identified targets, 12 not known to be thioredoxin-linked, function in a spectrum of processes: metabolism (12 targets), protein storage (three), oxidative stress (three), protein degradation (two), protein assembly/folding (one) and unknown reactions (two). In addition to formulating metabolic pathways functional in the endosperm, the results suggest that thioredoxin acts in redox regulation throughout the life cycle of the seed.

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Key words: Thioredoxin; Redox regulation; Wheat; *Triticum aestivum*; Endosperm; Proteomics

1. Introduction

Aside from the hydrolysis of stored starch and protein, the metabolic processes taking place in the starchy endosperm of germinating cereal grains are not well understood. Many of the proteins of this biochemically active, genetically inactive tissue that are oxidized to the disulfide (S–S) state during maturation and drying are reduced to the sulfhydryl (SH) state during germination. There is a growing body of evidence that thioredoxin *h*, reduced by NADPH via NADP-thioredoxin reductase, is a participant in this process. In addition to reducing the stored protein, thioredoxin interacts with enzymes catalyzing its breakdown as well as the hydrolysis of starch, thereby facilitating the mobilization of nitrogen and carbon for the developing seedling [1–6]. Recent evidence suggests that thioredoxin also acts in a transmembrane network whereby the starchy endosperm communicates with the embryo and aleurone [7].

To gain further insight into its regulatory role in cereals, we have used a fluorescent thiol-specific probe, monobromobi-

mane (mBBR), coupled with proteomics and enzyme assays to identify additional soluble thioredoxin-linked proteins of the starchy endosperm of mature wheat grain [8,9]. Building on our recent proteomic studies with peanut seeds [9] and spinach chloroplasts [10], this approach has led to the identification of 23 proteins linked to thioredoxin, 12 not previously recognized as targets. In addition to uncovering these thioredoxin targets, the results give insight into biochemical processes of the cereal endosperm.

2. Materials and methods

2.1. Plant material

Wheat plants (*Triticum aestivum* L., cv. Butte 86) were grown in a climate-controlled greenhouse with an average maximum daytime temperature of 25°C and nighttime temperature of 17°C [8]. Starchy endosperm was harvested from grain 36 days post anthesis, frozen in liquid nitrogen and stored at –80°C.

2.2. Protein isolation

Endosperm was suspended in cold (4°C) KCl buffer (50 mM Tris–HCl, 100 mM KCl, 5 mM EDTA, pH 7.8) using 200 µl of buffer per 50 mg endosperm, incubated on ice for 5 min with intermittent mixing (Vortex Genie 2, Scientific Industries, Bohemia, NY, USA), and centrifuged for 15 min at 4°C at 14 000 rpm (Eppendorf 5415C, Brinkman Instruments, Westbury, NY, USA). The protein in the supernatant fraction was used for reduction as described below.

2.3. In vitro protein reduction

Soluble thioredoxin-linked proteins in the starchy endosperm were identified using a fluorescent thiol-specific probe, mBBR, coupled with proteomics [5,9–11]. Reduction of the protein disulfide bonds was carried out with: (i) the NADP/thioredoxin system, consisting of 0.125 µmol NADPH, 0.7 µg NTR, 0.8 µg thioredoxin (both from *Escherichia coli*) [12], (ii) the NADP/glutathione system, composed of 0.125 µmol NADPH, 0.1 µmol reduced glutathione (GSH), and 2.0 µg yeast GSH reductase (Sigma, St. Louis, MO, USA), and (iii) buffer control. The reductant was pre-incubated at 37°C for 15 min in 30 mM Tris–HCl buffer, pH 7.5, in a volume of 40 µl, then 10 µl (18 µg protein) of the KCl extract was added and incubated for another 20 min at 37°C. To stop the enzymatic reaction and label the targets, 0.1 µmol mBBR in 5 µl acetonitrile was added to each sample, which was then incubated for an additional 15 min at room temperature. The labeling reaction was terminated by adding 1.0 µmol of 2-mercaptoethanol in 10 µl.

2.4. Protein precipitation

The proteins in the reaction mixture were precipitated by the addition of four volumes of ice-cold 0.1 M ammonium acetate in methanol and incubation overnight at –20°C. Following centrifugation at 14 000 rpm for 15 min and 4°C, the pellet was rinsed with acetone, dried, and the proteins solubilized in urea buffer (9 M urea, 4% NP-40, 1% dithiothreitol (DTT) and 2% ampholytes).

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Abbreviations: GSH, reduced glutathione; DTT, dithiothreitol; mBBR, monobromobimane; PFP, fructose 6-phosphate 1-phosphotransferase; PPI, inorganic pyrophosphate; PDI, protein disulfide isomerase; PPK, pyruvate kinase.

2.5. Proteomic analysis

The mBBR-labeled proteins were separated as described [11] and identified by comparison to an extensive 2-D map of KCl-soluble endosperm proteins using computer analysis of digitized gel images (Progenesis Workstation version 2002.01, Nonlinear Dynamics, Newcastle upon Tyne, UK). The map was constructed using mass spectroscopy (MS) and tandem MS [11]. Spectra were transferred to a PC and identification was carried out using a suite of programs from Genomic Solutions (Madison, WI, USA); the Knexus automation client was used for peptide mass mapping and the Sonar package for MS/MS data. Reported mass spectrometer identifications from the plant protein database search had expectation values of less than 1×10^{-3} (one chance in 1000 that the match was due to a random event).

2.6. Enzyme assays

In addition to proteomic analysis, the identity of several of the enzymes as thioredoxin targets was confirmed by activity assays. KCl-soluble proteins were extracted from Butte 86 flour as described above for endosperm and aliquots of the protein extract incubated for 10 min at room temperature in water (Control), 4 mM glutathione (GSH), 2 mM DTT or 2 mM DTT and 2 μ M thioredoxin from *E. coli* (DTT+Trx). Aliquots of these reactions were used to measure enzyme activities with substrates and coupling enzymes from Sigma (St. Louis, MO, USA). Protein concentration was adjusted for each enzyme so that activities were in the linear range. Formation (or disappearance) of NADH was measured at 340 nm (Ultrospec 4000 Spectrophotometer, Amersham Biosciences, Piscataway, NJ, USA) in a 1 ml cuvette containing 100 mM Tris-HCl, pH 7.5, and the following for the in-

dicated enzymes. Alanine aminotransferase: 2 mM alanine, 2 mM α -ketoglutarate, 0.2 mM NADH, 0.01 mM pyridoxal phosphate and 1 U/ml lactate dehydrogenase; aldolase: 2 mM fructose 1,6-bisphosphate, 0.2 mM NADH and 2 U/ml glycerol 3-phosphate dehydrogenase; enolase: 4 mM 3-phosphoglycerate, 1 U/ml pyruvate kinase and 1 U/ml lactate dehydrogenase (the latter mixture was incubated 5 min with the endosperm extract before the addition of 0.2 mM NADH to enable the endogenous phosphoglycerate isomerase to convert added 3-phosphoglycerate to the 2-phosphoglycerate substrate); NAD-dependent glyceraldehyde 3-phosphate dehydrogenase: 10 mM sodium arsenate, 2 mM glyceraldehyde 3-phosphate and 0.4 mM NAD; NAD-dependent malate dehydrogenase: 2 mM oxaloacetate and 0.2 mM NADH; pyruvate, Pi dikinase (PPDK): 2 mM MgCl_2 , 5 mM AMP, 5 mM inorganic pyrophosphate (PPi), 2 mM phosphoenolpyruvate, 0.2 mM NADH and 1 U/ml lactate dehydrogenase; and triose phosphate isomerase: 2 mM glyceraldehyde 3-phosphate, 0.2 mM NADH and 2 U/ml glycerol 3-phosphate dehydrogenase.

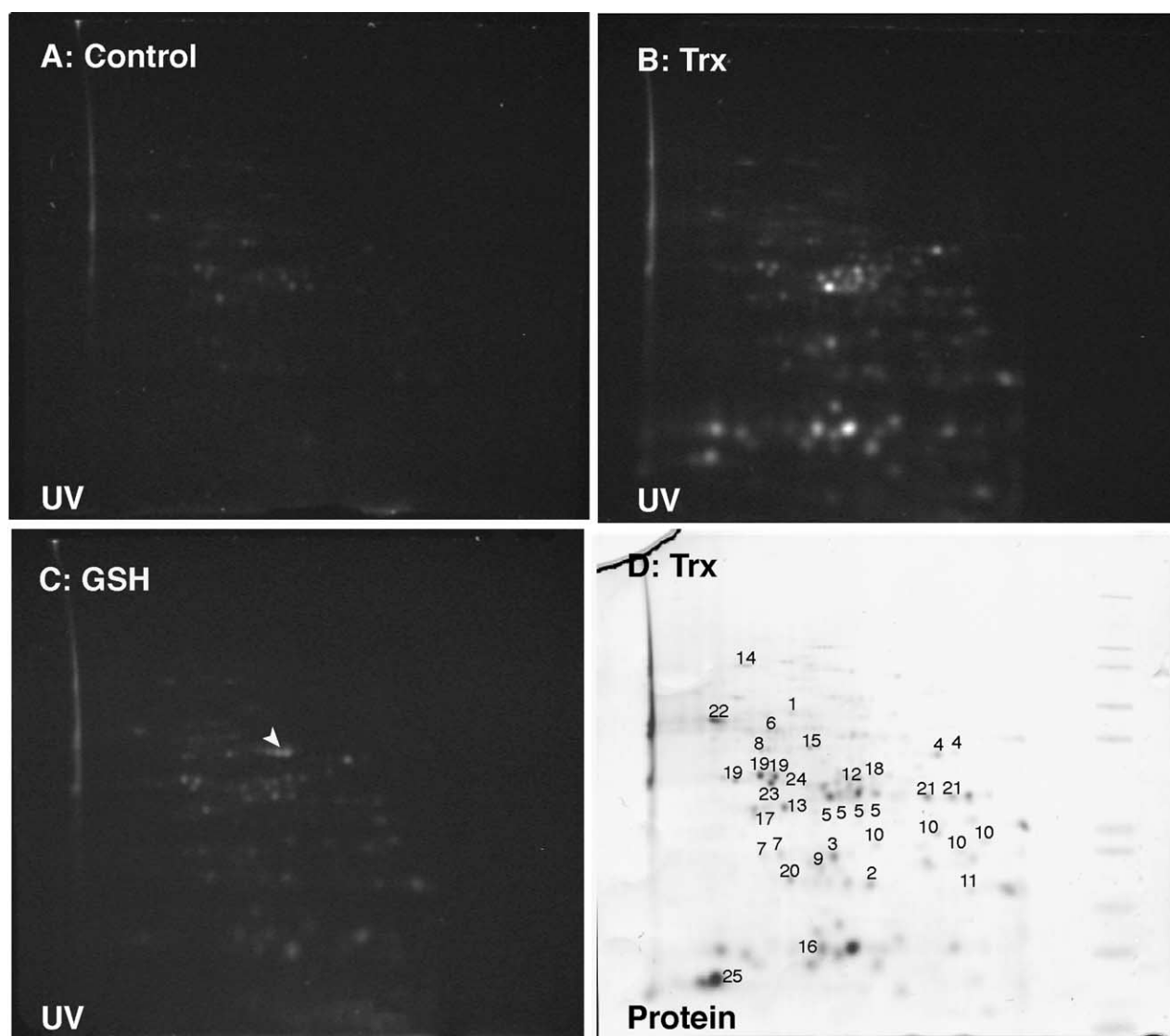
3. Results

As seen previously with cereal and other seeds [1,6,12–15], thioredoxin, reduced with NADPH via NADP-thioredoxin reductase, converted numerous proteins of the wheat starchy endosperm from disulfide (S–S) to sulfhydryl (–SH) form (Fig. 1A vs. B). By contrast, also as before, GSH, reduced by NADPH and GSH reductase, was much less effective (Fig.

Table 1
Potential thioredoxin target proteins of wheat endosperm

	Conserved cysteines
I. POTENTIAL NEW TARGETS	
Metabolism	
Glyceraldehyde 3-phosphate dehydrogenase (NAD) (4 isoforms)	2
Aldolase	4
Malate dehydrogenase (NAD)	6
Pyruvate, Pi dikinase (PPDK)	10
Alanine aminotransferase	8
Glyoxalase	2–3
Protein degradation	
26S proteasome regulating subunit S6	2–3
Serpin (4 isoforms)	nd
Stress-related proteins	
GSH-dependent dehydroascorbate reductase	2
Peroxidase (2 isoforms)	8
Protein assembly/folding	
Protein disulfide isomerase (PDI)	4
Unknown function	
Reversible glycosylated polypeptide (2 isoforms)	8
IIA. CONFIRMED TARGETS: WHEAT ENDOSPERM	
Metabolism	
PPi, fructose 6-phosphate 1-phosphotransferase (PFP)	7
α -Amylase inhibitor 0.19	10
α -Amylase/subtilisin inhibitor	4
Storage proteins	
Avenin precursor	8
LMW glutenin subunit b	6–7
IIB. CONFIRMED TARGETS: OTHER SYSTEMS	
Metabolism	
ADP-glucose pyrophosphorylase	4–5
Triose phosphate isomerase	4
Enolase	4
Stress-related protein	
1-Cys peroxiredoxin	2
Storage protein	
Seed globulins (4 isoforms)	nd
Unknown function	
Cyclophilin 2a	3

The SwissProt ID numbers of the proteins from top to bottom in I, starting with glyceraldehyde 3-phosphate dehydrogenase, are P26517, Q40676, Q9FWH5, O82032, P52894, Q948T6, Q8W425, P93692, Q9MB31, Q05855, P52589, Q9ZR33; in IIA, starting with PFP, P21343, P01085, P16347, P27919, Q8W3X1; in IIB, starting with ADP-glucose pyrophosphorylase, P12299, Q9FS79, Q42971, Q9AXH7, Q8LKV8, Q40673. nd = not determined.



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|--|-----------------------------------|--|
| 1. PPI, fructose-6-P, 1-phosphotransferase (PFP) | 10. Seed globulins (4 isoforms) | 19. Serpin (4 isoforms) |
| 2. Alpha-amylase/subtilisin inhibitor | 11. Cyclophilin 2a | 20. GSH-dependent dehydroascorbate reductase |
| 3. Avenin precursor | 12. Aldolase | 21. Peroxidase (2 isoforms) |
| 4. LMW glutenin subunit | 13. Malate dehydrogenase (NAD) | 22. Protein disulfide isomerase (PDI) |
| 5. Glyceraldehyde 3-P dehydrogenase (NAD) [4 isoforms] | 14. Pyruvate, Pi dikinase (PPDK) | 23. Reversibly glycosylated polypeptide |
| 6. ADP-glucose pyrophosphorylase | 15. Alanine aminotransferase 2a | 24. Reversibly glycosylated polypeptide b |
| 7. Triose phosphate isomerase (2 isoforms) | 16. Alpha-amylase inhibitor 0.19 | 25. <i>E. coli</i> thioredoxin |
| 8. Enolase (2 isoforms) | 17. Glyoxalase | ▲ Added GSH reductase |
| 9. 1-Cys peroxiredoxin | 18. Proteasome regulating subunit | |

Fig. 1. Reduction of proteins of wheat starchy endosperm by thioredoxin and GSH using the mBBR 2-D electrophoresis procedure and stained with Coomassie blue (D). KCl-soluble proteins were either not treated (A) or reduced by thioredoxin (B) or GSH (C), using NADPH and NADP-thioredoxin reductase and GSH reductase, respectively. Following reduction, the proteins were derivatized with mBBR and subjected to 2-D electrophoresis [9–11]. The white arrow in C designates added GSH reductase. Trx = thioredoxin.

1A vs. C). As in earlier cases, the fluorescent proteins in Fig. 1B appear to represent targets whose disulfide groups are specific for thioredoxin. It is noted that, while the gel in Fig. 1B was obtained using the *E. coli* NADP/thioredoxin system, similar but less intense results were obtained with the corresponding thioredoxin system from barley (*Hordeum vulgare*) aleurone that was overexpressed in *E. coli* (M.-J. Cho, unpublished results).

After Coomassie blue staining (Fig. 1D), the thioredoxin-

reduced proteins labeled with mBBR were identified by comparison to a 2-D map of KCl-soluble endosperm proteins. This approach led to the identification of 23 endosperm proteins linked to thioredoxin (Table 1). These proteins are participants in three metabolic processes: starch breakdown, protein breakdown and oxidative stress response. Included are 12 potential new targets functional in metabolism (four isoforms of NAD-glyceraldehyde 3-phosphate dehydrogenase, aldolase, NAD-malate dehydrogenase, PPDK, alanine aminotransfer-

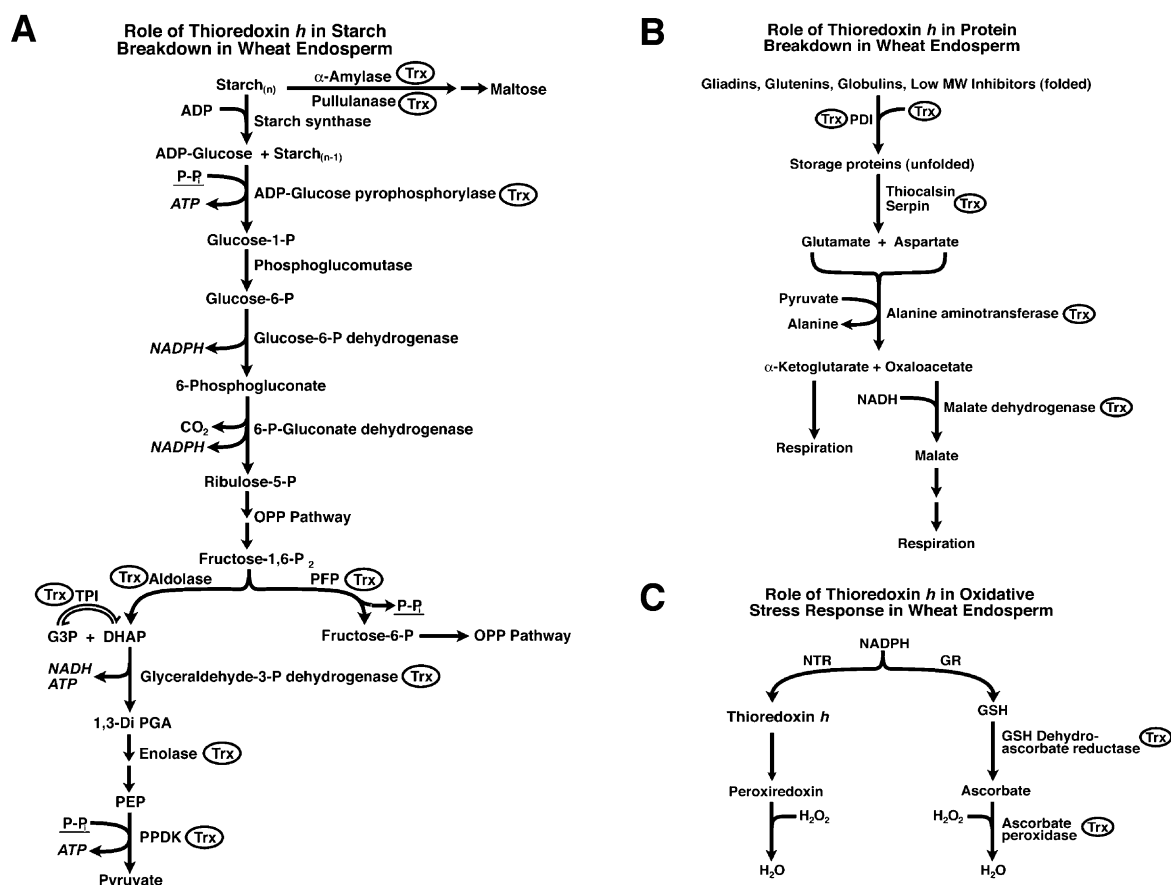


Fig. 2. Proposed role of thioredoxin *h* in regulating biochemical processes of wheat starchy endosperm. A: Starch breakdown. B: Protein breakdown. C: Oxidative stress response. The reductant specificity of the endosperm peroxidase identified in Table 1 is not known. It is assumed in C that the enzyme utilizes ascorbate as substrate as is the case for many peroxidases. TPI = triose phosphate isomerase.

ase, and glyoxalase), protein degradation (26S proteasome regulating subunit S6, serpin), stress response (GSH-dependent dehydroascorbate reductase and two peroxidase isoforms), protein assembly and folding (protein disulfide isomerase or PDI) and unknown reactions (two reversible glycosylated polypeptide isoforms). Five of the proteins in Table 1 have previously been identified as thioredoxin targets of the wheat endosperm, including representatives functional in metabolism (PPi, fructose 6-phosphate 1-phosphotransferase or fructose 6-phosphate 1-phosphotransferase (PFP), α -amylase/trypsin inhibitor and α -amylase/subtilisin inhibitor) [15,16] and as storage proteins (avenin precursor and LMW glutenin subunit b) [1,6,17]. Finally, six of the proteins are

known thioredoxin targets based on work with systems other than wheat grain. These include targets functional in metabolism (ADP-glucose pyrophosphorylase, triose phosphate isomerase and enolase) [10,18,19], oxidative stress response (1-cys peroxiredoxin) [20], as a storage protein (seed globulin, four isoforms) [5] and in unknown reaction(s) (cyclophilin 2a) [21]. Significantly, except for serpin and seed globulins for which the databases were not sufficient for sequence alignment, each of the potential target proteins identified in Table 1 contained at least two conserved cysteines – a feature characteristic of thioredoxin-linked proteins [10].

The identification of several of the proteins in Table 1 as thioredoxin targets was confirmed by enzymatic analysis. The

Table 2
Effect of DTT-reduced thioredoxin on the activity of enzymes identified as targets by proteomics

Enzyme	Control	GSH	DTT	DTT+Trx
Alanine aminotransferase	1.00	1.04	3.45	6.70
Aldolase	1.00	1.35	1.57	1.64
Enolase	1.00	1.86	3.15	4.61
Glyceraldehyde 3-phosphate dehydrogenase (NAD)	1.00	2.66	11.00	12.65
Malate dehydrogenase (NAD)	1.00	0.98	1.14	1.46
Pyruvate, Pi dikinase (PPDK)	1.00	1.02	1.08	1.27
Triose phosphate isomerase	1.00	1.22	2.64	2.93

The basal activity (Control) has been arbitrarily set to 1. Values represent the average activity observed in at least three assays. The activity recorded for the control of each enzyme tested was (nmol of NADH produced or consumed per min per mg of protein): alanine aminotransferase, 1.56; aldolase, 22; enolase, 1.4; glyceraldehyde 3-phosphate dehydrogenase, 0.32; malate dehydrogenase, 3225; PPDK, 41; triose phosphate isomerase, 1112.

activity of enzymes associated with glycolysis and amino acid breakdown was enhanced following preincubation with DTT-reduced thioredoxin: alanine aminotransferase, aldolase, enolase, NAD-glyceraldehyde-3-phosphate dehydrogenase, NAD-malate dehydrogenase, PPDk and triose phosphate isomerase (Table 2). Activation by thioredoxin ranged from 1.3- (pyruvate kinase) to 12-fold (NAD-glyceraldehyde 3-phosphate dehydrogenase). No attempt was made to optimize conditions to increase the effect of thioredoxin – a factor recognized as being important since early studies on chloroplast fructose 1,6-bisphosphatase, the first enzyme shown to be regulated by thioredoxin [22,23]. It should also be noted that, in addition to assay conditions, the extent of activation by thioredoxin could depend on the degree to which the seed becomes oxidized during maturation and drying – a factor that varies with respect to stage of development [24] and possibly environmental growth conditions. Typical of known thioredoxin-linked counterparts, each of the enzymes in Table 2 showed an increase in activity with DTT, but little if any response to reduced GSH.

4. Discussion

Recent work with peanut seeds [9], chloroplasts [10,21] and barley embryos [5] has resulted in the identification of approximately 35 previously unrecognized potential thioredoxin target proteins functional in a spectrum of processes, some not known to be thioredoxin-linked. In the present study, we have built on the recent work to elucidate additional thioredoxin-linked processes of wheat starchy endosperm – a tissue of scientific interest and commercial importance. In addition to identifying potential new thioredoxin targets, the present results permit the construction of a metabolic framework for the understanding of processes and pathways operative during wheat grain development. In the discussion below, earlier results [1–3,6,12,13,15,25,26] have been melded with those in Table 1 to formulate thioredoxin-linked biochemical processes of the starchy endosperm, notably starch breakdown, protein breakdown and oxidative stress response (Fig. 2). It is noted that confirmation of the effect of thioredoxin on certain of the enzymes shown as targets in Fig. 2 awaits more detailed study: (1) enzymes in Table 2 that showed a relatively low activation by thioredoxin (aldolase, NAD-malate dehydrogenase and PPDk), and (2) proteins not yet studied in this respect (PDI, serpin, GSH-dependent dehydroascorbate reductase and ascorbate peroxidase).

With respect to starch, thioredoxin appears to regulate breakdown directly at the α -amylase and pullulanase sites (via inactivation of specific inhibitor proteins and enhanced pullulanase synthesis) as well as downstream via ADP-glucose pyrophosphorylase, possibly the plastidic [18] or the recently identified wheat cytosolic form [27] (Fig. 2A). Thioredoxin also appears to act in the interconversion of fructose 1,6-bisphosphate and fructose 6-phosphate (activation of PFP) and at multiple points in a modified glycolytic pathway (activation of aldolase, triose phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, enolase and PPDk). It is of interest that three of the target enzymes either release PPi (PFP) or utilize PPi (ADP-glucose pyrophosphorylase, PPDk). It seems possible, therefore, that, like carbon, the associated metabolism of PPi is linked to the redox state of the endosperm.

In addition to extending its function in carbon transformations, the new findings strengthen the earlier recognized role of thioredoxin in regulating protein degradation [1,2,5,12,14,15,25]. Thus, thioredoxin not only facilitates the degradation of storage proteins directly via reductive unfolding (PDI) and protease activation (thiocalsin, serpin), but also by activating downstream enzymes (alanine aminotransferase and NAD-malate dehydrogenase) (Fig. 2B). In this way, redox state controls the conversion of stored nitrogen progressively from storage protein to respiratory substrate.

Finally, the present results have uncovered a role for thioredoxin in regulating the response of the starchy endosperm to oxidative stress (Fig. 2C). In addition to serving as an electron donor for peroxiredoxin, thioredoxin appears to facilitate the removal of hydrogen peroxide via regulation of GSH-dependent dehydroascorbate reductase and ascorbate peroxidase. It remains to be seen whether such a link to hydrogen peroxide and related oxidants has direct regulatory consequences for additional thioredoxin-linked proteins, e.g. transcription factors, as is the case for other systems [28,29].

5. Concluding remarks

By revealing previously unrecognized links to enzymes of carbon, nitrogen and oxygen metabolism, the mBBR/proteomic approach has amplified the role of thioredoxin *h* and led to the formulation of pathways functional in the starchy endosperm of cereals. Critical sulfhydryl groups of target proteins participating in these processes appear to become reversibly oxidized during grain maturation and drying [24], thereby either (a) suppressing activity (enzymes), (b) enhancing activity (enzyme inhibitors) or (c) lowering enzymatic accessibility (storage proteins). As a result of change from reducing to oxidizing redox status, activity of the biochemical machinery of the endosperm is decreased, in some cases to a minimum, and the grain enters a quiescent state to preserve nutrients and energy for subsequent germination and growth of new seedlings. When suitable environmental conditions develop, thioredoxin becomes reduced, and, in turn, reduces critical disulfide bonds of targets, thereby, dependent on the protein, changing either activity or solubility to promote germination. By reflecting the redox status of the endosperm, thioredoxin, which also occurs in adjoining tissues [5,30], appears to function in regulating metabolic activities associated with the life of the seed as it proceeds from the developing (reductive) to quiescent (oxidative) to the germination (reductive) state [1,5,24]. In fulfilling this function, thioredoxin responds to the changing redox status of endosperm essentially as in chloroplasts where, according to recent evidence [10], it appears to act in a similar capacity in oxidative regulation in addition to its established role in light/dark transition. While similar fundamentally, oxidative regulation in the two compartments would differ in one respect: chloroplasts could respond to oxidants repeatedly as a result of environmental fluctuations, whereas the endosperm would typically respond only once, as the developing seed becomes quiescent.

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