REGULAR ARTICLE

Developmental changes in the metabolic protein profiles of wheat endosperm

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A combined two-dimensional gel electrophoresis-mass spectrometry approach was utilized to identify over 250 proteins of wheat (*Triticum aestivum* L., cv. Butte 86) starchy endosperm that participate in 13 biochemical processes: ATP interconversion reactions, carbohydrate metabolism, cell division, cytoskeleton, lipid metabolism, nitrogen metabolism, protein synthesis/assembly, protein turnover, signal transduction, protein storage, stress/defense, transcription/translation, and transport. Endosperm protein populations were compared at early (10 days post-anthesis, dpa) and late (36 dpa) stages of grain development. Analysis of protein number and spot volume revealed that carbohydrate metabolism, transcription/translation, and protein synthesis/assembly were the principal endosperm functions at 10 dpa followed by nitrogen metabolism, protein turnover, cytoskeleton, cell division, signal transduction, and lipid metabolism. Carbohydrate metabolism and protein synthesis/assembly were also major functions at 36 dpa, but stress/defense and storage were predominant. The results provide insight into biochemical events taking place during wheat grain development and highlight the value of proteomics in characterizing complex biochemical processes. Further, the proteome maps will facilitate future studies addressing the effects of genetic and environmental factors on the development and quality of wheat grain.

1 Introduction

Proteins and carbohydrates that accumulate during seed development are not only essential reserves that support germination and early seedling growth in plants, but are critical to humans and animals as a major source of food. In wheat, the major protein reserves are the gluten proteins, a mixture of monomeric gliadins and polymeric glutenins located in the starchy endosperm (henceforth "endosperm"). 2-DE continues to be a principal tool for studying the gliadins, glutenins, and other complex protein families, because it allows the simultaneous separation and quantification of hundreds of proteins. Thus, 2-DE has been successfully used for the identification of wheat storage protein classes in extracts of endosperm or derived flour and the chromosomal location of the corresponding genes [1–8].

In contrast to the gliadins and glutenins, the other major protein families of the wheat endosperm, the albumins and globulins, have not been well characterized. In part, this is because the role of albumins and globulins in flour quality is not as well defined as that of the gliadins and glutenins. In addition, although the albumins and globulins are the prin-

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Abbreviation: dpa, days post-anthesis

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cipal storage proteins in dicot seeds, they are relatively minor components in cereal grain and few representatives of these families are detected by 2-DE analysis of total protein extracts of wheat grain. For example, Skylas *et al.* [9] were able to identify only the prominent α-amylase inhibitors and a small number of other soluble proteins, because their 2-DE patterns were dominated by the gliadins and glutenins. In a later study, Singh *et al.* [10] identified 19 albumins and globulins by Edman sequencing. More recently, we have identified 68 thioredoxin targets [11, 12] and 57 members of the methanol-soluble, albumins and globulins, endosperm proteins with unusual solubility properties [13]. However, despite this progress, incomplete knowledge of the enzymes and related proteins of the endosperm limits our understanding of biochemical events taking place in the grain.

Accordingly, we have undertaken a 2-DE/MS/proteomics study to identify the albumins and globulins of wheat endosperm. Our goal was to identify the less abundant endosperm proteins – notably enzymes of metabolism and related processes – and, based on their function, gain an understanding of biochemical processes taking place in the endosperm. Toward this end, metabolic proteins were separated as a KCl-soluble, methanol-insoluble subfraction of the albumins and globulins [12]. Because of our interest in development, we also established accumulation profiles of the metabolic proteins at early and late stages of grain maturation. In identifying the relevant proteins, we established not only a picture of the dynamic biochemical processes occurring during development, but also reference maps of the endosperm proteome that will facilitate future in-depth studies, including the effect of environmental conditions on grain-fill and the composition and quality of the mature grain. The present work complements other recent proteomic studies of seeds, including those noted above for wheat as well as similar work on *Hordeum* [14], *Arabidopsis* [15], and *Medicago* [16].

2 Materials and methods

2.1 Plant material

Triticum aestivum, L. cv. Butte 86, was grown in a climate-controlled greenhouse at a maximum daytime temperature of 24°C and a minimum night time temperature of 17°C. Water and fertilizer (Plantex 20-20-20, 500 mL of 0.6 g/L *per* pot *per* day) were applied by drip irrigation. Natural light was supplemented with 100 W high-pressure sodium lamps to maintain a day length of 16 h. Heads were tagged at anthesis and grains from three heads were harvested at 10 and 36 days post-anthesis (dpa). The embryo region of each grain was excised with a razor blade and the endosperm was squeezed through the cut end of the pericarp/testa, leaving the aleurone layer of the endosperm attached to the testa. Endosperm was frozen in liquid nitrogen and stored at −80°C.

2.2 Isolation of the metabolic proteins

Endosperm proteins were fractionated using a KCl buffer to separate the gliadins and glutenins from the albumins and globulins [12]. Endosperm from one head was ground to a fine powder in liquid nitrogen using a mortar and pestle. Fifty milligrams of endosperm was suspended in 200 μL of cold (4°C) KCl buffer (50 mM Tris-HCl, 100 mM KCl, 5 mM EDTA, pH 7.8) containing protease inhibitors (1 tablet *per* 10 mL, Mini Complete Protease Inhibitor Cocktail, Roche Applied Science, Indianapolis, IN, USA). The suspension was incubated on ice for 5 min with intermittent mixing and centrifuged at 14 000 rpm for 15 min at 4°C. The supernatant was collected and three samples (5 μL for 10 dpa and 10 μL for 36 dpa) were removed for protein determination. Five volume of 0.1 M ammonium acetate in methanol at room temperature were added to the remaining supernatant and the three samples. Following incubation overnight at −20°C, the proteins were pelleted by centrifugation at 14 000 rpm for 15 min at 4°C (Tomy MRX-151; Peninsula Laboratories, Belmont, CA, USA) [12]. The pellets were rinsed with cold (−20°C) acetone by pipetting the solvent onto the pellet, centrifuging at 14 000 rpm for 10 min at room temperature, and pipetting the solvent off the pellet. The pellets were dried by vacuum centrifugation (Speed Vac DNA 110; Savant Instruments, Farmingdale, NY, USA). The pellets from the samples removed for protein analysis were assayed by the method of Lowry *et al.* [17].

2.3 2-DE

The KCl-soluble protein pellet was suspended in urea buffer (9 M urea, 4% NP-40, 1% DTT, and 2% carrier ampholytes) and incubated for 1 h at room temperature. Insoluble material was removed by centrifugation at 14 000 rpm for 10 min at room temperature (Eppendorf 5415C; Brinkman Instruments, Westbury, NY, USA). Proteins were separated in the first dimension by IEF (Mini Protean II tube cell; Bio-Rad Richmond, CA, USA); the gels contained 9.2 M urea, 4% (total monomer) acrylamide: Bis, 2% NP-40, 2% 3–10 Iso-Dalt Grade Servalyts (Crescent Chemical, Islandia, NY, USA), 0.015% ammonium persulfate, and 0.125% TEMED. The gels were prefocused at 200 V for 10 min, 300 V for 15 min, and 400 V for 15 min. Samples with 18 μg of protein were loaded at the acidic end of the IEF gel and overlaid with 5 M urea. For protein p*I* determinations, 3 μL of 2-D SDS-PAGE standards (Bio-Rad) were added to the sample. The upper electrode (anode) buffer was 0.2% v/v sulfuric acid and the lower electrode buffer (cathode) was 0.5% v/v ethanolamine. Because the anode buffer was acidic, the leads from the electrophoresis cell were reversed at the power supply. IEF gels were run at 500 V for 10 min and then at 750 V for 1 h. Gels were extruded into microcentrifuge tubes using a syringe and equilibration buffer (2.3% SDS, 10% glycerol, 0.05% DTT, 62.5 mM Tris-Cl pH 6.8) added. Gels were frozen

immediately by placing the tubes in dry ice and then stored at −70°C. Proteins were separated in the second dimension by SDS gel electrophoresis (XCell Sure-Lock Mini-Cell Electrophoresis System; Invitrogen, Carlsbad, CA, USA). IEF gels were thawed, placed in the 2-D sample well of Novex NuPAGE Bis-Tris 4–12% acrylamide 2-D gels (Invitrogen), and overlaid with 45 μL of equilibration buffer. Five microliters of molecular weight markers (Mark 12 Unstained Standard; Invitrogen) were loaded into the reference well of the 2-D gel. The SDS gels were run with NuPAGE MES SDS running buffer (Invitrogen) for 48 min at 200 V. The 2-D gels were stained with colloidal CBB G-250 (Sigma, St. Louis, MO, USA) by the method of Kasarda *et al.* [18], destained in water for 3–5 h, and stored at 4°C in 20% ammonium sulfate. The gels were digitized with a calibrated scanner (Epson Expression 800; Long Beach, CA, USA) at 300 dpi with the same settings for all gels. Computer software (Phoretix 2D v. 5.01; Non-Linear Dynamics, Newcastle upon Tyne, UK) was used to match and analyze three gels *per* time point to create average gels for the 10 and 36 dpa endosperm proteins. Normalized spot volumes (individual spot volume/total spot volume × 100) were determined for each spot in the average gels; these values were included in Table 1 and were used to designate the 10 and 36 dpa abundant proteins in Table 1 and in the analysis depicted in Fig. 3B. With respect to the normalized spot volumes, the SEM ranged from 0 to 0.194 for 10 dpa and 0 to 0.674 for 36 dpa. Spot numbers in Figs. 1 and 2 were generated by the software.

2.4 Mass spectrometry

During the course of identifying the proteins in the wheat endosperm proteome, different mass spectrometers were utilized to collect mass spectra in the positive ion mode. Initial identifications by MALDI-TOF MS (peptide mass mapping) were carried out using a Bruker Reflex II (Bruker Daltonics, Billerica, MA, USA) as described previously [19]. Subsequent identifications by MS/MS were performed using either a Finnigan LCQ Classic (Thermo Finnigan, San Jose, CA, USA) as described [20] or with an API QSTAR Pulsar *i* hybrid quadrupole-TOF instrument (Applied Biosystems/ MDS Sciex, Toronto, Canada). MS and MS/MS experiments were performed with the QSTAR configured either with the ESI source or the o-MALDI source. Sample preparation by in-gel digestion was as described previously [19], except that ACN was omitted from the final extraction step and 10% formic acid used instead. For o-MALDI analyses, the extract from in-gel protein spot digestion was trapped on a Millipore C-18 ZipTip (Millipore, Billerica, MA, USA) by repeated (15– 20) sample aspiration. The sample-loaded ZipTip was washed repeatedly with 0.1% TFA prior to elution with 30% dihydroxy benzoic acid (DHB) dissolved in 60% ACN containing 0.1% TFA. The eluate was spotted directly on the surface of a 96-well stainless steel target and allowed to dry. The target was inserted into the o-MALDI source and spectra were acquired with the Analyst QS software operating under

control of the supplied o-MALDI server software. The laser was operated at a rate of 20 Hz and at a calibrated intensity of 40 μJ. An initial survey (peptide mass map) scan of the singly charged peptide ions was acquired over the course of 1 min with the mass range set from 800 to 2500 *m/z*. Acquisition of survey scans was performed with Q1 and Q2 set to operate in the full transmission mode. MS/MS spectra were then acquired with Q1 set to allow the complete isotope spectrum of the selected ion to pass into Q2 where CID assisted MS/ MS was performed in the presence of argon. The Analyst QS software (ABI/MDS Sciex) was used to set the lower mass range of the MS/MS experiments to 50 *m/z* and the upper limit to that of the parent ion. Typically the five most intense ions were selected. ESI/MS was performed using a Proxeon Biosystems (Odense, Denmark) nano-electrospray source. In-gel digest (20 μL) was loaded automatically onto a C-18 trap cartridge and chromatographed on a reversed-phase column (Vydac 238EV5.07515, 75 μm × 150 mm; Hesperia, CA, USA) fitted at the effluent end with a coated spray tip (FS360–50–5-CE, New Objective, Woburn, MA, USA). An LC Packings nano-flow LC system (Dionex, Sunnyvale, CA, USA) 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% ACN, 0.5% acetic acid). Samples were eluted at 220 nL/min with the following gradient 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 with external calibration. The acquisition cycle time of 4 s consisted of a single 1 s MS "survey" scan followed by a 3 s 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 determined by the Analyst QS software. Nitrogen was used for the collision gas and the pressure in the collision cell ranged from 3×10^{-6} to 6×10^{-6} Torr.

2.5 Protein identification

Spectra were converted to a text format and analyzed using the KNEXUS data automation client (Genomic Solutions, Ann Arbor, MI, USA). The following constraints were used to search the databases: tryptic peptides with a maximum of one missed cleavage, iodoacetamide modification of sulfhydryl groups, partial oxidation of methionine residues, mass range of 2–120 kDa, and a p*I* for the intact protein of 5–9. Proteins were identified through either peptide mass mapping (MS) or peptide fragmentation (MS/MS) data, necessitating the use of two different search algorithms. KNEXUS uses ProFound for MS experiments and Sonar for MS/MS experiments. ProFound incorporates a Bayesian algorithm to identify proteins from databases of protein amino acid

Table 1. Proteins of wheat endosperm identified by MS

Spot no.	Swiss-Prot no.	Protein	Spot volume		Method	E-value[a)]
			10 dpa	36 dpa		
6	Q8S1A5	Carbamoyl phosphate synthetase	0.197	0.013	M[g)]	9.60E-06
10	Q8L5C2	4SNc-Tudor protein, (NTPase)	0.207	0.047	QE[h)]	1.90E-17
11	Q8L5C2	4SNc-Tudor protein, (NTPase)	0.465	0.029	M	2.00E-04
25	Q42669	Aconitase	0.349	0.099	F[i)]	1.60E-03
26	Q9LZF6	Cell division cycle protein	0.724	<0.004[f)]	M	7.10E-07
27	P49608	Aconitase	0.246	0.080	M	1.00E-05
31	O23927	Pyruvate Pi dikinase	2.029	0.539	F	1.90E-27
33	O23755	Elongation factor 2	0.225	0.15	QM[j)]	2.50E-05
35	O98447	ClpC protease	0.190	0.013	F	1.10E-10
42	Q43638	Heat shock protein, 82K, precursor	0.171	<0.004	QM	4.70E-03
43	Q9LF88	Late embryogenesis abundant protein-like	0.022	0.051	QE	3.30E-04
48	Q8W0Q7	Methionine synthase	0.418	0.145	QE	5.10E-11
49	Q9XGF1	Heat shock protein 80–2	0.241	0.044	QM	2.50E-05
53	Q9M6E6	Poly(A)-binding protein	0.133	<0.004	QE	1.20E-04
55	Q9AT32	Poly(A)-binding protein	0.162	0.009	QM	9.40E-05
57	Q9M6E6	Poly(A)-binding protein	0.137	<0.004	QE	2.90E-07
60	P93616	Poly(A)-binding protein	0.145	0.015	QE	5.10E-14
61	P93616	Poly(A)-binding protein	0.076	<0.004	QE	2.70E-02
63	P93616	Poly(A)-binding protein	0.126	0.012	QE	1.10E-20
65	Q39641	Heat shock protein 70	0.360	0.079	M	2.30E-05
66	P93616	Poly(A)-binding protein	0.157	0.01	QE	8.70E-13
69	P93616	Poly(A)-binding protein	0.277	0.072	QE	1.70E-13
70	Q01899	Heat shock protein 70	0.317	0.052	QE	5.50E-31
72	Q9SPK5	10-Formyltetrahydrofolate synthetase	0.420	0.091	QE	1.40E-10
74	Q40058	DNAK-type molecular chaperone HSP70	1.973	0.568	QE	4.60E-56
77[b)]	Q95I76	F23N19.10, putative stress-induced protein	0.204	0.056	QE	5.70E-10
77[b)]	P93616	Poly(A)-binding protein	0.204	0.056	QE	2.70E-06
82	Q8L724	Stress-induced protein, sti1-like	0.250	0.050	QE	7.00E-22
93	Q9FME2	RNA-binding protein, similarity	0.152	0.018	QE	1.10E-14
94	Q9FME2	RNA-binding protein, similarity	0.237	<0.004	QM	2.20E-09
96	Q95NX2	Phosphoglucomutase	0.149	0.040	F	2.30E-18
97	Q944F5	Fructokinase	0.175	0.037	M	4.40E-03
98	O65305	Acetohydroxyacid synthase	0.220	0.016	M	2.60E-05
102	Q9ZR86	Protein disulfide isomerase-like protein	0.166	0.040	QE	6.80E-08
103	Q94DV7	Phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent	0.426	0.075	QE	2.60E-49
104	Q94DV7	Phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent	0.312	0.059	M	6.10E-10
110	Q8GWX8	2-Isopropylmalate synthase	0.249	0.108	F	4.20E-05
111	Q8W4M5	PPi-fructose-6-P 1-phosphotransferase	0.085	0.182	M	3.40E-09
112	Q41141	PPi-fructose 6-P 1-phosphotransferase beta subunit	0.565	0.125	F	1.30E-09
117	Q9LWT6	Chaperonin 60 kDa beta subunit	0.532	0.079	M	7.60E-04
118	P52589	Protein disulfide isomerase	8.808	3.348	M	2.90E-14
119	O49485	Phosphoglycerate dehydrogenase-like protein	0.286	<0.004	QE	3.30E-12
121	O82783	Importin α-2 subunit	0.703	1.974	F	2.90E-28
125	P16098	β-Amylase	0.009	0.641	F	1.80E-14
128	P12299	ADP-glucose PPase, LS	0.245	0.049	M	1.50E-05
130	P12299	ADP-glucose PPase, LS	0.216	0.058	F	1.60E-31
132	P12299	ADP-glucose PPase, LS	0.661	0.159	M	1.20E-11
135	P30184	Leucine amino peptidase	0.592	0.157	QE	7.50E-33
137	P30184	Leucine amino peptidase	<0.004	0.185	QE	9.20E-13
138	Q8RZF3	Ketol-reductoisomerase	0.599	0.446	M	9.50E-09
141	Q9M7E0	Elongation factor 1-α	0.170	0.295	F	1.50E-07
145	Q8RZW7	Selenium binding protein	0.286	0.034	QE	2.00E-30
147	Q8GU01	Globulin-2	0.019	0.286	QE	1.20E-25

Table 1. Continued

Spot no.	Swiss-Prot no.	Protein	Spot volume		Method	E-value[a]
			10 dpa	36 dpa		
148	P55307	Catalase isozyme 1	0.260	0.116	M	1.60E-05
149	Q93YR3	Heat shock associated protein	0.161	0.142	QE	5.00E-22
151	Q8RZW7	Selenium binding protein	0.204	0.165	QE	1.90E-05
153	Q8LST6	Aldehyde dehydrogenase	0.246	0.050	QE	1.10E-41
154	Q9FPK6	Aldehyde dehydrogenase	0.158	0.189	QE	4.20E-16
155	Q8GU01	Globulin-2	<0.004	0.392	M	4.80E-07
156[b]	Q9M4Z1	ADP-glucose PPase, SS	<0.004	0.224	QE	2.10E-21
156[b]	O49218	Methylmalonate-semialdehyde dehydrogenase	<0.004	0.224	QE	7.30E-08
157	Q43772	UDP-glucose PPase	0.412	0.138	M	1.10E-08
158	Q43772	UDP-glucose PPase	0.608	0.216	QE	4.70E-53
159	Q9M4Z1	ADP glucose PPase, SS	0.343	0.145	M	6.10E-05
161	Q9ASP4	Dihydrolipoamide dehydrogenase	0.178	0.111	QE	2.30E-55
163	Q93YR3	Heat shock associated protein	0.132	0.190	QE	1.60E-23
165	Q8W3W6	LMW glutenin subunit group 3 type II	0.315	0.420	QM	9.60E-06
166	Q8GU18	LMW glutenin subunit	0.659	1.365	QE	1.10E-10
168	Q9ASP4	Dihydrolipoamide dehydrogenase	nd[e]	0.314	QE	7.30E-18
171	Q8GU18	LMW glutenin subunit group 3 type II	0.101	0.302	QE	7.20E-04
173	P52894	Alanine amino transferase 2	0.351	0.093	M	2.70E-06
175	Q8GU01	Globulin-2	0.038	0.201	QE	7.50E-04
176	P52894	Alanine amino transferase 2	1.533	0.694	F	2.20E-16
178	Q42971	Enolase	0.230	0.123	M	7.60E-08
179	Q42971	Enolase	0.877	0.322	F	1.80E-33
182	Q9ZRR5	Tubulin α-3 chain	0.369	0.032	M	1.90E-08
184	Q9ZRB0	Tubulin β-3 chain	0.467	0.008	M	2.30E-06
185	Q42971	Enolase	2.053	0.917	F	3.60E-08
193	O81237	6-Phosphogluconate dehydrogenase	0.325	nd[e]	QE	3.00E-02
195[b]	Q8W3N9	26S Proteasome regulatory particle triple-A ATPase subunit 3	0.287	0.027	QE	7.80E-12
195[b]	Q8VZ47	Argininosuccinate synthase-like protein	0.287	0.027	QE	2.70E-13
195[b]	Q9AUV6	UDP-glucose dehydrogenase	0.287	0.027	QE	6.70E-19
199	Q08837	Triticin	0.089	0.209	M	4.60E-04
201	Q8W516	SGT1	0.265	0.078	M	2.70E-06
203	Q8W3W4	LMW glutenin subunit group 4 type II	0.186	0.675	QE	4.30E-02
205	Q9FXT8	26S proteasome regulatory particle triple-A ATPase subunit 4	0.085	0.251	QE	4.00E-27
206	P41378	Eukaryotic initiation factor 4A	0.301	0.129	M	2.00E-05
212	Q40058	DNAK-type molecular chaperone HSP70	<0.004	0.567	QE	6.00E-21
220[b]	Q9FXT8	26S Proteasome regulatory particle triple-A ATPase subunit 4	0.351	0.369	QE	7.60E-04
220[b]	P37833	Aspartate amino transferase	0.351	0.369	QE	1.70E-49
225	Q9SAU8	Heat shock protein 70	0.042	0.318	QE	6.90E-20
228[c]	Q9FXT8	26S Proteasome regulatory particle triple-A ATPase subunit 4b (10 dpa)	0.182	0.280	QE	4.20E-08
228[c]	Q9XGU8	Isocitrate dehydrogenase (NAD) (36 dpa)	0.182	0.280	QE	4.30E-26
229	Q8GU01	Globulin-2	0.068	0.196	M	1.60E-04
230[b]	Q9ZRI8	Formate dehydrogenase	1.059	0.148	M	8.40E-07
230[b]	P37833	Aspartate amino transferase	1.059	0.148	QE	2.10E-31
231	P93693	Serpin WZS2	0.490	1.960	M	3.00E-08
232	P37833	Aspartate amino transferase	0.197	0.166	QE	7.70E-28
233	Q9ST58	Serpin	<0.004	0.366	QE	1.50E-04
234	Q43492	Serpin homolog WZS3	0.085	0.022	QE	2.90E-05
236	P37833	Aspartate amino transferase	0.222	0.682	QE	9.70E-48
237	P93692	Serpin homolog WZS3	0.111	0.826	QE	1.20E-40
238	P04727	α/β-Gliadin clone PW8142	0.129	0.126	QE	5.40E-04
240[b]	Q9FUS4	Actin	0.430	0.283	F	1.50E-35

Table 1. Continued

Spot no.	Swiss-Prot no.	Protein	Spot volume		Method	E-value[a]
			10 dpa	36 dpa		
240[b]	P93692	Serpin homolog WZS3	0.430	0.283	QE	9.70E-10
244	Q40676	Aldolase	0.570	0.259	QE	2.30E-36
245	P93692	Serpin homolog WZS3	<0.004	0.632	QE	5.90E-25
246	Q41593	Serpin	0.157	1.115	M	7.50E-06
247[c]	Q9ZR33	Reversibly glycosylated polypeptide (10 dpa)	0.671	0.148	M	1.30E-07
247[c]	P93692	Serpin homolog WZS3 (36 dpa)	0.671	0.148	QM	7.30E-05
249	Q9ZRI8	Formate dehydrogenase	1.159	0.472	QE	1.20E-13
250	Q9ZR33	Reversibly glycosylated polypeptide	0.162	0.168	M	6.90E-08
251	Q9ZRI8	Formate dehydrogenase	0.379	0.105	QM	2.90E-05
252	Q40676	Aldolase	1.418	0.293	QE	1.30E-19
255	Q8LK23	Peroxidase 1	0.091	1.330	M	1.80E-07
256[b]	O81221	Actin	0.354	0.045	F	1.20E-21
256[b]	Q41319	Acyl-acyl-carrier protein desaturase	0.354	0.045	F	9.80E-04
256[b]	Q93Y71	Protein disulfide-isomerase precursor	0.354	0.045	QE	1.10E-09
257	Q40676	Aldolase	<0.004	0.572	QE	9.40E-21
259	Q8LK23	Peroxidase 1	0.110	1.494	QM	1.30E-09
262	Q9M4V4	Glyceraldehyde-3-P dehydrogenase (NAD)	1.816	1.907	QE	8.10E-43
263	Q40069	Peroxidase BP1	0.200	2.242	F	2.20E-16
264	P25861	Glyceraldehyde 3-P dehydrogenase (NAD)	4.518	3.130	QM	1.50E-09
265[b]	Q8LK23	Peroxidase 1	0.045	0.605	QE	1.40E-09
265[b]	Q9ST58	Serpin	0.045	0.605	QE	5.10E-07
272	Q94CS6	Legumin-like protein	0.025	0.247	QE	4.90E-13
273[c]	Q942N5	Auxin-induced protein (10 dpa)	0.275	2.569	QE	8.60E-15
273[c]	Q9M4V4	Glyceraldehyde-3-P dehydrogenase (NAD) (36 dpa)	0.275	2.569	QE	1.10E-10
274	Q9M4V4	Glyceraldehyde-3-P dehydrogenase (NAD)	0.421	0.490	QE	1.10E-33
275	P26517	Glyceraldehyde-3-P dehydrogenase (NAD)	0.023	0.282	F	2.80E-03
278	Q94KS2	TGF-β receptor-interacting protein 1	0.233	0.213	QE	5.60E-25
279	Q9XGC6	Adenosine kinase	0.291	0.082	F	1.20E-22
281	Q03678	Globulin Beg 1	0.027	0.240	QM	3.40E-03
282	Q9FRV1	Chitinase-a	0.078	0.199	QM	2.40E-12
283	Q94KS2	TGF-β receptor-interacting protein 1	0.064	0.078	QE	7.60E-06
284[b]	P41095	60S Acidic ribosomal protein P0	0.209	0.055	QE	7.40E-09
284[b]	Q94CS6	Legumin-like protein	0.209	0.055	QE	3.40E-08
285	Q94CS6	Legumin-like protein	0.175	0.144	M	7.00E-06
289[b]	Q9C774	26S Proteasome regulatory subunit S12	0.617	0.535	F	1.50E-05
289[b]	Q09114	Avenin N9	0.617	0.535	QE	3.20E-14
294	Q9FT00	Malate dehydrogenase (NAD)	2.266	1.348	QM	4.50E-17
295	Q94JA2	Malate dehydrogenase (NAD)	0.587	0.223	QE	2.80E-12
301	P49027	Guanine nucleotide-binding protein β subunit-like protein	0.807	0.174	QE	7.10E-27
303[b]	Q945R5	Ascorbate peroxidase	0.293	<0.004	M	6.90E-06
303[b]	Q42988	PPi-fructose-6-P 1-phosphotransferase	0.293	<0.004	F	6.90E-05
305	Q9ZWJ2	Glyoxalase I	0.790	0.769	M	5.30E-07
306	Q40676	Aldolase	0.299	0.209	QE	5.30E-14
308	Q07810	Tritin	0.165	0.773	QE	1.60E-08
310	T06212[d]	Glucose and ribitol dehydrogenase	nd[e]	0.226	QE	8.80E-05
311	Q9C5Y9	Initiation factor 3g	0.367	0.205	QE	4.70E-04
312	Q8W5L9	Purple acid phosphatase	0.068	0.202	QE	1.20E-05
314	Q7X653	OSJNBb0118P14.5	<0.004	0.165	QE	9.10E-08
318	Q8L5C6	Xylanase inhibitor protein I	nd[e]	0.880	QE	1.60E-24
324	Q7X653	OSJNBb0118P14.5	0.035	0.084	QE	3.30E-08
325	Q8L5C6	Xylanase inhibitor protein I	0.133	0.587	QE	2.30E-11
327	Q8LKV8	Seed globulin	0.085	0.482	QE	1.70E-03
330	P29305	14–3-3 Protein homolog	0.301	0.047	QE	1.20E-14
335	Q8LKV8	Seed globulin	0.177	0.565	QM	2.10E-26
336	Q8LKV8	Seed globulin	0.083	0.466	M	1.90E-03

Table 1. Continued

Spot no.	Swiss-Prot no.	Protein	Spot volume		Method	E-value[a]
			10 dpa	36 dpa		
337	Q8LKV8	Seed globulin	0.059	0.482	QM	1.80E-10
338	Q04832	DNA-binding protein HEXBP	0.293	0.057	QE	5.10E-07
341	Q9FRV0	Chitinase-c	<0.004	0.662	QM	1.90E-08
342	O23983	Ascorbate peroxidase	0.351	0.044	M	7.20E-05
344	O23983	Ascorbate peroxidase	0.480	0.026	M	3.10E-04
347	Q9FER4	20S Proteasome α-subunit	0.124	0.243	QE	8.10E-14
348	O23983	Ascorbate peroxidase	0.227	<0.004	QM	6.40E-06
350	Q944C6	Small ras-related GTP-binding protein	0.132	0.058	QM	6.50E-06
351	P11955	Chitinase-a	nd[e]	0.726	F	3.10E-10
353	P27919	Avenin	0.340	1.321	QM	8.20E-04
354	Q9FS79	Triosephosphate isomerase	0.518	0.505	QM	3.30E-11
355	Q8LKV8	Seed globulin	0.009	0.371	QE	6.00E-05
356	Q9FS79	Triosephosphate isomerase	0.618	0.724	M	4.40E-11
357	P34937	Triosephosphate isomerase	0.383	0.040	F	2.70E-20
359	Q8LK23	Peroxidase 1	<0.004	0.209	QE	1.50E-10
360[b]	Q8LKV8	Seed globulin	0.024	1.055	QE	4.20E-09
360[b]	Q8S4P7	Thaumatin-like protein TLP7	0.024	1.055	QE	5.90E-07
361	Q9FER4	20S Proteasome α-subunit	0.163	0.053	QE	2.10E-02
362	P29546	Elongation factor 1-β	0.307	0.064	QE	1.70E-25
364	Q945R5	Ascorbate peroxidase	0.219	0.060	QM	7.20E-10
368[b]	Q9LMK1	F10K1.21/F7A7_100 protein, similarity	0.128	0.802	QE	1.10E-03
368[b]	Q9AXH7	1-Cys peroxiredoxin	0.128	0.802	QE	3.20E-11
369	Q09114	Avenin N9	0.097	0.269	QE	2.20E-05
371	P52572	Peroxiredoxin	nd[e]	0.304	QE	2.30E-17
373	Q9LSU2	20S Proteasome α-subunit B	0.146	0.083	F	1.40E-12
376	Q96185	Superoxide dismutase [Mn]	0.053	0.162	QE	2.70E-29
378	Q9MB31	GSH-dependent dehydroascorbate reductase 1	0.530	0.088	F	5.90E-04
379	Q93VQ6	Expressed protein	0.032	0.580	QM	2.30E-05
381	P16347	α-Amylase/subtilisin inhibitor	0.314	1.516	M	6.40E-07
382	Q84UH6	Dehydroascorbate reductase	0.865	1.446	F	2.70E-54
383	Q93XQ6	Cyclophilin A-2	0.610	0.280	QE	1.20E-08
384	Q09114	Avenin N9	0.335	0.943	QM	1.00E-02
385[b]	Q9LMK1	F10K1.21/F7A7_100 protein, similarity	nd[e]	0.276	QE	8.10E-21
385[b]	Q9MB31	GSH-dependent dehydroascorbate reductase 1	nd[e]	0.276	QE	8.70E-14
386	Q9LMK1	F10K1.21/F7A7_100 protein, similarity	0.105	1.006	QE	1.20E-30
394[b]	Q9LST9	β-1 Subunit of 20S proteasome	0.684	0.065	QE	2.00E-08
394[b]	Q9MB31	GSH-dependent dehydroascorbate reductase 1	0.684	0.065	QE	5.80E-28
394[b]	Q9ZSU2	Translation initiation factor 5A	0.684	0.065	QE	1.00E-18
399	Q8LRM8	Translationally controlled tumor protein	0.393	0.141	QE	1.40E-10
405	Q93XQ8	Protein disulfide isomerase 2 precursor	nd[e]	0.249	QE	5.10E-17
407	Q41561	Heat shock protein 16.9C	0.022	0.163	QE	6.20E-13
411	Q41518	Glycine-rich RNA-binding protein	0.424	0.025	QM	3.00E-03
412	Q41518	Glycine-rich RNA-binding protein	0.295	0.015	QE	8.00E-23
415	Q42973	Ubiquitin-protein ligase	0.271	0.029	QE	2.30E-03
416[c]	P35686	40S Ribosomal protein S20 (10 dpa)	0.635	0.314	QE	3.10E-06
416[c]	Q43659	Grain softness protein 1b (36 dpa)	0.635	0.314	QM	9.20E-06
417	P17314	α-Amylase/trypsin inhibitor, CM3	0.098	0.561	M	2.70E-03
418	P23345	Superoxide dismutase [Cu-Zn]	0.039	0.200	QM	1.70E-12
419	Q43472	Glycine-rich RNA-binding protein	0.290	nd[e]	M	4.40E-04
420	Q02254	Nucleoside diphosphate kinase I	0.745	1.454	QM	3.70E-07
422	Q9XHS0	40S Ribosomal protein S12	0.238	0.017	QM	1.10E-12
427	Q95QG8	Pathogenesis-related protein 4	nd[e]	0.721	QE	2.10E-04
430	P01085	α-Amylase inhibitor 0.19	0.072	0.695	M	3.00E-03
432[b]	Q8S3L1	Glutaredoxin	0.177	0.601	QE	1.20E-06

Table 1. Continued

Spot no.	Swiss-Prot no.	Protein	Spot volume		Method	E-value[a]
			10 dpa	36 dpa		
432[b]	Q43723	Trypsin inhibitor CMx precursor (clones pCMx1 and pCMx3)	0.177	0.601	QE	6.90E-08
433	Q41518	Glycine-rich RNA-binding protein	0.191	nd[e]	QE	3.00E-06
434	P01084	α-Amylase inhibitor 0.53	0.050	0.138	QE	3.10E-28
436	P01084	α-Amylase inhibitor 0.53	<0.004	0.191	M	6.80E-06
440	O49956	α-Amylase inhibitor Ima 1, monomeric	nd[e]	0.224	QE	1.40E-10
441	Q09114	Avenin N9	<0.004	0.202	QE	1.10E-03
444	P35687	40S Ribosomal protein S21	0.230	0.036	QE	6.00E-06
447	Q40641	Polyubiquitin 6	0.406	<0.004	M	2.90E-04
471	Q40058	DNAK-type molecular chaperone HSP70	0.718	<0.004	F	1.80E-34
493	Q944R8	UDP-glucose dehydrogenase	0.296	0.035	F	1.00E-15
529	P40978	40S Ribosomal protein S19	0.696	nd[e]	QE	6.50E-12
530	Q93W25	Cyclophilin A-1	0.497	nd[e]	M	3.90E-03
550	Q43223	Sucrose synthase type 2	<0.004	0.047	QE	8.20E-42
551	Q9LF88	Late embryogenesis abundant protein-like	nd[e]	0.280	QE	1.90E-07
557	Q8S7U3	Embryo-specific protein	nd[e]	0.099	QE	5.40E-10
559	Q8GU01	Globulin-2	nd[e]	0.440	QE	1.10E-31
561	Q8GU01	Globulin-2	nd[e]	0.148	QE	5.20E-16
577	Q41551	LMW glutenin (fragment)	nd[e]	0.125	QE	1.90E-03
579	P26517	Glyceraldehyde 3-P dehydrogenase (NAD)	nd[e]	0.302	F	9.70E-13
582	P26517	Glyceraldehyde 3-P dehydrogenase (NAD)	nd[e]	0.646	QM	1.90E-05
583[b]	Q8LK23	Peroxidase 1	<0.004	0.112	QE	1.70E-03
583[b]	Q947H4	Plasmodesmal receptor	<0.004	0.112	QE	6.20E-05
591	T06212[d]	Glucose and ribitol dehydrogenase	nd[e]	0.176	QE	1.90E-18
620	Q03678	Globulin Beg 1	nd[e]	0.803	QE	5.00E-03
631	Q8L8I0	Globulin-like protein	nd[e]	0.199	QM	3.70E-05
633	P24296	Nonspecific lipid-transfer protein precursor	nd[e]	0.182	QM	6.10E-04
639	Q9ST58	Serpin	nd[e]	0.927	M	9.00E-05
640	P12783	Phosphoglycerate kinase	0.445	<0.004	F	2.30E-46
655	P93438	*S*-Adenosylmethionine synthetase 2	0.282	nd[e]	M	5.60E-04
656	Q9FXT8	26S Proteasome regulatory A subunit	0.136	<0.004	QE	5.00E-04
657	P50299	*S*-Adenosylmethionine synthetase 1	0.219	<0.004	QE	5.60E-19
666	Q945R5	Ascorbate peroxidase	0.405	<0.004	M	2.90E-05
667	Q945R5	Ascorbate peroxidase	0.178	nd[e]	QE	5.50E-12
672	Q9ZR33	Reversibly glycosylated polypeptide	0.339	nd[e]	M	9.10E-08
842	Q9LF88	Late embryogenesis abundant protein-like	nd[e]	0.262	QE	5.00E-05
851	Q8GU01	Globulin-2	nd[e]	0.360	QM	8.80E-04
852	Q8GU01	Globulin-2	nd[e]	0.163	QM	2.60E-03
860	P26517	Glyceraldehyde 3-P dehydrogenase (NAD)	<0.004	0.353	QE	4.00E-10
861	Q43247	Glyceraldehyde 3-P dehydrogenase (NAD)	<0.004	0.582	QM	2.30E-09
869	Q9FF52	60S Ribosomal protein L12	nd[e]	1.659	QE	1.30E-14
871	P28814	Barwin	nd[e]	0.274	QE	1.80E-02

a) E-value, expectation value, see Section 2.
b) Spot numbers that contain more than one protein.
c) Spot numbers that contain one protein at 10 dpa and a different protein at 36 dpa.
d) NCBI accession number; Swiss-Prot number not available.
e) Not detected.
f) Lower limit of volume detection due to scan resolution and software settings.
g) Bruker MALDI-TOF.
h) QSTAR ESI/MS/MS.
i) Finnigan ESI/MS/MS.
j) QSTAR MALDI/MS/MS.

sequences using the observed peptide masses [21]. Sonar incorporates *a*, *b*, and *γ* ions and uses vector alignment and spectrum dot products to calculate correlations between mass spectra and database peptide sequences [22]. A number of different databases were searched: HarvEST: Wheat Version 1.04 (http://harvest.ucr.edu), NCBI nonredundant green plant database, NCBI *Triticum aestivum*: UniGene Build #37, and wEST Database (http://wheat.pw.usda.gov/wEST) [23]. Both ProFound and Sonar report protein identifications along with expectation values, the number of matches expected if the matches were completely random. An expectation value of 1 means that at least one match would be expected when searching a database that did not include the sequence of the true protein. An expectation value of 1×10^{-3} can be interpreted as 1 chance in 1000 that the match was random. Thus the lower the expectation value, the more likely the match is true and not random. A detailed description of the expectation score using ProFound and Sonar can be found at http://129.85.13.70/prowl/profound_help.html and http://129.85.13.70/prowl/sonar/sonar_cntrl_help.html, respectively. Initial experiments using MALDI-TOF MS allowed the identification of only about 30% of the gel spots analyzed. In contrast, over 80% of the gel spots were identified using MS/MS. Thus, the added information that was obtained from MS/MS peptide fragmentation substantially increased gel spot identification.

3 Results

3.1 Identification of proteins

In this study, the KCl-soluble, albumins and globulins were first separated from the KCl-insoluble, gliadins and glutenins. The albumins and globulins were further separated into methanol-soluble and methanol-insoluble fractions. The methanol-insoluble, albumin and globulin fraction contained principally metabolic proteins (Table 1). Significantly, only six gluten proteins, one gliadin and five glutenins, were identified in this fraction, confirming the effectiveness of the separation procedure. The KCl-soluble, methanol-insoluble fraction permitted an analysis of the largely unexplored albumins and globulins that function in metabolism and related biochemical processes. Due to an interest in grain development, we analyzed the metabolic proteins present in the endosperm at early and late stages of grain-fill, that is, 10 dpa (Fig. 1) and 36 dpa (Fig. 2). The 2-DE patterns differed at these two developmental stages. The majority of proteins at 10 dpa had a mass of 14–120 kDa and an acidic to neutral p*I* (Fig. 1). In contrast, 36 dpa endosperm contained fewer proteins with mass greater than 60 kDa and more with a basic p*I* (Fig. 2). Of the approximately 450 proteins detected in the 2-D gels, we identified a total of 254. Proteins not identified were present in insufficient quantities in the 2-D gels or were refractory to trypsin digestion.
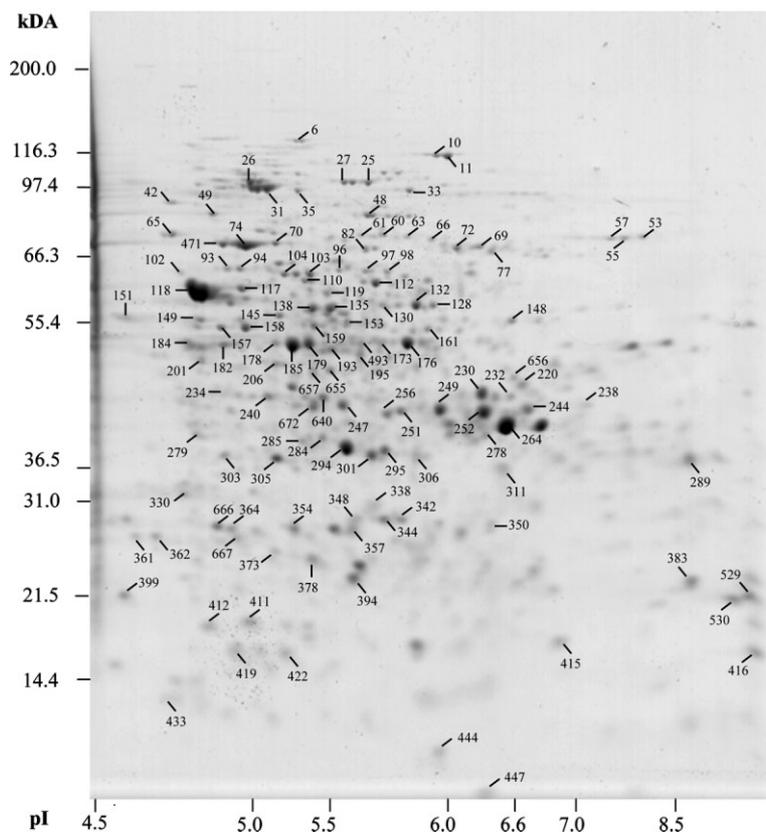

Figure 1. Proteome map of 10 dpa wheat endosperm. Proteins were separated using 2-DE and numbered proteins were identified by MS (see Table 1). Normalized spot volumes were used to quantify the proteins.

Figure 2. Proteome map of 36 dpa wheat endosperm. Conditions as in Figure 1.

Table 1 shows the accession numbers and putative names of proteins that correspond to specific spots shown in Figs. 1 and 2, along with the expectation values and method used for protein identification. The proteins in Table 1 were also classified as abundant at 10 or 36 dpa based on normalized spot volumes. A total of 136 proteins had greater normalized spot volumes at 10 dpa than at 36 dpa and 118 had greater spot volumes at 36 dpa than at 10 dpa; the differences in spot volumes were at least two-fold for 89% of the identified proteins. Computer matching of the 10 and 36 dpa patterns revealed a relatively small number of proteins unique to each, that is, those detected at one time point, but not at the other (Table 1). Eight proteins were detected only at 10 dpa and 26 only at 36 dpa. Some spots with identical mobilities at 10 and 36 dpa contained more than one protein with the allowable expectation values (identified with a "b" in Table 1). These proteins were also classified by developmental stage, although individual protein volumes within the spot could not be quantified. Proteins in this category are found in nine spots at 10 dpa (77, 195, 230, 240, 256, 284, 289, 303, and 394) and eight spots at 36 dpa (156, 220, 265, 360, 368, 385, 432, and 583). At 10 dpa, for example, spot 77 contained two proteins (F23N19.10, putative stress-induced protein, and a poly(A)-binding protein) and spot 195 contained three proteins (26S proteosome regulatory particle triple-A ATPase subunit 3, argininosuccinate synthase-like protein, and UDP-glucose dehydrogenase). Other spots with

identical mobilities contained different proteins at 10 and 36 dpa (identified with a "c" in Table 1). This set included four spots: 228, 247, 273, and 416. Spot 228, for example, was identified as 26S proteasome regulatory particle at 10 dpa and isocitrate dehydrogenase at 36 dpa (Table 1).

3.2 Protein function

To gain insight into biochemical processes occurring in the endosperm, proteins were classified according to putative function (Table 2). The list was simplified by entering related proteins and isoforms under a single name. For this purpose, we defined isoforms as proteins related by function and sequence; they often had a different M_r and/or pI. Interestingly, isoforms of some proteins accumulated at only one time point (*e.g.*, ADP glucose pyrophosphorylase or PPase, LS, at 10 dpa; chitinase at 36 dpa) and others at both time points (*e.g.*, aldehyde dehydrogenase, aldolase). The proteins identified were grouped into 13 biochemical processes of the endosperm: ATP interconversion reactions, carbohydrate metabolism, cell division, cytoskeleton, lipid metabolism, nitrogen metabolism, protein synthesis/assembly, protein turnover, signal transduction, protein storage, stress/defense, transcription/translation, and transport. Four of the proteins identified had no assigned function and were thus classified as unknown.

Table 2. Proteins of wheat endosperm categorized by function and stage of development. Normalized spot volumes (Table 1) were used to identify proteins more abundant at 10 dpa than at 36 dpa. Multiple entries under 10 and 36 dpa indicate related proteins and iso-forms

Function	10 dpa	36 dpa
ATP interconversion		
Adenosine kinase	279	
Nucleoside diphosphate kinase I		420
Carbohydrate metabolism		
6-Phosphogluconate dehydrogenase	193	
Aconitase	25, 27	
ADP glucose PPase, LS	128, 130, 132	
ADP glucose PPase, SS	159	156[a]
Aldehyde dehydrogenase	153	154
Aldolase	244, 252, 306	257
β-Amylase		125
Dihydrolipoamide dehydrogenase	161	168
Enolase	178, 179, 185	
Fructokinase	97	
Glucose and ribitol dehydrogenase		310, 591
Glyceraldehyde 3-P dehydrogenase (NAD)	264	262, 273, 274, 275, 579, 582, 860, 861
Isocitrate dehydrogenase (NAD)		228
Malate dehydrogenase (NAD)	294, 295	
Phosphoglucomutase	96	
Phosphoglycerate dehydrogenase-like protein	119	
Phosphoglycerate kinase	640	
Phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent	103, 104	
PPi-fructose 6-P 1-phosphotransferase	112, 303[a]	111
Pyruvate Pi dikinase	31	
Reversibly glycosylated polypeptide	247, 672	250
Sucrose synthase type 2		550
Triosephosphate isomerase	354, 357	356
UDP-glucose dehydrogenase	195[a], 493	
UDP-glucose PPase	157, 158	
Cell division		
Cell division cycle protein	26	
Tubulin α-3 chain	182	
Tubulin β-3 chain	184	
Cytoskeleton		
4SNc-Tudor protein (NTPase)	10, 11	
Actin	240[a], 256[a]	
Lipid metabolism		
Acyl-acyl-carrier protein desaturase	256[a]	
Nitrogen metabolism		
10-Formyltetrahydrofolate synthetase	72	
2-Isopropylmalate synthase (leu)	110	
Acetohydroxyacid synthase (leu, ile, val)	98	
Alanine amino transferase 2 (ala)	173, 176	
Argininosuccinate synthase-like protein (arg)	195[a]	
Aspartate amino transferase (asp)	232	220[a], 230[a], 236
Carbamoyl phosphate synthetase (glu)	6	
Formate dehydrogenase (gly, ser, met)	230[a], 249, 251	
Ketol-reductoisomerase (val)	138	
Methionine synthase (met)	48	
Methylmalonate-semialdehyde dehydrogenase (val)		156[a]
S-Adenosylmethionine synthetase (met)	655, 657	

Table 2. Continued

Function	10 dpa	36 dpa
Protein synthesis/assembly		
40S Ribosomal protein	416, 422, 444, 529	
60S Ribosomal protein	284[a]	869
Chaperonin 60 kDa β-subunit	117	
Cyclophilin	383, 530	
Elongation factor 1-α		141
Elongation factor 1-β	362	
Elongation factor 2	33	
Heat shock associated protein	149	163
Heat shock protein 16.9C		407
Heat shock protein 70	65, 70, 74, 471	212, 225
Heat shock protein 80–2	49	
Heat shock protein, 82K	42	
Protein disulfide isomerase	102, 118, 256[a]	405
Protein turnover		
20S Proteasome α-subunit	361, 373	347
20S Proteasome β-subunit	394[a]	
26S Proteasome regulatory particle subunit	195[a], 228, 289[a], 656	205, 220[a]
ClpC protease	35	
Leucine amino peptidase	135	137
Polyubiquitin 6	447	
Ubiquitin-protein ligase	415	
Signal transduction		
14–3-3 Protein homolog	330	
Guanine nucleotide-binding protein β subunit-like protein	301	
TGF-β receptor-interacting protein 1	278	283
Storage protein		
α/β-Gliadin	238	
Avenin		353
Avenin N9		289[a], 369, 384, 441
Globulin-2		147, 155, 175, 229, 559, 561, 851, 852
Globulin Beg 1		281, 620
Globulin-like protein		631
Grain softness protein 1b		416
Legumin-like protein	284[a], 285	272
LMW glutenin		165, 166, 171, 203, 577
Seed globulin		327, 335, 336, 337, 355, 360[a]
Triticin		199
Stress/defense		
α-Amylase inhibitor		381, 417, 430, 434, 436, 440
Ascorbate peroxidase		303[a], 342, 344, 348, 364, 666, 667
Barwin		871
Catalase isozyme 1	148	
Chitinase		282, 341, 351
Dehydroascorbate reductase		382
Glutaredoxin		432[a]
Glyoxalase I	305	
GSH-dependent dehydroascorbate reductase	378, 394[a]	385[a]
Late embryogenesis abundant protein		43, 551, 842

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Table 2. Continued

Function	10 dpa	36 dpa
OSJNBb0118P14.5		314, 324
Pathogenesis-related protein 4		427
Peroxidase		255, 259, 263, 265[a], 359, 583[a]
Peroxiredoxin		368[a], 371
Purple acid phosphatase		312
Selenium binding protein	145, 151	
Serpin	234	231, 233, 237, 240[a], 245, 246, 247, 265[a], 639
SGT1	201	
Stress-induced protein	77[a], 82	
Superoxide dismutase [Cu-Zn]		418
Superoxide dismutase [Mn]		376
Thaumatin-like protein TLP7		360[a]
Tritin		308
Trypsin inhibitor CMx		432[a]
Xylanase inhibitor protein I		318, 325
Transcription/translation		
DNA-binding protein HEXBP	338	
Glycine-rich RNA-binding protein	411, 412, 419, 433	
Poly(A)-binding protein	53, 55, 57, 60, 61, 63, 66, 69, 77[a]	
RNA-binding protein, similarity	93, 94	
Translation initiation factor	206, 311, 394[a]	
Translationally controlled tumor protein (mRNP)	399	
Transport		
Nonspecific lipid-transfer protein		633
Importin α-2 subunit		121
Plasmodesmal receptor		583[a]
Small ras-related GTP-binding protein	350	
Unknown		
Auxin-induced protein	273	
Embryo-specific protein		557
At1g07080/F10k1_15 protein		379
F10K1.21/F7A7_100 protein	368[a]	385[a], 386

a) Spots that contain more than one protein

The timing of events occurring during endosperm development can be seen more clearly when the number of proteins in each category is compared at 10 *versus* 36 dpa. Thus, carbohydrate metabolism, protein synthesis/assembly, transcription/translation, stress/defense, nitrogen metabolism, and protein turnover were the principal functions at 10 dpa (Fig. 3A). As a percentage of total proteins identified, the majority functioned in carbohydrate metabolism (26%), protein synthesis/assembly (15%), and transcription/translation (15%). Proteins involved in stress/defense (13%), nitrogen metabolism (11%), and protein turnover (8%) were also prominent at 10 dpa. Although relatively few proteins participating in cell division, cytoskeleton, lipid metabolism, and signal transduction were identified in this study, they were more abundant at 10 dpa (8%) than at 36 dpa (4%). Stress/defense, storage, and carbohydrate metabolism were the principal functions of the endosperm at 36 dpa (Fig. 3A). Compared to 10 dpa, proteins involved in carbohydrate metabolism continued to be prominent (18%) and a greater percentage of proteins were involved in stress/defense (38%) and storage (25%). In addition, relatively few proteins functioned in transport at 36 dpa (3%), but they were more abundant than at 10 dpa (1%). The number of ATP interconversion enzymes was relatively low (1%) at both developmental stages.

The trends in the timing of developmental events were further explored by graphing the normalized spot volumes of identified proteins against function (Fig. 3B). Spots for
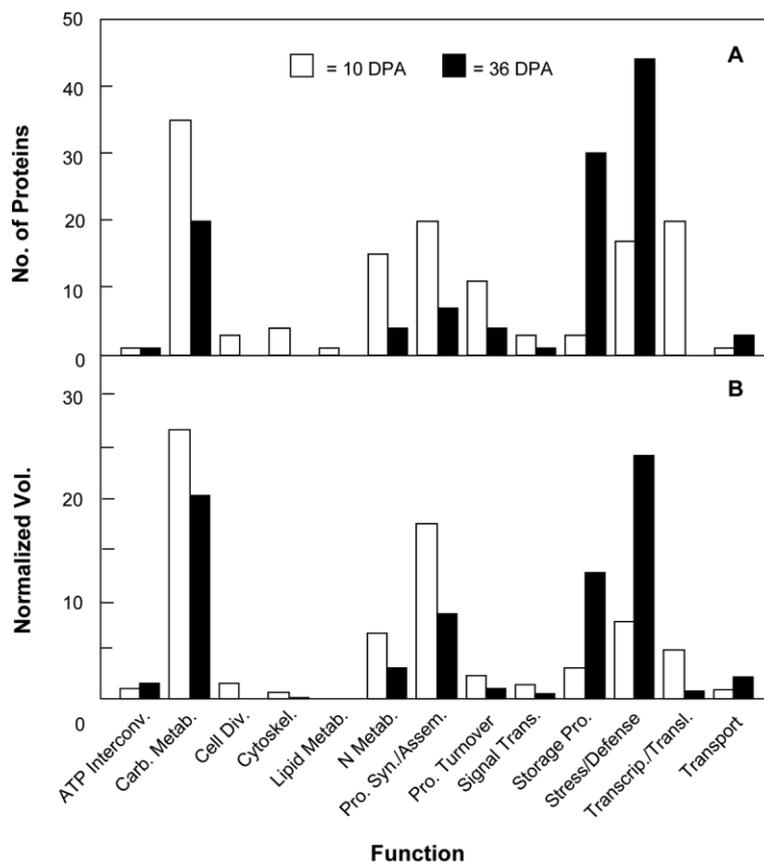
Figure 3. Timing of biochemical processes of wheat endosperm during grain development. A: Profiles based on protein number. B: Profiles based on normalized spot volume. □, 10 dpa; ■, 36 dpa.

which different proteins were identified at 10 and 36 dpa in Table 1 were included in this analysis. Spots containing more than one protein were excluded (designated with the letter "b" in Table 1), since volumes for individual components could not be determined. For this reason, lipid metabolism is present in Fig. 3A, but not in Fig. 3B. One result of the volume analysis is that two functional classes, cytoskeleton and transcription/translation, have protein volumes (Fig. 3B) but no protein members (Fig. 3A). The basis for this difference is that all of the cytoskeleton and transcription/translation protein members had higher volumes at 10 dpa than at 36 dpa. The 36 dpa proteins were, as noted in Section 3.1, excluded, and, therefore, are not shown in Fig. 3A. The volumes of these members, however, were included in Fig. 3B. Overall, the results of the normalized spot volume analysis (Fig. 3B) complement and confirm the protein number analysis (Fig. 3A). At 10 dpa, carbohydrate metabolism, protein synthesis/assembly, stress/defense, nitrogen metabolism, and transcription/translation were the principal functions (Fig. 3B). As a percentage of total normalized volume, proteins involved in carbohydrate metabolism (37%) and protein synthesis/assembly (23%) were the most prominent at 10 dpa. Proteins involved in nitrogen metabolism (11%) and stress/defense (11%) were also abundant. Those involved in protein turnover, cell division, cytoskeleton, and signal transduction, were more evident at

10 dpa and accounted for 9% of total spot volume. Transcription/translation was less prominent based on volume (7%) than on protein number (15%) as a result of the relatively low abundance of these proteins. Proteins involved in stress/defense (33%) and protein storage (17%) were predominant at 36 dpa as were those in carbohydrate metabolism (27%) and protein synthesis/assembly (11%). Proteins involved in ATP interconversion reactions and transport were quantitatively minor (2%) at both time points.

4 Discussion

4.1 Metabolic protein fraction

The albumins and globulins of wheat endosperm have received relatively little attention, because of their low abundance and perceived secondary role in flour quality. In previous studies, we found that the insoluble gliadins and glutenins account for more than 85% of the extractable endosperm protein [24]. Accordingly, analyses of total protein extracts by 2-DE revealed relatively few albumins and globulins. We, therefore, developed a sequential fractionation procedure that first separated the KCl-soluble albumins and globulins from the KCl-insoluble gliadins and glutenins and then sub-fractionated the albumins and globulins into the methanol-insol-

uble, metabolic proteins, and a methanol-soluble fraction that contained principally low molecular weight disulfide proteins (α-amylase, α-amylase/trypsin and WCI proteinase inhibitors, lipid transfer proteins, γ-thionins) and storage proteins (γ- and ω-gliadins, low-molecular-weight glutenin subunits, avenins) [13]. The method proved successful in that there were only 6 gluten proteins (1 gliadin and 5 LMW glutenin subunits) among the more than 250 proteins identified in the metabolic protein fraction. The method was applied previously in demonstrating the dramatic increase in hydrophilicity of the methanol-soluble albumins and globulins following reduction by thioredoxin or DTT [13].

4.2 Metabolic proteins characteristic of 10 dpa endosperm

In the current study, a combined 2-DE-MS approach allowed us to identify over 250 proteins in the metabolic protein fraction of wheat endosperm. The protein identification, together with the analyses of early and late stages, provides a dynamic picture of events that accompany grain development (Fig. 4). Overall there was a shift from active biosynthesis and metabolism to maintenance and storage. Thus, proteins involved in several processes were present early in grain development: cell division, signal transduction, transcription/translation, protein synthesis/assembly, and components of the cytoskeleton. A single lipid enzyme, acyl-acyl-carrier protein desaturase that functions in the synthesis of triacylglycerols, the major storage lipids of seeds, was also identified at 10 dpa. Related enzymes were not detected, likely due to their relatively low abundance in developing endosperm.

Enzymes of nitrogen metabolism needed for the extensive synthesis of storage proteins were also more abundant at 10 dpa. We were able to identify enzymes participating in the synthesis of 10 amino acids – alanine, arginine, aspartate, glutamic acid, glycine, isoleucine, leucine, methionine, serine, and valine as well as an enzyme functional in myriad transmethylation reactions, *S*-adenosylmethionine synthetase. This accumulation pattern is consistent with amino acid of the gliadins and glutenins, storage proteins integral to grain maturation.

4.3 Metabolic proteins characteristic of 10 and 36 dpa endosperm

Carbohydrate metabolism was a principal process at both 10 and 36 dpa. Enzymes were identified that functioned in glycolysis, citric acid cycle, sucrose synthesis, and starch synthesis. Consistent with the shift from active metabolism to maintenance and storage, the number of enzymes involved in carbohydrate metabolism decreased during the transition from 10 to 36 dpa. Enzymes involved in glycolysis decreased from 7 to 4, those in the citric acid cycle decreased from 3 to 2, and those in the oxidative pentose phosphate pathway decreased from 1 to 0. Glyceraldehyde-

3-phosphate dehydrogenase was a notable exception in that the number of isoforms increased strikingly from 1 to 11 as the grain matured. This result is consistent with an earlier report that dehydration strongly increased the level of this enzyme in leaves and callus tissue of the resurrection plant, *Craterostigma plantagineum*, a species capable of withstanding severe desiccation [25]. The increase in glyceraldehyde-3-phosphate dehydrogenase is also consistent for the developing wheat grain, which desiccates as it reaches maturity. Enzymes catalyzing ATP interconversion reactions, though low in number, resembled proteins involved in carbohydrate metabolism in that they were present at both 10 and 36 dpa.

4.4 Storage proteins characteristic of 36 dpa endosperm

Nearly all storage proteins detected in the metabolic protein fraction of the endosperm were present only at 36 dpa. Due to the fractionation procedure, gliadins and glutenins, the principal storage proteins of the endosperm, were noticeably absent. Globulins, the principal storage proteins in the seeds of dicot species, were the most prevalent class of storage proteins in the metabolic protein fraction. This is not surprising, since the globulins are by definition salt-soluble. Avenin, legumin-like protein, and triticin share sequence similarity with the 11/12S globulins [26–28] and globulin Beg 1 with a wheat 7S globulin [29]. The 11/12S globulins are known residents of the endosperm and the 7S globulins of the embryo and aleurone layer [30, 31]. Although embryos were removed and aleurone is minimal in our endosperm preparation, the tissue location of the globulin Beg 1-like protein remains to be confirmed. Another storage protein, grain softness protein, is a lipid-binding component that is associated with starch granules and may be correlated with grain texture [32].

4.5 Stress- and defense-related proteins characteristic of 36 dpa endosperm

The endosperm proved to be rich in defense- and stress-related proteins, particularly at 36 dpa. Many of the proteins identified protect against pathogens such as insects and fungi by acting as inhibitors of digestive enzymes, for example, α-amylase inhibitors, bifunctional α-amylase/trypsin and /subtilisin inhibitors, and thaumatin-like protein. Also detected were members of the serpin family of serine protease inhibitors that are believed to protect seeds against insects [33], but can be considered storage proteins. In view of the former function, we classified the serpins as stress/defense proteins in Table 2. Other types of proteins identified are also believed to offer protection against biological stress: barwin, which is closely related to wound-induced proteins and binds a chitin analog [34]; chitinase which hydrolyzes the structural carbohydrate of fungal cell

walls; and xylanase, which specifically inhibits fungal counterparts that degrade plant cell walls. Other defense proteins found include SGT1, a component of R-gene triggered disease resistance [35], and tritin, a ribosome-inactivating protein that may play a role in defense against viruses [36].

Proteins related to abiotic environmental stresses were also found primarily in the 36 dpa endosperm. Consistent with the grain undergoing desiccation at this stage of development, accumulated proteins include those related to drought stress. Representatives include glyceraldehyde-3-phosphate dehydrogenase (noted above), glyoxalase I [37], selenium-binding protein [38], and a group of LEA or late embryogenesis abundant proteins [39]. Although the embryo was removed in the present study, it remains to be seen whether the LEA proteins observed in our analysis are true residents of the endosperm. Proteins that respond to elevated temperature, HSP chaperones [40] and stress inducible protein [41], together with a representative involved in nutritional status, purple acid phosphatase [42], were also present.

Yet another major set of proteins protects the endosperm against oxidative stress, notably reactive oxygen species such as hydrogen peroxide and superoxide radicals. Overall, these proteins were more abundant at 36 dpa. However, ascorbate peroxidase, catalase, and glyoxalase were in much greater abundance at 10 dpa, whereas, glutaredoxin, peroxidase, and peroxiredoxins were in greater abundance at 36 dpa. This suggests that different metabolic processes generate hydrogen peroxide during early and late stages of endosperm development. Superoxide dismutases, metalloenzymes that scavenge reactive superoxide radicals, were found only at 36 dpa. Superoxide radicals are destructive oxidants that can be present at low levels under normal growth conditions, but increase dramatically in response to both biotic and abiotic stresses [43]. Since superoxide radicals increase in response to dehydration and the grain is undergoing desiccation at 36 dpa, the increase in superoxide dismutases at 36 dpa is consistent with this final stage of maturation. Since hydrogen peroxide is a by-product of the dismutation of superoxide by these enzymes, peroxidases would be expected to be present at 36 dpa.

4.6 Marker proteins

The question arises as to whether there are specific markers of early and late stages of grain development among the proteins identified. Proteins present at 10 dpa that were absent or had normalized spot volumes less than 0.004 at 36 dpa (Table 1) are potential markers of early development. Likewise, proteins present at 36 dpa that were absent or had normalized spot volumes less than 0.004 at 10 dpa are potential markers for late development. A marker protein list, including proteins present or quantifiable only at one time point, would contain 23 candidates for early development and 45 candidates for late development. Experiments in which endosperm is analyzed at additional developmental time points are necessary to determine if these proteins are indeed markers of early and late stages of grain development.

4.7 Concluding remarks

In summary, we identified many of the low abundance proteins present in wheat endosperm. More importantly, we obtained an overview of processes taking place in the grain and how they change during development (Fig. 4). Consistent with the ultimate role of the mature grain in reproduction, there was a shift in the accumulation of proteins during maturation from those active in biosynthesis and metabolism to those with roles in storage and protection against biotic and abiotic stresses. In concert with this striking alteration in accumulation profiles, endosperm proteins are known to undergo a change in thiol redox state as the grain matures, that is, conversion from an active (sulfhydryl) state to a quiescent, stable (disulfide) state as indicated in Fig. 4 [45–46]. During germination, proteins of the endosperm of wheat [47] and other cereals [48, 49] show a reversal of this redox change, that is, reduction and conversion back to an activated state, thereby facilitating the mobilization of nitrogen and carbon for the developing seedling. Although the KCl/methanol fractionation procedure allowed us to identify many endosperm metabolic proteins for the first time directly, it is noted that these are only the most prominent members. Less abundant proteins will need to be identified
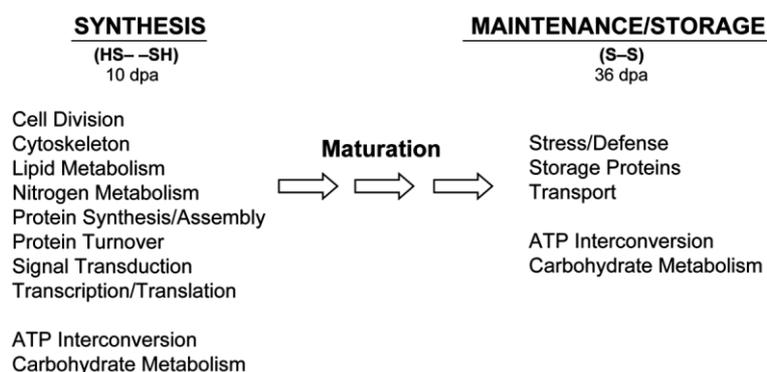

Figure 4. Changes in major biochemical processes of the endosperm as the grain develops.

by other methods such as those used recently for the successful identification of thioredoxin targets: gel fluorescence and affinity chromatography for seeds [11–13, 50–52] and affinity chromatography for chloroplasts [53, 54] cyanobacteria and green algae [55–57], and *Arabidopsis* [58].

In this study, we obtained not only a picture of the dynamic processes taking place during grain maturation, but also reference maps of the developing endosperm proteome. With the formulation of these maps, the stage is set for elucidating further regulatory details of the reactions governing biochemical processes in cereals such as the growing number found to be controlled by redox regulation by way of thioredoxin [11, 44, 59–61]. Additional areas awaiting exploration include the effect of environmental conditions on grain-fill and on the composition and quality of mature grain.

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