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Thioredoxin targets of developing wheat seeds identified by complementary proteomic approaches

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Abstract

The role of thioredoxin in wheat starchy endosperm was investigated utilizing two proteomic approaches. Thioredoxin targets were isolated from total KCl-soluble extracts of endosperm and flour and separated by 2-DE following (1) reduction of the extract by the NADP/thioredoxin system and labeling the newly generated sulfhydryl (SH) groups with monobromobimane (mBBr), and, in parallel, (2) trapping covalently interacting proteins on an affinity column prepared with mutant thioredoxin *h* in which one of the active site cysteines was replaced by serine. The two procedures were complementary: of the total targets, one-third were observed with both procedures and one-third were unique to each. Altogether 68 potential targets were identified; almost all containing conserved cysteines. In addition to confirming known interacting proteins, we identified 40 potential thioredoxin targets not previously described in seeds. A comparison of the results obtained with young endosperm (isolated 10 days after flowering) to those with mature endosperm (isolated 36 days after flowering) revealed a unique set of proteins functional in processes characteristic of each developmental stage. Flour contained 36 thioredoxin targets, most of which have been found in the isolated developing endosperm.

Keywords: *Triticum aestivum*; Wheat; Disulfide proteins; Disulfide proteome; Monobromobimane; Proteomics; Thioredoxin; Wheat endosperm; Wheat flour

1. Introduction

Redox regulation leads to an alteration in the activity of target proteins of a growing number of processes via thiol-disulfide exchange. The redox state of these proteins, including enzymes and regulatory components, is controlled by cellular redox agents. Paramount among these is thioredoxin, a family of small, 12 kDa proteins containing a redox-active disulfide group that is found in virtually all organisms (Holmgren, 1985, 1989). The active site of thioredoxin has two redox active cysteine residues in a highly conserved sequence [–Trp–Cys–Gly(Pro)–Pro–Cys–]. In heterotrophic systems, thioredoxin is reduced by NADPH via a flavin enzyme, NADP-thioredoxin reductase (NTR) (Berstermann et al., 1983; Florencio et al., 1988; Johnson et al., 1987a,b; Suske et al., 1979). Reduced thioredoxin transfers its electrons to a target disulfide, thereby, providing reducing equivalents for either regulating activity or reducing a substrate (Buchanan, 1980, 1991; Buchanan et al., 1994; Jacquot et al., 1997; Meyer et al., 1999).

Plants contain several forms of thioredoxin that differ in their subcellular localization and thus in the target proteins with which they interact (Baumann and Juttner, 2002; Besse and Buchanan, 1997; Buchanan, 1991; Schürmann and Jacquot, 2000). The emergence of improved proteomic techniques and development of new isolation procedures (Yano et al., 2001a; Motohashi et al., 2001) has led to a dramatic increase in the number of proteins targeted by thioredoxin in organelles – chloroplasts and mitochondria (Balmer et al.,

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2003, 2004; Motohashi et al., 2001) – as well as tissues – starchy endosperm, henceforth, endosperm, and embryos of mature seeds (Maeda et al., 2004; Marx et al., 2003; Wong et al., 2003, 2004). As a consequence, our understanding of thioredoxin function in these systems has greatly increased. These advances were made possible by the development of two procedures for the isolation of thioredoxin targets that are identified by proteomics: fluorescent gel electrophoresis (Yano et al., 2001a) and mutant thioredoxin affinity column chromatography (Balmer et al., 2003, 2004; Motohashi et al., 2001).

Thioredoxin (*h*-type) has also been found in developing wheat seeds (Gobin et al., 1997; Serrato and Cejudo, 2003) that actively undergo oxidation-reduction change during development (Gobin et al., 1997; Rhazi et al., 2003; De Gara et al., 2003). However, its role in the early life of the seed is not clear. To help fill this gap we have examined thioredoxin targets in the endosperm of wheat seeds harvested as soon as this tissue could be isolated – i.e., 10 days after flowering (days post anthesis or dpa). By applying both the fluorescence gel electrophoresis (Yano et al., 2001a) and mutant thioredoxin affinity column procedures (Balmer et al., 2003, 2004; Motohashi et al., 2001), proteomic analysis led to the identification of 68 total targets, including 40 new members functional in enzyme reactions not previously known to be linked to thioredoxin in cereal endosperm. In addition, to gain insight into their advantages and limitations, we applied both approaches to the analysis of isolated endosperm and flour. The approaches were found to be complementary: about one-third of the proteins were identified by both procedures, while an additional third were identified uniquely by each. Almost all of the potential targets contained conserved cysteines.

2. Results and discussion

The in vitro reduction/mBBr fluorescence labeling procedure used in this study is depicted in Fig. 1(a) and the mutant thioredoxin affinity column procedure is shown in Fig. 1(b). The starting material was the total KCl-soluble extract, containing mostly metabolic proteins (Evans et al., 1975; Wong et al., 2003, 2004), obtained from either 10 dpa endosperm or flour. Potential thioredoxin target proteins isolated by the mBBr labeling procedure were identified from a proteomic reference map of KCl-soluble extract of wheat endosperm that was developed over a period of three years (Venzel et al., in preparation). Numerous analyses of endosperm were conducted at several stages of development. Identification was based on mobility of proteins according to p*I* in the first dimension and according to molecular weight in the second dimension. For the mutant thioredoxin procedure the concentration of KCl in the sample was diluted 5-fold, to 20 mM, before application to the column. Owing to this additional chromatographic step, the protein pattern obtained with the fraction eluted from the affinity column differed from the reference map for the endosperm. To be confident of their identity, the spots were excised, digested by trypsin and identified by mass spectrometry.

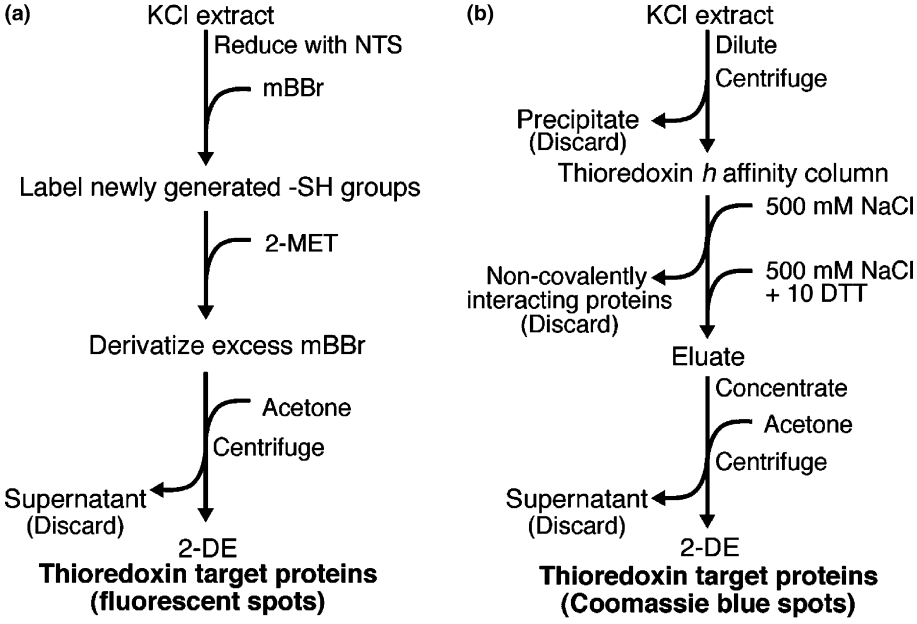

Fig. 1. Isolation of thioredoxin-linked proteins: (a) mBBr fluorescence gel procedure. (b) Mutant thioredoxin affinity column procedure. NTS = NADP/thioredoxin system.

2.1. Isolation of thioredoxin target proteins by in vitro reduction and the mBBr fluorescence labeling procedure

The proteins in the different preparations were reduced by the *E. coli* thioredoxin system and then subjected to 2-DE after labeling with mBBr. Similar patterns of protein reduction are observed when plant thioredoxin systems replaced that of the *E. coli* (Balmer et al., 2004; Wong et al., 2004; Yano et al., 2001a). The fluorescence and protein patterns from 10 dpa endosperm and flour are shown in Figs. 2 and 3, respectively. Overall, 39 proteins were reduced by thioredoxin and detected as fluorescent spots in the 2-DE gel of 10 dpa endosperm (Fig. 2), whereas only 15 were observed in flour extracts (Fig. 3). (A spot with its own Swiss-Prot number was assigned an independent number in the gels in this study.) The identity and number of conserved cysteines of each of these proteins are presented in Table 1. More than 80% of the total potential target proteins, i.e., 32 spots, were detected in 10 dpa endosperm extract and not in flour (Fig. 2 and Table 1).

It is noted that, of the 68 potential targets in Table 1, 28 were identified previously. Wheat endosperm: Nos. 1, 6, 11, 17, 20, 24, 28, 32, 35, 36, 37, 41, 42, 46, 49, 51, 52,

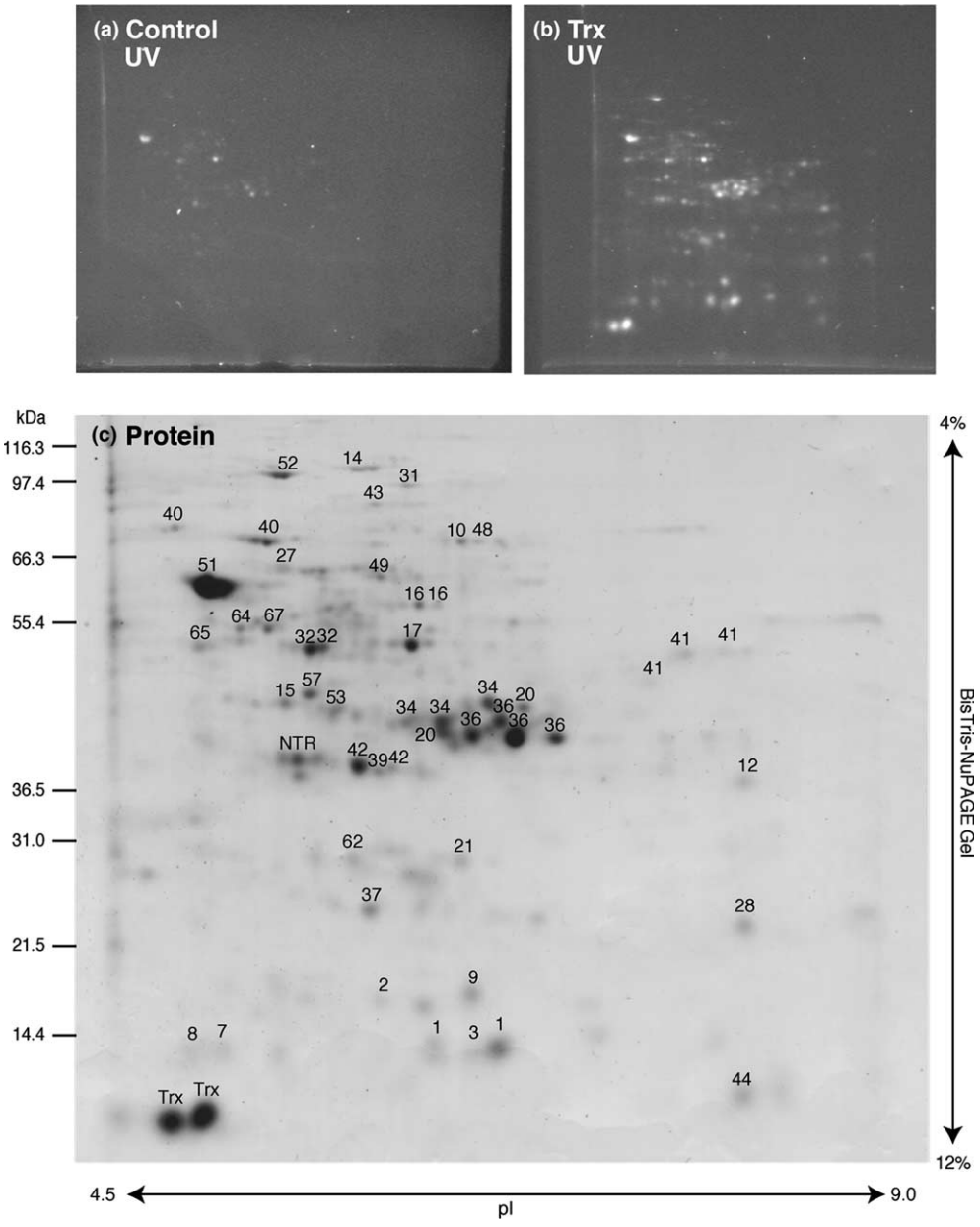

Fig. 2. Thioredoxin targets in endosperm of 10 dpa seeds identified by the mBBr fluorescence procedure: (a) Fluorescent image of control; sample treated with buffer without thioredoxin. (b) Fluorescent image of sample treated with reduced *E. coli* thioredoxin. (c) Image of fluorescent gel stained with Coomassie Blue. A spot with its own Swiss-Prot number was assigned its own protein number. The same number was used for a single protein showing multiple spots. Numbers correspond to the proteins identified in Table 1.

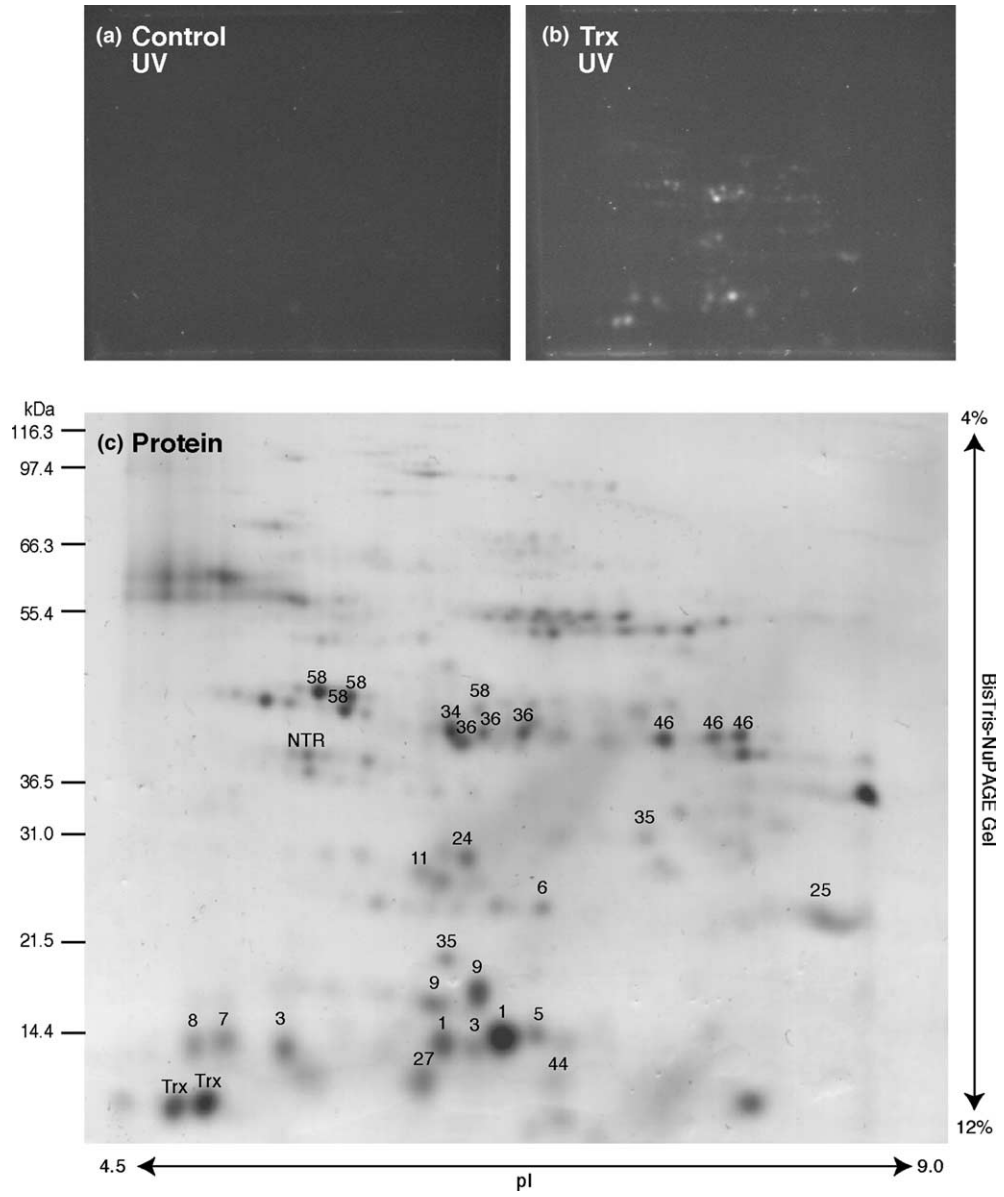

Fig. 3. Thioredoxin targets in flour identified by the mBBr fluorescence procedure. Experimental conditions and protein identification as in Fig. 2. (a) Fluorescent image of control; sample treated with buffer without thioredoxin. (b) Fluorescent image of sample treated with reduced *E. coli* thioredoxin. (c) Image of fluorescent gel stained with Coomassie Blue. Other conditions as in Fig. 2.

58, 62 (Wong et al., 2003); Nos. 2, 3, 4, 5 (Wong et al., 2004); mature and germinating barley: Nos. 7, 8, 9, 44, 61 (Maeda et al., 2004). Many of the identified targets not previously observed are involved in cell division, amino acid and protein synthesis – processes that characterize early endosperm development (Bewley and Black, 1994; Evans et al., 1975). Eight of the fifteen targets identified in flour extract (Fig. 3 and Table 1) were not detected in young tissue, notably storage and related proteins that develop later in the life of the seed (Bewley and Black, 1994; Evans et al., 1975). It is noted that while most of the proteins detected appeared to be Trx targets, several were not labeled with mBBr. Included were a number of weakly fluorescently stained spots in the 10 dpa preparation and several major proteins in flour (4 o'clock from #46; 3 o'clock from #1 in Fig. 3). The latter proteins were seen as minor spots at 10 dpa.

2.2. Isolation of thioredoxin target proteins by mutant thioredoxin affinity column procedure

Target proteins from 10 dpa endosperm and flour were found to interact with thioredoxin *h* and to bind to the mutant derivative on an affinity column (Figs. 4 and 5, respectively). Approximately the same number of major proteins were identified by the column procedure in two preparations – i.e., 28 proteins from 10 dpa en-

Table 1
Identification of potential thioredoxin targets in endosperm of 10 dpa seeds and flour using the mBBr fluorescence gel and affinity column procedures

Spot #	Protein	mBBr		Column		CYS	Swiss-Prot
		E	F	E	F		
1	α-Amylase inhibitor 0.19	•	•		•	10(5)	P01085
2	α-Amylase inhibitor 0.28	•				10(5)	P01083
3	α-Amylase inhibitor 0.53	•	•		•	9(4)	P01084
4	α-Amylase inhibitor Ima1		•			7(2)	O49956
5	α-Amylase/chymtotrypsin inhibitor WCI		•			6	P83207
6	α-Amylase/subtilisin inhibitor (WASI)		•			3	P16347
7	α-Amylase/trypsin inhibitor CM16	•	•			7(2)	P16159
8	α-Amylase/trypsin inhibitor CM17	•	•			7(2)	Q41540
9	α-Amylase/trypsin inhibitor CM3	•	•		•	7(2)	P17314
10	10-Formyltetrahydrofolate synthase	•		•		10	Q9SPK5
11	1-Cys peroxiredoxin		•		•	1	Q9AXH7
12	26S Proteasome regulatory S12 subunit	•				0	Q9C774
13	2-Cys peroxiredoxin			•		2	P80602
14	Aconitase	•		•		10	Q8L784
15	Actin	•				4	Q9FUS4
16	ADP-glucose PPase, LS	•				11	P12299
17	Alanine aminotransferase	•		•	•	8	P52894
18	Aldehyde dehydrogenase			•		3	Q8LST6
19	Aldehyde oxidase				•	20	064418
20	Aldolase	•		•	•	4	Q8VWM9
21	Ascorbate peroxidase	•		•		1	O23983
22	ATPase -subunit			•	•	2	Q41534
23	Auxin-induced protein	•				5	Q942N5
24	Avenin		•			nd	P27919
25	Avenin N9		•			nd	Q09114
26	Catalase			•		5	P55307
27	Chaperonin 60 KD β-subunit	•				4	Q9LWT6
28	Cyclophilin A	•		•	•	3(1)	Q93XQ6
29	Dessication-related protein				•	0	Q7X653
30	Elongation factor 1			•		1	Q9M3U8
31	Elongation factor 2	•				4	O23755
32	Enolase	•		•	•	4	Q42971
33	ER membrane fusion protein, CDC 48-like				•	11	QX7E16
34	Formate dehydrogenase, mitochondrial	•		•	•	1	Q9ZRI8
35	Globulin	•			•	8	Q7XYC3
36	Glyceraldehyde 3-P dehydrogenase (NAD)	•	•	•	•	2	Q9M4V4
37	GSH dehydroascorbate reductase	•			•	2	Q84UH6
38	GSH peroxidase			•	•	1	Q9SME4
39	Guanine nucleotide-binding protein	•				6	P49027
40	Heat shock protein, 70	•		•	•	2	Q40058
41	LMW glutenin subunit	•			•	6	Q8GU18
42	Malate dehydrogenase (NAD)	•		•	•	5	Q9FWH5
43	Methionine synthase	•				5	Q8W0Q7
44	Nonspecific lipid-transfer protein precursor	•	•			8	P24296
45	P0460c04.20				•	nd	Q8LPY3
46	Peroxidase		•		•	8	Q8LK23
47	Phosphoglycerate mutase			•		2	P30792
48	Poly(A)-binding protein	•				2	P93616
49	PPi-fructose-6-P 1-phosphotransferase	•				10	Q41141
50	Profilin			•		2	P49232
51	Protein disulfide isomerase	•		•	•	4	Q93XQ8
52	Pyruvate Pi dikinase	•		•		8	Q41847
53	Reversibly glycosylated polypeptide	•				9	Q9ZR33
54	Ribonuclease-like protein 1, regulator			•		5	Q9M8R8
55	Ribosomal protein S12			•	•	5	Q9XHS0
56	RNA binding protein			•		1	Q41518
57	S-adenosylmethionine synthetase	•				4	P50299
58	Serpin		•			0	P93693
59	Stress-induced protein, sti-1 like			•		5	Q9STH1
60	Sulfite reductase				•	3	Q8LP96

(*continued on next page*)

Table 1 (*continued*)

Spot #	Protein	mBBr		Column		CYS	Swiss-Prot
		E	F	E	F		
61	Superoxide dismutase [Cu-Zn]				•	2	Q8LIB7
62	Triosephosphate isomerase	•		•		4	Q9FS79
63	Triticin				•	4	Q08837
64	Tubulin α-3 chain	•				10	Q9ZRR5
65	Tubulin β-3 chain	•				10	Q9ZRB0
66	Ubiquitin conjugating enzyme E2			•		3	P35133
67	UDP-glucose PPase	•				2	Q43772
68	WD-repeat protein RBAP1			•		7	Q9ASE6

Numbers under CYS indicate the number of conserved cysteines; the number of known structural disulfide bonds is designated in parentheses. E = isolated endosperm, F = flour; • indicates presence of protein.

dosperm and 27 from flour (Table 1). Despite the similarity in the number of targets retained on the column with the two samples, only 12 proteins shared common identity in the two preparations (Table 1). The affinity column trapped an additional 16 potential thioredoxin targets in 10 dpa endosperm that were not obvious in flour. Similarly, the column retained 15 flour proteins not detected in the young endosperm. These results demonstrate that the mutant thioredoxin *h* interacted with individual targets prevalent in endosperm tissues at different stages of development.

2.3. Comparison of the two isolation procedures

The mBBr fluorescence and affinity column procedures were found to be complementary. Of the 68 total proteins identified in Table 1, approximately one-third (22) were detected by both methods and one-third were unique to each, i.e., 25 to the fluorescence and 21 to the affinity column procedure (Table 2). There are several possibilities to explain differences between two approaches (Fig. 2 vs. Fig. 4). Owing to the limited amount of protein that can be loaded on the gels, the in vitro

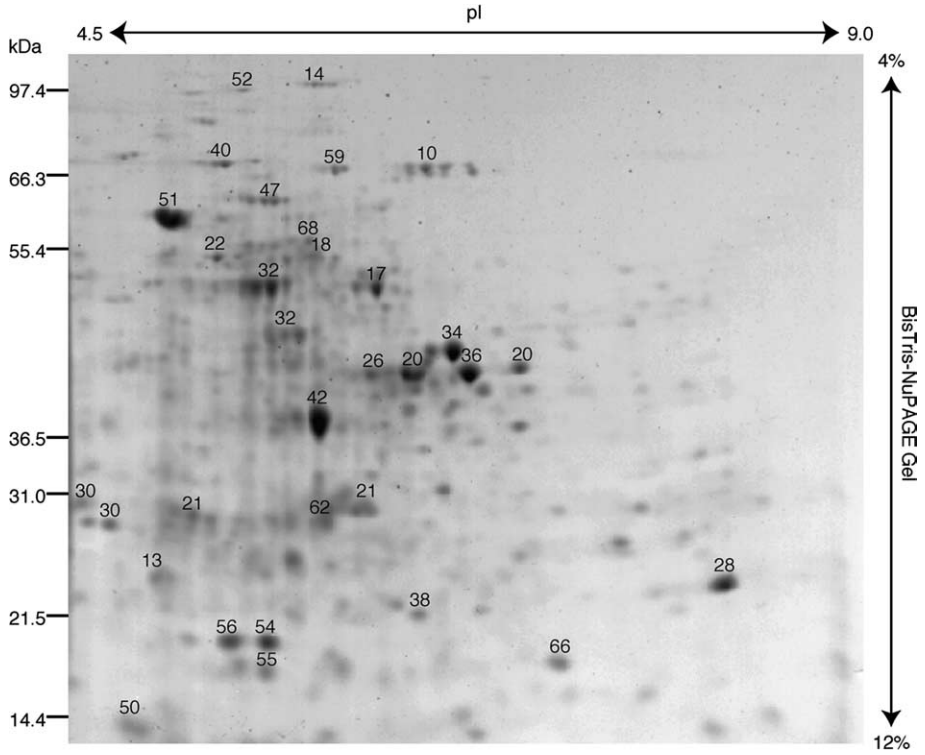

Fig. 4. Thioredoxin targets in endosperm of 10 dpa seeds identified by the affinity column procedure. Proteins were eluted from the column with DTT, resolved by 2-DE and stained with Coomassie Blue. Protein identification as in Fig. 2.

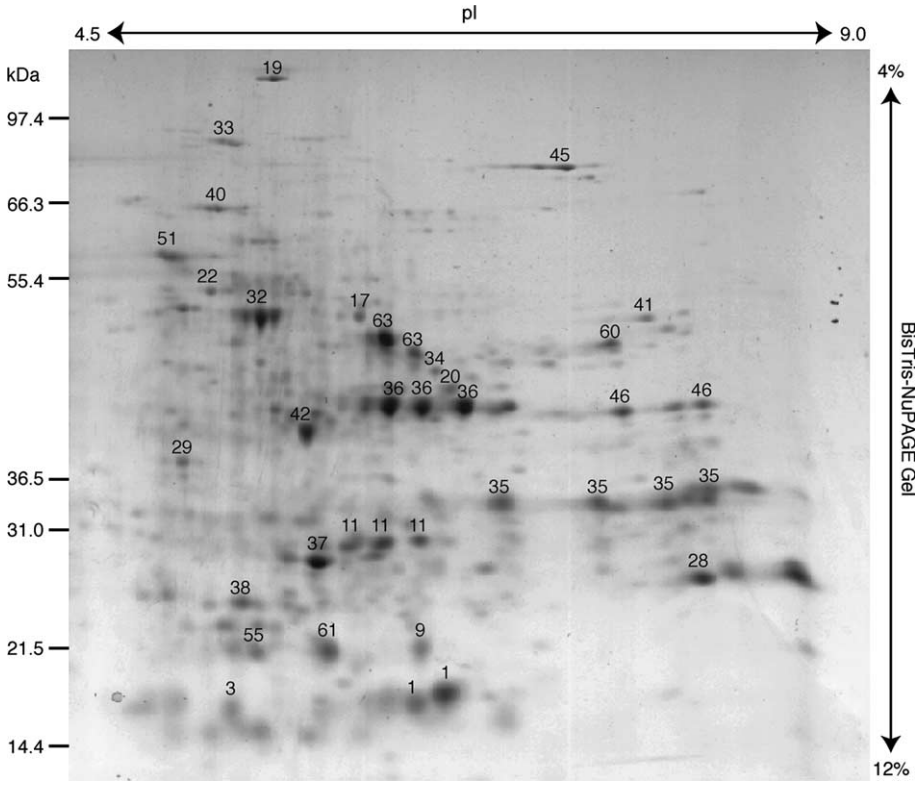

Fig. 5. Thioredoxin targets in flour identified by the affinity column procedure. Experimental conditions as in Fig. 4; protein identification as in Fig. 2.

Table 2
Comparison of thioredoxin targets identified by the mBBr fluorescence and affinity column procedures

mBBr fluorescence	Affinity chromatography	Both procedures
α-Amylase inhibitor 0.28	2-Cys peroxiredoxin	α-Amylase inhibitor 0.19
α-Amylase inhibitor Ima1	Aldehyde dehydrogenase	α-Amylase inhibitor 0.53
α-Amylase/chymtotrypsin inhibitor WCI	Aldehyde oxidase	α-Amylase/trypsin inhibitor CM3
α-Amylase/subtilisin inhibitor (WASI)	ATPase β-subunit	10-Formyltetrahydrofolate synthase
α-Amylase/trypsin inhibitor CM16	Catalase	1-Cys peroxiredoxin
α-Amylase/trypsin inhibitor CM17	Dessication-related protein	Aconitase
26S Proteasome regulatory S12 subunit	Elongation factor 1β	Alanine aminotransferase
Actin	ER membrane fusion protein, CDC 48-like	Aldolase
ADP-glucose PPase, LS	GSH peroxidase	Ascorbate peroxidase
Auxin-induced protein	P0460c04.20	Cyclophilin A
Avenin	Phosphoglycerate mutase	Enolase
Avenin N9	Profilin	Formate dehydrogenase, mitochondrial
Chaperonin 60 KD β-subunit	Ribonuclease-like protein 1, regulator	Globulin
Elongation factor 2	Ribosomal protein S12	Glyceraldehyde 3-P dehydrogenase (NAD)
Guanine nucleotide-binding protein	RNA binding protein	GSH dehydroascorbate reductase
Methionine synthase	Stress-induced protein, sti-1 like	Heat shock protein, 70
Nonspecific lipid-transfer protein precursor	Sulfite reductase	LMW glutenin subunit
Poly(A)-binding protein	Superoxide dismutase [Cu–Zn]	Malate dehydrogenase (NAD)
PPi-fructose-6-P 1-phosphotransferase	Triticin	Peroxidase
Reversibly glycosylated polypeptide	Ubiquitin conjugating enzyme E2	Protein disulfide isomerase
S-adenosylmethionine synthetase	WD-repeat protein RBAP1	Pyruvate Pi dikinase
Serpin		Triosephosphate isomerase
Tubulin β-3 chain		
Tubulin a-3 chain		
UDP-glucose PPase		

Proteins as listed were identified only by mBBr fluorescence or by affinity chromatography. The indicated proteins were identified in both procedures. Results are included for both seed endosperm and flour.

reduction/mBBr labeling method seemingly highlights the more abundant targets – e.g., protein disulfide isomerase, enolase and glyceraldehyde 3-P dehydrogenase. By contrast, the mutant thioredoxin affinity column traps interacting proteins irrespective of abundance, allowing the enrichment of many potential targets, including ones such as 2-cys peroxiredoxin, ubiquitin conjugating enzyme E2 and elongation factor 1B that are barely visible on 2-DE gels developed with the complete KCl-soluble extract.

Another difference between the two procedures is inherent in the nature of the techniques. Comparison of control and thioredoxin-reduced samples labeled with the thiol-specific probe mBBr ensures that reduction took 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. Differences could also result from the possibility that: (i) a single spot may contain more than one protein and the one identified is not a target; (ii) the defined sequence of the protein identified may not be fully accurate, leading to an apparent deficiency in Cys content although the amino acid is actually present; (iii) the mutant thioredoxin, normally forming a mixed disulfide with an authentic target on the column, could potentially also bind a glutathionylated or oxidized cysteine residue (Kumar et al., 2002; Rouhier et al., 2002); (iv) the mutant thioredoxin could trap a non-target protein with a free, exposed cysteine; (v) dilution of the extract that is required to achieve a salt concentration suitable for the affinity protocol may lead to differences in content of hydrophobic proteins (Fig. 1(a) vs. (b)) – e.g., α-amylase inhibitors and storage proteins (avenins) that are sensitive to salt concentration (Kim and Bushuk, 1995) seem to have precipitated and were observed to a lesser extent with the affinity column preparation (Table 2). Overall, the results show that a combination of the two approaches is best for obtaining a comprehensive overview of potential targets ranging from less to more abundant.

2.4. Developmental changes

A comparison of the thioredoxin-targets in Table 1 reveals major differences in the profiles observed in early development (10 dpa) and in flour, an end-product of mature seeds consisting of essentially pure endosperm (Pomeranz, 1964). As seen in the fluorescence gels, proteins from 10 dpa endosperm extract were more reduced than those in flour (Fig. 2(a) vs. Fig. 3(a)). This finding is in keeping with the conclusion that the cysteines initially present become progressively oxidized to disulfides as the seed matures and dries (De Gara et al., 2003; Gobin et al., 1997; Rhazi et al., 2003). Further, following treatment with reduced *E. coli* thioredoxin, noticeably more proteins were reduced and labeled in the 10 dpa preparation than in flour (Fig. 2(b) vs. 3(b)). A general shift in the p*I* of the protein population from the acidic range for 10 dpa endosperm to the more basic range for flour was also observed (Fig. 2(c) vs. 3(c)). The basis and significance of this shift are not clear. It is noted, however, that external processing procedures, e.g., conditioning and milling, can affect flour proteins, and may make comparison difficult.

To assess the involvement of thioredoxin during seed development, it is desirable to compare the same tissue (endosperm) at two or more stages of maturation using a single method of identification. The results obtained with the fluorescence gel procedure in an earlier study describing thioredoxin targets in mature, 36 dpa, endosperm (Wong et al., 2003) are, therefore, suitable for comparison to the 10 dpa counterparts (Table 1). It is noted that in the present experiments we have identified 14 additional thioredoxin targets in 36 dpa endosperm, thereby increasing the previous number of 23 to 37. A compilation of the data for 36 dpa endosperm (previous and updated) together with those for 10 dpa tissue is presented in Table 3. It is noted that isoforms of the different proteins are grouped together, thus reducing the number of entries from 68 to 49. The 49 proteins listed can be assigned to 7 functional groups: carbohydrate metabolism, cell division, nitrogen metabolism, protein degradation, protein synthesis, protein storage and stress-related reactions in addition to those of unknown function. Whereas an approximately equal number of proteins were identified in the two samples – 35 for 10 dpa and 37 for 36 dpa samples, of which 12 and 14 were, respectively, unique to each age. As expected, young endosperm was characterized by an emphasis on biosynthesis (protein synthesis and nitrogen metabolism) and cell division – a process not previously known to be linked to thioredoxin in seeds. Targets of mature endosperm, on the other hand, highlighted functions related to stress, protein storage and degradation. The shift in the processes regulated by thioredoxin reflects the evolution of biological events characterizing seed development: an emphasis in young endosperm on building the machinery needed for grain filling, growth, cell expansion and division vs. storing and potentially degrading protein in the mature endosperm. On the other hand, while there were differences in individual targets, the number of thioredoxin-linked proteins affecting carbohydrate metabolism was the same at both ages (Table 3). This uniform distribution of the enzymes involved in carbohydrate metabolism may be indicative of their importance to both synthesis and degradation. The increase in stress and defense-related proteins in the older tissue could account for the seed's need to survive harsh conditions until and when germination commences. We were surprised that such a large number of proteins appear to be thioredoxin targets. Perhaps this finding emphasizes the importance of

Table 3
Differences in thioredoxin targets as a function of seed development

	10 dpa	36 dpa
Carbohydrate metabolism		
α-Amylase inhibitor	•	•
α-Amylase inhibitor Ima1		•
α-Amylase/chymtotrypsin inhibitor WCI		•
α-Amylase/subtilisin inhibitor (WASI)		•
α-Amylase/trypsin inhibitor	•	•
Aconitase	•	•
ADP-glucose PPase, LS	•	•
Aldolase	•	•
Enolase	•	•
Formate dehydrogenase, mitochondrial	•	•
Glyceraldehyde 3-P dehydrogenase (NAD)	•	•
Malate dehydrogenase (NAD)	•	•
PPi-fructose-6-P 1-phosphotransferase	•	•
Pyruvate Pi dikinase	•	•
Triosephosphate isomerase	•	•
UDP-glucose Ppase	•	
Cell division		
Actin	•	
Tubulin α-3 chain	•	
Tubulin β-3 chain	•	
Nitrogen metabolism		
10-Formyltetrahydrofolate synthase	•	
Alanine aminotransferase	•	•
Ketol-acid reductoisomerase		•
Methionine synthase	•	•
S-adenosylmethionine synthetase	•	
Protein degradation		
26S Proteasome regulatory S12 subunit	•	•
Chaperonin 60 KD β-subunit	•	•
Leucine aminopeptidase		•
Protein synthesis		
Elongation factor 2	•	
Heat shock protein, 70	•	•
Poly(A)-binding protein	•	
Protein disulfide isomerase	•	•
Storage proteins		
Avenin		•
Avenin N9		•
Globulin	•	•
LMW glutenin subunit	•	•
Stress-related		
1-Cys peroxiredoxin		•
Ascorbate peroxidase	•	
Glyoxalase		•
GSH dehydroascorbate reductase	•	•
Peroxidase 1		•
Serpin		•
Thaumatin-like protein		•
Unknown		
Auxin-induced protein	•	
Cyclophilin A	•	•
Guanine nucleotide-binding protein	•	
Lipid-transfer protein precursor		•
Nonspecific lipid-transfer protein precursor	•	•
Protein F7A7_100, similarity		•
Reversibly glycosylated polypeptide	•	

The summary for 10 and 36 dpa endosperm taken, respectively, from the current study and Wong et al. (2003). • Indicates presence of protein. The proteins shown each has its own Swiss-Prot number.

disulfide proteins in the cereal endosperm in reactions ranging from synthesis and assembly to regulation and response to stress.

Overall, the results suggest that thioredoxin plays a broad role in young, biosynthetically active endosperm and regulates processes somewhat different from those in the mature tissue (Wong et al., 2003). These results are to be compared with the study by Skylas et al. (2000), who in a proteomic analysis of wheat endosperm, found that the emphasis on protein synthesis observed in young endosperm tissue decreased as the grain matured.

3. Concluding remarks

A dual experimental approach based on the mBBr gel fluorescence and affinity column procedures led to a near tripling (from 23 to 68) in the number of potential thioredoxin target proteins in wheat endosperm. Approximately one-third of the targets were identified by both approaches, whereas an additional third were identified individually by each. The results show that the procedures are complementary. If possible, both should be applied in the search for new targets. For extracts rich in sulfhydryl groups, it is advantageous to use the fluorescence procedure in which *N*-ethylmaleimide (NEM) is added to decrease background fluorescence prior to reducing target proteins with thioredoxin (Yano, 2003). This modification proved successful in identifying thioredoxin targets in plant mitochondria (Balmer et al., 2004).

The newly identified seed proteins function in a spectrum of processes, many not previously known to be redox regulated in cereals. Commensurate with a link to thioredoxin, almost all of the potential targets contain conserved cysteines. In some cases, the proteins are also known to contain structural disulfide bonds.

In addition to revealing new functions for thioredoxin, the present work confirms the earlier deduced sequence of redox changes taking place in cereal endosperm: proteins are synthesized in the reduced state early in seed development and oxidized during maturation and drying (Fig. 6) (Gobin et al., 1997). Upon

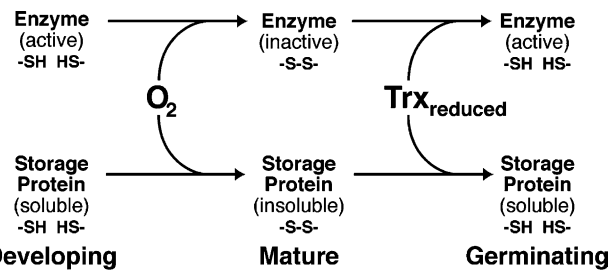

Fig. 6. Role of oxygen and reduced thioredoxin on enzymes and storage proteins in grain development and germination.

germination, thioredoxin reduces the oxidized proteins, thereby leading to increased solubility, proteolysis and, ultimately, nitrogen and carbon mobilization (Besse et al., 1996; Kobrehel et al., 1992; Lozano et al., 1996; Marx et al., 2003; Wong et al., 2002, 2004; Yano et al., 2001b) (Fig. 6).

4. Experimental

4.1. Biological 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 (Altenbach et al., 2003). Starchy endosperm was harvested from 10 dpa grain, frozen in liquid nitrogen and stored at −80 °C. Mature grain was tempered and milled to flour with a Brabender Quadramat Junior (South Hakensack, NJ) using standard procedures at the Western Wheat Quality Laboratory (US Department of Agriculture, Agricultural Research Service, Pullman, WA). Recombinant mutant poplar Trx *h*1 C42S was overexpressed in *E. coli* and purified from cell extracts as described (Behm and Jacquot, 2000).

4.2. Protein isolation

Proteins from endosperm or flour were separated into two fractions based on solubility in KCl (Wong et al., 2004). Endosperm was ground and flour 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 or flour. For endosperm fractionation, protease inhibitors (Mini Complete Protease Inhibitor Cocktail, Roche Applied Science, Indianapolis, IN) were added to the KCl buffer (1 tablet/10 ml). The mixture was incubated on ice for 5 min with intermittent mixing (Vortex Genie 2, Scientific Industries, Inc., Bohemia, NY) and centrifuged at 4 °C for 15 min at 14,000 rpm (Tomy MRX-151, Peninsula Laboratories, Inc., Belmont, CA). The pellet, containing the gliadins and glutenins, was not further analyzed. The proteins of the supernatant fraction, designated the KCl-soluble fraction, were subjected to in vitro reduction or affinity column fractionation as described below.

4.3. Protein determination

Proteins in the KCl-soluble fraction were precipitated with ammonium acetate/methanol (Wong et al., 2004) and the pellet rinsed with acetone and dried. The Bio-Rad assay based on the dye-binding procedure (Bradford, 1976) was used to quantify protein amount. Bovine γ-globulin served as the standard.

4.4. Labeling of protein sulfhydryls by in vitro protein reduction

The disulfide proteins of the KCl-soluble fraction from endosperm or flour were identified after reduction with thioredoxin using a fluorescent thiol-specific probe, monobromobimane (mBBr), coupled with proteomics (Balmer et al., 2004; Marx et al., 2003; Wong et al., 2003; Yano et al., 2001a). Reduction of protein target disulfides was effected with the NADP/thioredoxin system, consisting of 0.125 μmol NADPH, 0.7 μg *E. coli* NTR, and 0.8 μg *E. coli* thioredoxin (Jiao et al., 1992). The reductant mixture 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 10 μl 2-mercaptoethanol (100 mM). Total reduction of disulfide proteins was carried out by incubating an aliquot of the extract with 2.5 mM DTT at 90–95 °C for 5 min and derivatizing with mBBr as described above. The in vitro labeling procedure is summarized in Fig. 1(a).

4.5. Separation of target proteins on thioredoxin–Sepharose affinity column

The preparation of the affinity resin was as described in (Balmer et al., 2003) except that a mutated poplar Trx *h*1 was used. The concentration of salt in the KCl-soluble fraction from endosperm or flour (5 mg of protein) was diluted to 20 mM before being applied to the thioredoxin *h* column that was equilibrated in buffer (50 mM Tris–HCl, pH 7.5 containing 50 mM NaCl). The column was then extensively washed with the same buffer and an increased salt concentration (500 mM NaCl) to remove noncovalently bound proteins. Subsequently, the potential target proteins trapped by the newly formed heterodisulfide were eluted with the above buffer containing 10 mM DTT and 500 mM NaCl. Each elution step was continued until the absorbance at 280 nm reached almost zero. The DTT-eluted fraction containing protein was concentrated and washed with buffer (50 mM Tris–HCl, pH 7.5) on Ultrafree centrifugal devices (Millipore, 5 kDa molecular mass cut-off). The affinity column procedure is summarized in Fig. 1(b).

4.6. Protein solubilization for 2-DE

After mBBr derivatization (Section 4.4) or concentration on the affinity column (Section 4.5), the protein fraction was precipitated by the addition of five volumes of cold acetone followed by incubation overnight at −20

°C. The proteins were collected by centrifugation at 14,000 rpm for 15 min at 4 °C. The pellet was rinsed with acetone, dried, and solubilized in urea buffer (9 M urea, 4% NP-40, 1% DTT and 2% ampholytes) for 2-DE.

4.7. 2-DE and proteomic analysis

Proteins in the KCl-soluble fraction were separated by 2-DE (Vensel et al., 2002; Wong et al., 2004). Fluorescence of the SH groups of protein spots on two-dimensional gels of the mBBr-labeled proteins was captured using a Gel Doc-1000 fitted with a UV 365 nm light-box and a Quantity One program, version 4.1 (BioRad Laboratories, Inc., Hercules, CA). Exposure (aperture and time) was determined under a non-saturated condition with a totally reduced DTT sample. The same exposure setting was used to capture fluorescent images of all other treatments. The gels were then stained with Coomassie Blue G-250 and protein patterns were captured using a scanner. Molecular weights on 2-DE gels were estimated using Mark 12 unstained protein standards (Invitrogen, Carlsbad, CA). Protein spots were identified using a KCl-soluble protein map (Vensel et al., in preparation). The major protein spots isolated by affinity chromatography that could not be identified from the map were excised, trypsin-digested, and identified by mass spectrometry (MS) (Vensel et al., 2002). Electrospray ionization (ESI) mass spectrometry was performed using a hybrid quadrupole-TOF LC/MS/MS instrument (API QStar Pulsar) (Applied Biosystems/MDS Sciex, Toronto, Canada) equipped with a Proxeon Biosystems (Odense, Denmark) nano-electrospray source. In-gel-digestion of protein spots was as described previously (Vensel et al., 2002), except that acetonitrile was omitted from the final extraction step and 10% formic acid used instead. For LC/MS, 20 μl of the resulting extract was loaded onto a C-18 trap cartridge and chromatographed on a reversed-phase column (Vydac 238EV5.07515, 75Q × 150 mm, Hesperia, CA) fitted at the effluent end with a coated spray tip (FS360-50-5-CE, New Objective Inc., Woburn, MA). An LC Packings nano-flow LC system (Dionex, Sunnyvale, CA) with autosampler, column switching device, loading pump, and nano-flow solvent delivery system was used to elute the column. Elution solvents were: A (0.5% acetic acid) and B (80% acetonitrile, 0.5% acetic acid). Samples were eluted into the mass spectrometer at 220 μl/min with the following profile: 8% B at 0 min to 80% B by 12 min through 13 min to 8% B by 14 min continuing at 8% B to 28 min. The QStar Pulsar was calibrated daily and operated above a resolution of 8000 with a mass accuracy of 10–50 ppm using external calibration. The acquisition cycle time of 4s consisted of a single 1s MS "survey" scan followed by a 3s MS/MS scan. The dynamic exclusion window was set to always exclude previously fragmented masses. Doubly or triply charged ions with intensities greater than 40 counts in the survey scan were selected for fragmentation. Collision energy optimized for charge state and m/z was calculated using Analyst QS software (ABI/MDS Sciex, Toronto, Canada). Nitrogen was used for the collision gas and the pressure in the collision cell ranged from 3 to 6×10^{-6} Torr. The mass spectra were recorded in the positive ion mode.

Spectra collected from the MS/MS scan over the m/z range of 70–2000 Da were used for peptide and protein identification. The spectra were converted to a text file format and analyzed using the KNEXUS data analysis package (Genomic Solutions, Ann Arbor, MI). The software was set to search for tryptic peptides allowing for one missed cleavage, modification of sulfhydryl groups was set to iodoacetamide and partial oxidation of methionine residues was allowed. The NCBI nonredundant green plant database was searched using a mass range of 2–120 kDa and a pI of the intact protein from 5 to 9. Mass spectrometer identification scores from the plant protein database search are reported as expectation values. For example an expectation score of 1×10^{-3} can be interpreted to mean that there was one chance in 1000 that the match was due to a random event. Proteins included in Table 1 all had expectation scores less than 1×10^{-3} and 3 or more supporting peptides.

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