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ORIGINAL RESEARCH

Proteomics Analysis of Embryo and Endosperm from Mature Common Buckwheat Seeds

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Abstract We used proteomics analysis to generate the profiles of proteins in the endosperm and embryo of common buckwheat grains. These differentially expressed proteins are potentially involved in seed metabolism. Extractions were done by trichloroacetic acid (TCA) precipitation. The resulting proteins were separated using SDS-PAGE coupled to LC-ESI-Q/TOF-MS/MS. This allowed us to detect and identify 67 proteins with isoforms, making this the most inclusive protein profile. The proteins were determined to be functionally involved in the central metabolic pathway of the seed, with metabolic interest being reflected in the occurrence of a tissue-specific enzyme balance. For a case in point, we found a tissue-specific and subcellular compartment-specific isoform of granule-bound starch synthase 1 in the chloroplast/amyloplast. This provided proteomic verification of the presence of a distinct regulatory mechanism for the biosynthesis of glycan and starch, which produce amylase and amylopectin. Furthermore, several previously characterized allergenic proteins such as 11S and 13S globulin seed storage protein were acknowledged in our seed samples, thus representing the potential for proteomics techniques that survey food

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sources for any incidence of allergens. This protein profile of common buckwheat grain is a new avenue for understanding its seed physiology in dormant stage as well as suggesting commercial applications for the buckwheat industry as buckwheat flour.

Keywords Common buckwheat · Embryo · Endosperm · Proteomics

Abbreviations

LC	Liquid chromatography
PAGE	Polyacrylamide gel electrophoresis
PIR	Protein information resource
Q/TOF	Quadruple/time of flight
SDS	Sodium dodecyl sulfate

Introduction

Buckwheat (*Fagopyrum esculentum*) is gaining worldwide importance as a future minor food source due to its high nutritive value. This dicotyledonous crop is cultivated under cool climates and is adapted to regions with high elevations and short growing seasons (Tsuji and Ohnishi 2000). De-hulled buckwheat kernels resemble other cereal grains in their general chemical composition and structure, having a non-starchy aleurone layer and starchy endosperm (Javornik 1986). Buckwheat is an important part of the diet in China, Japan, Korea, Europe, Canada, and other western countries. It is praised as a functional food in Asia as material for noodles, tea, and sprouts and is marketed as an ingredient of pancake mixes in the United States and Canada. Its grains contain 12.6% protein; the bulk is easily soluble albumin and globulin fractions that are readily assimilated by the human body. These proteins have a well-balanced amino acid composition and, compared with other bread grains, they contain many indispensable amino acids, including lysine and threonine (Cepkova and Dvoracek 2006; Dvoracek and Cepkova 2006; Cepkova et al. 2007). Of the important amino acids that are abundant in other cereals, only leucine is lacking in buckwheat. With their high content of essential histidine, buckwheat grains also have a positive influence on infant growth (Guozhu et al. 2007).

Buckwheat seeds are an important source of natural antioxidants that can effectively inhibit oxidation in food and reduce the risk of age-dependent diseases (Burda and Oleszek 2001; Zou et al. 2004). As well as being rich in several types of flavonoids—rutin, quercitin, kaempferol, and kaempferol-3-rutinoside—this cereal is also high in starch and Vitamins B and E (Li et al. 2001; Zhao et al. 2001; Yoon et al. 2007). Buckwheat is astringent, acrid, bitter, cardiotonic, hypotensive, galactogogue, and prophylactic. It dilates blood vessels, lowers blood pressure, and treats fragile capillaries and reduces their permeability (Guozhu et al. 2007; Matejova et al. 2007). This material can help cure gout, varicose veins, and retinal hemorrhages, and it is used for treating circulatory problems (Fu 2001; Sisuo et al. 2007) as well as chilblain, retinitis, eczema, and liver disorders. Grains contain abundant iron, copper, cobalt, manganese, and other trace elements (Bonafaccia et al. 2003). It can be successfully cultivated for such purposes after other haymaking and crop harvesting are completed, and its vegetative mass can be fed as green fodder or used for ensilage (Anon 2002).

1D-PAGE is a popular approach for sample fractionation of a biological sample; LC-ESI-Q/TOF mass spectrometry analysis is then performed on the enzyme digests of selected bands (Wu et al. 2002). Large-scale genomics has enabled the newer field of proteomics research to be successful by creating a sequence infrastructure that can be used with high-throughput mass spectrometry data to identify previously indistinguishable enzymes and proteins that rely on recent information about genomic and protein sequences. For example, the digestion of a 1D-PAGE-separated protein by trypsin is then followed by the direct analysis of the resulting peptides by conducting high-throughput capillary multidimensional liquid chromatography coupled to ESI double mass spectrometry. Thus, an MS/MS spectrum can be algorithmically compared with predicted peptide spectra from a sequence database to identify a particular protein. Shotgun techniques are also being developed not only to discover the proteins within a mixture but also to compare the relative levels of protein expression from diverse samples. These proteomics techniques are now used to improve various biological systems (MacCoss et al. 2002). Proteome investigation in plants is issue to the same obstacles and restrictions found with other organisms. However, plant tissues contain their highly rigid cell walls and a complex variety of secondary metabolites resulting to its difficult separation and identification. To the best of our knowledge, no studies of common buckwheat have yet been reported using buckwheat embryo and endosperm coupled to proteome approach. Herein, we investigated the proteome diversity of the common buckwheat gene via protein expression in the seed using 1D-PAGE coupled to LC-ESI-Q/TOF mass spectrometry.

Materials and Methods

Experimental Materials

Seeds of common buckwheat (*Fagopyrum esculentum* Moench L.) were collected from the field at Chungbuk National University, Cheong-ju, Korea. Plants had been

grown during the summer when the temperature was 28°C to 30°C. The physiology of this species is timed so that plants will bloom and set seed when the hot, dry weather is over. Seeds were harvested and stored at 10°C. For sample preparation, those seeds were separated with a scalpel into their embryo and endosperm components.

Protein Preparation

Mature seeds (endosperm and embryo) were ground to flour in a mortar with liquid nitrogen. Using a modified method, we fractionated the seed proteins to exploit the solubility and precipitation properties of endosperm proteins in trichloroacetic acid (TCA)/acetone (Damerval et al. 1986; Porubleva et al. 2001; Kim et al. 2010a, b). Each 200 mg of frozen powder was suspended in TCA solution (10% TCA/acetone solution containing 0.07% 2-mercaptoethanol with a 0.04% protease inhibitor cocktail for plant cell and tissue extracts). After vortexing, the solution was frozen at –20°C for 1 h then centrifuged at 14,000×*g* for 30 min at 4°C. The precipitate was suspended in wash buffer (acetone containing 0.07% 2-mercaptoethanol and a 0.04% protease inhibitor cocktail). After standing for 12 h at –20°C, the suspension was centrifuged at 14,000×*g* for 30 min at 4°C. The precipitate was dried in vacuo, and part of the resultant powder was suspended in 400 μl of solubulization solution containing 6 M urea, 2 M thio-urea, 10 mM Tris-HCl, 0.75% ampholine (pH 3–10), 50 mM DTT, 4% CHAPS, and 0.4% protease inhibitor cocktail. After incubating at room temperature for 2 h with continuous vortexing, the suspension was centrifuged at 14,000×*g* for 30 min at 4°C. The protein concentration in each sample was determined by the method of Bradford (1976), using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) and bovine serum albumin (BSA) as a standard, and stored at – 80°C until further use.

Gel Electrophoresis

Protein samples were mixed with an equal volume of SDS reducing buffer containing 0.625 mM Tris–HCl (pH 6.8), 2% SDS, 0.5% 2-mercaptoethanol, 30% glycerol, and 0.002% Bromophenol Blue for 1D-PAGE, separated by SDS-PAGE (Laemmli 1970; Yun et al. 2008; Kim et al. 2010b). The 4% stacking gel was overlaid on a separating gel (12% polyacrylamide at an acrylamide/*bis* acrylamide ratio of 30.0:0.8). Equal volumes of protein extracts from endosperm and embryo were loaded in each lane. The running buffer consisted of 0.25 M Tris, 1.92 M glycine, and 1% SDS (pH 8.3). Gels were run in a Mini-protein III dual-slab cell (Bio-Rad) at a constant current of 100 V per slab gel from a Power PAC 300 (Bio-Rad). The gels were fixed and the proteins visualized by highly sensitive silver staining. Images were taken by a flatbed scanner (HP Scanjet G4010) (Fig. 1).

In Gel Digestion and LC-ESI-Q/TOF-MS/MS

Silver-stained proteins were destained with chemical reducers to remove the silver as described previously with the following slight modifications (Sinha et al. 2001; Richert et al. 2004). The reactive substances of the chemical reducers are potassium ferricyanide and sodium thiosulfate. These chemical agents were prepared prior to digestion as two stock solutions of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate, both dissolved in water. A working solution was prepared by mixing a 1:1 ratio of the above stock solutions and was kept at 4°C. Thirty to 50 μl of working solution were added to cover the gel bands and occasionally vortexed. The gel color was monitored until the brownish color disappeared, then the gel band was rinsed a few times with water to stop the reaction. At that time, 200 mM ammonium bicarbonate was added to cover the gel for 20 min and was then discarded. Consequently, the gel was cut into small pieces, washed with water, and dehydrated repeatedly with changes of acetonitrile until the gel pieces turned opaque white. The gel pieces were dried in a vacuum centrifuge for 30 min (Modulspin 3,080 C, BioTron, Inc., Korea). The protein was reduced with 10 mM DTT in 100 mM NH_4CO_3 at 56°C for 1 h and alkylated with 55 mM IAA (iodoacetamide) in 100 mM NH_4CO_3 in the dark for 40 min. The gel pieces were minced, lypolized, and then rehydrated in 50 mM NH_4CO_3 with 11.9 ng/μl sequencing-grade modified trypsin (Promega Corp.) at 37°C overnight for 16 h. The digested peptides were then recovered through two extraction steps with a solution containing 50 mM ABC, 50% (*v*/*v*) ACN, and 5% (*v*/*v*) TFA. These extracts were used for MS analysis

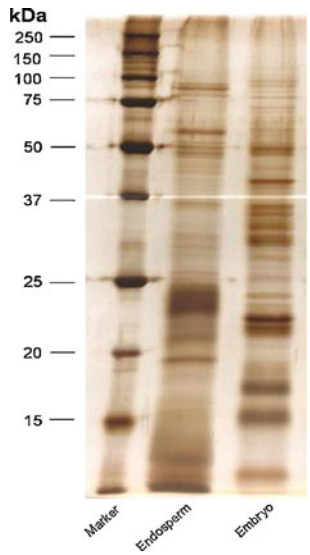

Fig. 1 Silver-stained SDS-PAGE gel of endosperm and embryo from common buckwheat

with an LC-ESI-Q/TOF-MS/MS (Thermo Finnigan, San Jose, CA, USA). A total of 10 μl of each peptide sample was resolved on an MGU30-C18 trapping column (LC Packing). Peptides were eluted from the column and directed onto a 10 cm×5 μm i.d. C_{18} reverse phase column (PROXEON, Odense, Denmark) at a flow rate of 120 nl/min. They were eluted by a gradient of 0~65% ACN for 70 min. All MS/MS spectra in the LC-ESI-Q/TOF ion trap mass spectrometer were acquired in a data-dependent mode. Each full MS scan (*m*/*z* range of 400 to 2,000) was followed by three MS/MS scans of the most abundant precursor ions in the MS spectrum, with dynamic exclusion enabled (Yun et al. 2008).

Bioinformatics

For protein identification, we searched the MS/MS spectra by MASCOT (www.matrixscience.com). Using that program, the spectra were transformed into mascot generic files (MGF) that were then used to search for protein candidates in the UniProt-Sprot databases (ver. 57.2) against the Viridiplantae (green plants) database. Green plants were employed in our homologous search because buckwheat has no complete genome sequence or identical databases.

The degree of modification was varied with carbamidomethyl cysteine and the oxidation of methionine; the peptide charge was 2+ and 3+. One missed trypsin cleavage was allowed, within 1.5 Da, for peptide and MS/MS tolerance. Protein hits with more than one peptide match were considered valid identifications. To ascertain our accuracy, all other hits were manually analyzed. The criterion used for true identification was that the masses of all the major peaks (typically more than seven) in an MS/MS spectrum had to match those of the theoretically calculated fragment ions.

Functional Classification Based on Gene Ontology

Protein Information Resources [http://pir.georgetown.edu] (PIR) is an integrated public bioinformatics source that supports genomics, proteomics, and systems biology research. It is used for identifying gene ontology-based molecular functions, cellular components, and biological functions; these are automatically classified in the data set according to gene ontology using Batch Retrieval with the *i*ProClass database (Huang et al. 2003).

Results

Identification of Mature Seed (Embryo and Endosperm) Proteins of Common Buckwheat

To determine the composition of seed proteins from common buckwheat, we analyzed embryo and endosperm samples (Fig. 1) via SDS-PAGE following LC-ESI-Q/TOF mass spectrometry. Through shotgun analysis, two fractions examined by tryptic digestion revealed 303 and 196 proteins from the embryo and endosperm, respectively. Out of 465 proteins (each with≥1 peptide matches), 270 were identified from the embryo, 163 from the endosperm, and 33 common to both tissues (data not shown). After screening all of the proteins according to peptide match, we focused on the 67 proteins with ≥2 peptide matches (Table 1). Selections were also based on protein resemblance, Mowse scores, and sequence coverage against green plants. Of those 67 proteins, 20 (29.8%) were identified in the endosperm, 29 (43.3%) in the embryo, and 18 (26.9%) shared between the two (Fig. 2).

Molecular weight (MW), p*I*, protein length, and exponential modified protein abundance index (emPAI) are the physical properties of protein, all of which are fundamental to organismal function. Here, the plots exhibited a bimodal distribution, with a lower pH appearing at approximately p*I* 3.71 and a higher pH at approximately p*I* 11.48 of identified proteins. Overall, 62 proteins (92.5%) were identified at p*I* 3 through 10 and 5 (7.5%) at p*I* >10; these were correlated to molecular weight. Of these, 57 proteins (85%) had molecular weights of 1~99.99 kDa (<100 kDa) while ten others (15%) were >100 kDa (Fig. 3a; Table 1). However, MW and emPAI are important parameters for measuring absolute protein content (% weight) (Ishihama et al. 2005). Here, 63 proteins (94%) revealed emPAI values while that parameter could not be calculated for four others (6%) when LC-ESI-Q/TOF mass spectrometry was used. Values of 0.01 to 0.90 for emPAI were found for 56 proteins (88.8%) while seven (11.11%) had values >1 (Fig. 3b; Table 1).

Functional Classification of the Detected Proteins

Our proteomics survey involved 67 unique proteins found in the buckwheat embryo and endosperm. All identified proteins were functionally classified according to PIR, coupled with the *i*ProClass batch retrieval system; these were based on gene ontology for molecular functions (15 categories), cellular components (15), and biological processes (18).

For molecular functions, 173 functional frequencies were found in total. These included 28 (16.18%) identified as nucleotide-binding proteins, 21 (12.14%) for nucleoside binding, 17 (9.83%) for hydrolase activity, 14 (8.09%) for transferase activity, 13 (7.51%) for nucleic acid binding, ten (5.78%) for protein binding, nine (5.20%) for ion binding, eight (4.62%) for catalytic activity, seven (4.05%) for oxidoreductase activity, six (3.47%) for cofactor binding, five (2.89%) for transporter activity, four (2.31%) for nutrient reservoir activity, and four (2.31%) for signal

Table 1 General features of proteins categorized from buckwheat endosperm and embryo

Uni_acc.	Protein description	GN	MW	PL	MS	PM	p*I*	emPAI	SC (%)
Embryo									
Q9XHP0	11S globulin seed storage protein 2	–	51,830	459	57	3	7.72	0.08	42.8
Q8LAH7	12-oxophytodienoate reductase 1	OPR1	41,167	372	54	2	6.32	0.1	33
O23878	13S globulin seed storage protein 1	FA02	64,517	565	1,255	63	5.68	3.86	37
O23880	13S globulin seed storage protein 2	FA18	57,043	504	941	45	6.12	2.61	41.1
A0ZZ70	30S ribosomal protein S8, chloroplastic	rps8	15,498	134	43	2	11.19	–	26.7
A4QKK1	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta	accD	55,032	484	45	2	6.93	0.16	41
Q8LFH6	Actin-depolymerizing factor 12	ADF12	15,884	137	80	3	5.57	0.28	28.8
Q84WU8	Cell division protease ftsH homolog 3, mitochondrial	FTSH3	89,352	809	62	3	6.8	–	31.6
O80983	Cell division protease ftsH homolog 4, mitochondrial	FTSH4	77,274	717	42	2	8.73	0.05	41.5
Q39211	DNA-directed RNA polymerase II subunit RPB3-A	RPB36A	35,461	319	40	2	4.63	0.12	25
O49169	Elongation factor 1-alpha	EF1	49,371	449	126	5	9.2	0.51	51.1
Q41803	Elongation factor 1-alpha	EF1A	49,232	447	140	5	9.19	0.51	23.6
Q9LID1	F-box/kelch-repeat protein At3g13680	At3g13680	45,171	395	43	2	6.94	–	33
Q9SHV1	Glutamate receptor 2.2	GLR2.2	102,845	920	48	2	7.51	0.04	22.2
P26520	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	GAPC	36,526	337	56	2	6.68	0.25	18.6
Q0J8A4	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	GAPC	36,412	337	50	2	6.61	0.25	17.7
P25840	Heat shock 70 kDa protein	HSP70	70,997	650	59	3	5.2	0.19	27.7
P22953	Heat shock cognate 70 kDa protein 1	HSC70-1	71,357	651	76	3	5.03	0.19	18.3
P22954	Heat shock cognate 70 kDa protein 2	HSC70-2	71,386	653	70	3	5.03	0.19	31.6
O65719	Heat shock cognate 70 kDa protein 3	HSC70-3	71,147	649	78	3	4.97	0.19	22.3
Q6F2Y7	Heat shock protein 101	HSP101	100,895	912	124	2	5.9	0.08	33.3
O49118	Histone H2B	HIS2B	16,045	145	75	2	10.11	0.63	20.3
Q6LAF3	Histone H4	hh4	11,409	103	59	3	11.48	1.77	26.2
Q1S053	Probable histone H2A.3	–	16,142	152	57	2	10.85	0.63	12.5
Q9LP77	Probable inactive receptor kinase At1g48480	RKL1	71,130	655	43	2	8.3	0.06	35.6
Q9SZ67	Probable WRKY transcription factor 19	WRKY19	210,320	1,895	52	2	6.35	0.04	31.3
Q9SZC9	Putative copper-transporting ATPase PAA1	PAA1	99,996	949	38	2	8.98	0.04	24.5
A8W3M6	Putative membrane protein ycf1	ycf1	197,704	1,676	61	2	9.74	0.02	30.7
Q08183	Ribulose bisphosphate carboxylase small chain 3, chloroplastic	RBCS-3	20,385	183	43	3	6.73	0.22	22.4
Embryo and endosperm									
Q9XFM4	13S globulin seed storage protein 3	FAGAG1	61,163	538	1,278	66	6	5.05	46.5
P19023	ATP synthase subunit beta, mitochondrial	ATPB	59,103	553	51	2	6.01	0.07	12.4
Q8W0Z9	Carbon catabolite repressor protein 4 homolog 1	CCR4-1	66,760	602	140	3	7.03	0.06	22.2
Q8W4R3	DEAD-box ATP-dependent RNA helicase 30	RH30	64,620	591	53	3	9.66	0.07	32.2
Q5SCX8	DNA-directed RNA polymerase subunit beta	rpoB	122,782	1,080	56	5	5.33	0.03	30.7
P93107	Flagellar WD repeat-containing protein Pf20	PF20	65,838	606	73	5	6.13	0.06	41.3
P11143	Heat shock 70 kDa protein	HSP70	70,572	645	81	5	5.22	0.34	14.4
Q9LE42	Histone acetyltransferase HAC5	HAC5	188,186	1,670	41	2	8.8	0.02	20.8
P46875	Kinesin-3	ATK3	85,029	754	57	3	5.9	0.05	31.2
Q9BBR9	Photosystem I assembly protein ycf4	ycf4	22,952	200	39	2	9.27	–	14.5
Q9SCQ7	Probable acidic leucine-rich nuclear phosphoprotein 32-related protein	At3g50690	49,565	447	66	4	3.71	0.18	13.1
P10091	Probable sulfate/thiosulfate import ATP-binding protein cysA	cysA	42,799	370	88	6	9.77	0.1	32.4

Table 1 (continued)

Uni_acc.	Protein description	GN	MW	PL	MS	PM	p*I*	emPAI	SC (%)
Q85A69	Probable sulfate/thiosulfate import ATP-binding protein cysA	cysA	43,654	381	84	5	9.62	0.1	42.4
P09975	Protein ycf2	ycf2	259,911	2,136	55	2	9.88	0.02	20.6
A1XGT3	Putative membrane protein ycf1	ycf1	213,427	1,788	62	4	9.48	0.02	50.4
Q0D3B6	Two-component response regulator-like PRR37	PRR37	79,917	742	51	2	8.87	0.11	24.6
P85925	Unknown protein 18 (fragment)	–	1,394	12	153	5	5.8	6.03	91.7
P85487	Unknown protein 3 (fragment)	–	1,158	10	124	6	4.21	9.51	100
Endosperm									
P50249	Adenosylhomocysteinase	SAHH	53,141	485	73	2	5.79	0.17	23.7
Q8L7B5	Chaperonin CPN60-like 1, mitochondrial	At2g33210	61,978	585	43	2	6.37	0.07	24.4
Q5VRY0	DEAD-box ATP-dependent RNA helicase 39	Os01g0184500	67,859	625	48	2	10.2	0.06	23
Q3EB08	F-box/kelch-repeat protein At3g24760	At3g24760	42,672	383	38	2	5.5	0.21	25.2
Q9SK28	Formin-like protein 18	FH18	123,225	1,111	43	2	8.65	0.07	43.9
P34924	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	GAPC	36,529	340	77	2	6.67	0.25	28.2
P09094	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic (fragment)	GAPC	35,533	326	96	2	6.14	0.26	18.6
P04713	Granule-bound starch synthase 1, chloroplastic/amyloplastic	WAXY	65,966	605	108	2	6.59	0.13	33.8
P0C585	Granule-bound starch synthase 1, chloroplastic/amyloplastic	WAXY	66,322	606	123	3	8.34	0.2	24.3
P27736	Granule-bound starch synthase 1, chloroplastic/amyloplastic	WAXY	67,750	615	164	4	8.25	0.27	25.9
Q43784	Granule-bound starch synthase 1, chloroplastic/amyloplastic	WAXY	66,968	608	125	2	8.26	0.13	14.3
Q85XY6	Maturase K	matK	60,281	506	45	2	9.91	0.07	13.2
P30571	Metallothionein-like protein 1	–	7,486	76	38	2	4.91	1.75	23.7
P37223	NADP-dependent malic enzyme	MOD1	64,357	585	43	2	6.06	0.14	5.8
Q9MAQ0	Probable granule-bound starch synthase 1, chloroplastic/amyloplastic	WAXY	66,879	610	59	2	8.76	0.13	11.8
P56785	Putative membrane protein ycf1	ycf1-A	213,725	1,786	44	2	9.75	0.04	21
P49039	Sucrose synthase	–	92,576	805	110	4	5.98	0.14	34
Q01390	Sucrose synthase	SS1	92,091	805	110	4	6.07	0.14	44
P49040	Sucrose synthase 1	SUS1	92,996	808	82	3	5.84	0.14	24
P49035	Sucrose synthase isoform 1	–	92,473	808	76	2	6.21	0.09	23

GN gene name, *MW* molecular weight, *PL* protein length, *MS* Mowse score, *PM* peptide match, p*I* isoelectric point, *emPAI* exponential modified protein abundance index, *SC* sequence coverage

transducer activity. Another 17 proteins (9.83%) are involved in other molecular functions, e.g., transcription regulation; activities for helicase, peptidase, ligase, lyase, and monooxygenase; and structural molecule and motor activities (Fig. 4a; Supplementary Table 1).

For cellular components, most of these proteins (24, 19.51%) were found in the plastid, followed by 16 (13.01%) in the membrane, 15 (12.20%) in the cytoplasm, ten (8.13%) in the nucleus, four (3.25%) in the mitochondrion, and three each (2.44%) in the cell wall, ribosome, extracellular region, chromosome, and nucleosome. Two proteins each (1.63%) were located to the thylakoid and nucleus, while another 15 (12.20%) were found in other cellular components, e.g., vacuole, periplasmic space, RNA polymerase complex, cilium, cytoskeleton, proton-transporting two-sector ATPase complex, DNA-directed RNA polymerase II, holoenzyme, protein complex, external encapsulating structure, flagellum, intracellular membrane-bounded organelle, cell envelope, acetyl–CoA carboxylase complex, ribonucleoprotein complex, and cell projections (Fig. 4b; Supplementary Table 1).

For biological processes, 18 proteins (10.47%) function in metabolism, 11 (6.40%) in response to stimulus, nine (5.23%) in carbohydrate metabolism, seven (4.07%) in oxidation reduction, six (3.49%) in transcription, five each (2.91%) in macromolecule biosynthesis, polysaccharide

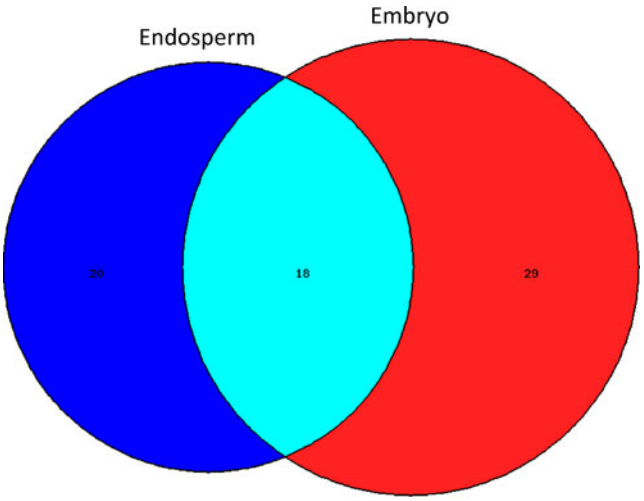

Fig. 2 Cross-correlation between embryo and endosperm proteins in common buckwheat (67 proteins identified from buckwheat seeds, including 29 from embryo, 20 from endosperm, and 18 common to both)

metabolism, and ion transport, and four each (2.33%) in regulation, metabolism of RNA proteins and alcohol, chromosome organization, generation of precursor metabolites and energy, and macromolecular complex assembly. An additional 57 proteins (33.14%) are involved in other biological processes (Fig. 4c; Supplementary Table 1). For other biological roles, all categories were annotated by single proteins. According to this database, many proteins are still unclassified, including ten (5.78%) for molecular functions, 18 (14.63%) for cellular components, and 16 (9.30%) for various biological processes.

Discussion

A comprehensive understanding of the metabolic actions that incorporate all the components of storage reserves in the seeds of crop plants is central to efforts for improving grain quality and yield. Given the diversity of metabolic events, an all-encompassing proteomics approach can be used to provide a global perspective on this complex system. We followed proteomics protocols that used SDS-PAGE with nano-LC-ESI-Q/TOF-MS/MS to analyze inclusively the proteins expressed in two major parts of common buckwheat grain. Of the 67 identified in total, 20 were found in the endosperm and 29 in the embryo, while the remaining 18 were common to both tissues. Samples were extracted directly by TCA precipitation and the proteins were separated by SDS-PAGE. We also performed the shotgun technique, whereby whole-cell protein extracts were instantly cleaved and the peptide mixture subjected to separation before mass spectrometry to generate peptide sequence data (Link et al. 1999; Smith et al. 2002). This method leads to a highly efficient detection rate compared

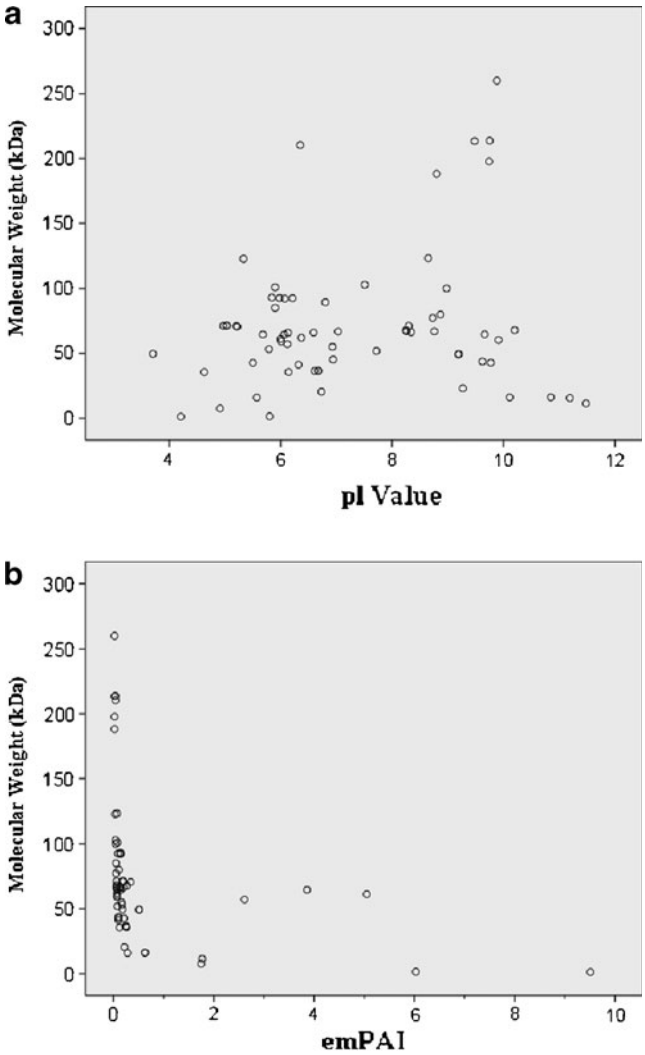

Fig. 3 Scatter plots of estimated molecular weight versus iso-electric points (p*I*) (**a**) and exponential modified protein abundance index (emPAI) (**b**) for all proteins identified from buckwheat endosperm and embryo

with conventional 2-DGE coupled to mass spectrometry (Koller et al. 2002).

The p*I* value has long been a standard measure for distinguishing among proteins. The trimodal character in eukaryotes is likely to be a general property of proteomes; thus, different p*I* values are needed depending upon subcellular localization (Schwartz et al. 2001). In large-scale proteome analyses, MW and emPAI are essential features for estimating the protein content in a digestive sample (Ishihama et al. 2005). Finally, p*I*, MW, and emPAI are critical to predicting and accurately identifying those proteins. emPAI is a measure to explain the protein composition in sample solutions. When the total protein amount in the sample is available, emPAI can be converted to the absolute amount of each protein in the sample. emPAI is derived from PAI, which is defined as the number

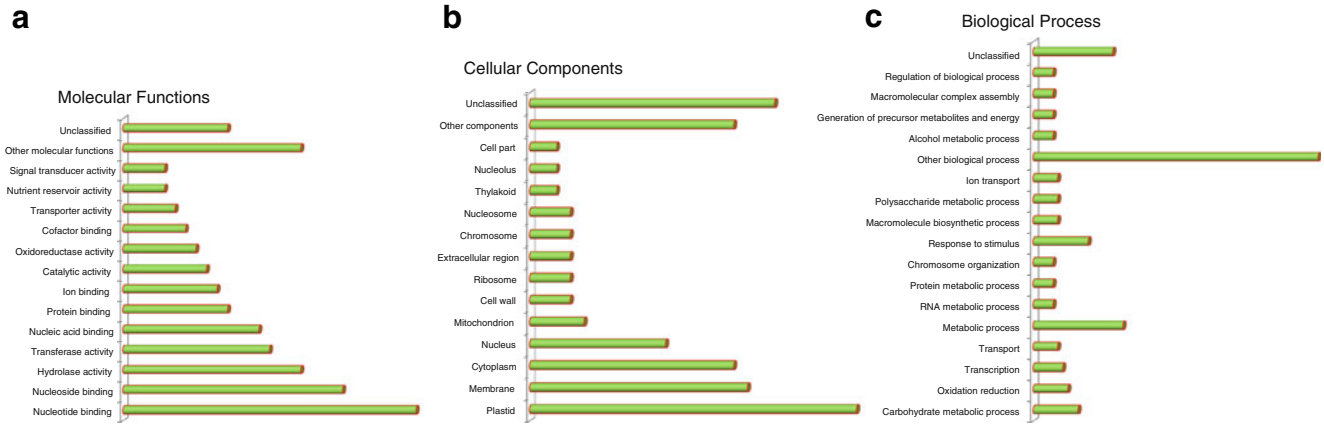

Fig. 4 Frequency distribution of identified proteins within functional categories based on molecular functions (**a**), cellular components (**b**), and biological processes (**c**). Classifications were made using *i*ProClass databases, and assignment by function was based on gene ontology (see Table 1 and Supplementary Table 1)

of observed peptides divided by the number of observable peptides per protein. It was previously reported that log (PAI) had a linear relationship to the protein amounts and that emPAI was proportional to the protein amounts for whole cell lysate digested by trypsin. The accuracy of this method was within factor 5, similar or better than the determination of abundance by protein staining. Since the emPAI index does not require further experimentation in protein identification experiments, it is only suggested to be used routinely in the reporting of proteomic results (Krokhin et al. 2004; Ishihama et al. 2005; Shinoda et al. 2010).

We functionally annotated buckwheat proteins into 15, 15, and 18 categories for molecular functions, cellular components, and biological processes, respectively, based on protein-encoded gene (GO slim) using *i*ProClass databases (Huang et al. 2003). The most abundant proteins were found in metabolic processes. For example, 12-oxophytodienoate reductase 1 (OPR1) specifically cleaves olefinic bonds in α,ß-unsaturated carbonyls. It may be involved in the detoxification or modification of those reactive compounds as well as the biosynthesis or metabolism of oxylipin-signaling molecules that produce jasmonic acid (Schaller and Weiler 1997). OPR1 is expressed in the roots, leaves, shoots, and flowers and even more abundantly in the cotyledons (Biesgen and Weiler 1999). Not only is it responsive to abiotic and biotic stresses from wounding or cadmium ions but it is also induced by cold and heat, senescence, or exposure to salicylate, jasmonate, and UV-A, B, and C (He and Gan 2001).

Glyceraldehyde-3-phosphate dehydrogenase (GAPC) gives rise to a cytoplasmic protein that is involved in carbohydrate degradation in the glycolysis pathway; it also participates in photosynthesis within the chloroplast (Martin et al. 1989). Adenosylhomocysteinase (SAHH), active in amino acid biosynthesis of one-carbon metabolism, is a competitive inhibitor of *S*-adenosyl-L-methionine-dependent methyl transferase reactions. Therefore, it may play a key role in controlling methylation by regulating the intracellular concentration of adenosylhomocysteine (Preisig-Mueller et al. 1995). Granule-bound starch synthase 1 (WAXY), found in the amyloplast and chloroplast, has an important role in starch synthesis and amylose in the endosperm (Wang et al. 1994a, b). Granule-bound starch synthase I is responsible for forming the extra-long chains in amylopectins, the major type α-glucans in starch (Hanashiro et al. 2008). Starch has two types of glucan polymers: amylose, which has predominantly linear α-1,4-linked glucan, and amylopectin, whose α-1,4-linked chains are branched extensively with 1,6-linkages. Starch branching enzyme, soluble starch synthase, and starch debranching enzyme are responsible for the synthesis of amylopectin (Ball et al. 1996), whereas amylose synthesis is catalyzed by granule-bound starch synthase (Preiss 1991). Silva et al. (1998) have also demonstrated that *O. catharinensis* seeds present a similar protein and carbohydrate concentration, and these proteins also bound to starch granules in various tissues from immature seeds of wheat (Fujita and Taira 1998). NADP-dependent malic enzyme (MOD1) is another cytoplasmic protein that is involved in malate metabolic processes, oxidation reduction, and stress responses; it also functions in CAM (crassulacean acid metabolism) photosynthesis (Cushman 1992). Sucrose synthase (EC=2.4.1.13), which is initiated in sucrose metabolism, provides UDP-glucose and fructose for various metabolic pathways in the amyloplast (Fu and Park 1995). It is the predominant sucrose cleavage enzyme in cereal endosperm (Wang et al. 1994a, b). Sucrose synthase is also concerned in meeting the increased glycolytic demand during anaerobic and cold stress as well as in supplying UDP-glucose for cell wall biosynthesis (Martin et al. 1993), associated with vascular tissues in a number of species (Tomlinson et al. 1991), and is localized specifically in the

companion cells in maize leaves and citrus fruits (Nolte and Koch 1993).

We also revealed globulin seed storage proteins such as 11S1, 13S1, 13S2, and 13S3. The embryo, which acts as a nutrient reservoir for buckwheat grains, contained 11S3, 13S1, and 13S2 (Tai et al. 1999; Fujino et al. 2001). Protein 13S3, also detected in the endosperm, has been shown to be directly responsive to allergen-related diseases (Nair et al. 1999; Nair and Adachi 2002). In fact, Licen and Kreft (2007) have reported that no proteins from that endosperm induce symptoms of buckwheat allergy. Interestingly, we identified 13S globulin seed storage protein 3 both of endosperm and embryo, which is directly related to allergen. The identification of a storage globulin in the seed at this early developmental stage prior to the accumulation of storage globulins in the seeds suggests a role for seeds in intermediate amino acid storage in buckwheat (Rout et al. 1997) and *Lotus japonicus* (Nautrup-Pedersen et al. 2010).

We identified heat shock 70-kDa protein (HSP70); heat shock cognate 70-kDa proteins 1, 2, and 3; heat shock protein 101; and chaperonin CPN60-like 1. HSP70s are a major class of chaperones involved in protein folding and organelle transport. They play an important role in biotic and abiotic stress responses to cadmium ions, cold, heat, and viruses. These proteins are localized to the nucleus, nucleolus, apoplast, chloroplast, cell wall, and plasma membrane when plants undergo stress and have been implicated in pre-ribosome assembly (Wu et al. 1988; Boston et al. 1996). Genes encoding HSP70s are also induced upon viral infection as a generalized response to the accumulation of cytosolic proteins, similar to the stress-induced accumulation of unfolded proteins (Aparicio et al. 2005).

In here, some identified proteins are involved in the transport of molecules, ions, or electrons across cell membranes, within cells, or in tissue fluid. For instance, a plastid protein (in the chloroplast) is a likely sulfate/thiosulfate import ATP-binding protein cysA, which is part of the ABC transporter complex for sulfate/thiosulfate import as well as functioning in energy coupling to the transport system (Ohyama et al. 1986). The glutamate receptor 2.2 (GLR2.2) is an ion channel ligand that acts in light signal transduction and calcium homeostasis by regulating calcium influx into cells (Chiu et al. 2002). ATP synthase subunit beta, in the mitochondria, produces ATP from ADP in the presence of a proton gradient across the membrane. This is generated by electron transport complexes in the respiratory chain. F-type ATPases consist of two structural domains: F_1—containing the extra membranous catalytic core—and F_0—containing the membrane proton channel. These are linked by a central stalk and a peripheral stalk. During catalysis, ATP synthesis in the catalytic domain of F_1 is coupled via a rotary mechanism in the central stalk subunits for proton translocation. Subunits alpha and beta form the catalytic core in F_1. Rotation of the central stalk against the surrounding $\alpha_3ß_3$ subunits leads to hydrolysis of ATP at three separate catalytic sites on the beta subunits (Winning et al. 1990).

With transcription proteins such as DNA-directed RNA polymerase II subunit RPB3-A, DNA-directed RNA polymerase subunit beta, carbon catabolite repressor protein 4 homolog 1, and two-component response regulator-like PRR37, genetic information is transferred from DNA to mRNA by DNA-directed RNA polymerase, which catalyzes the transcription of DNA into RNA using four ribonucleoside triphosphates as substrates, plus synthesized mRNA precursors and many functional non-coding RNAs. Polymerase II is the central component of the basal RNA polymerase II transcription machinery (Yamada et al. 2003; Murakami et al. 2005). Here, we identified translational proteins such as elongation factor 1-alpha (Suhandono et al. 2001) and chloroplastic 30S ribosomal protein S8 (Ibrahim et al. 2006), which are involved in the biosynthesis of proteins from mRNA molecules. Other translational proteins, e.g., elongation factor 1 in the cytoplasm and 30S ribosomal S8 in the chloroplast, are essential for protein synthesis, promoting the GTP-dependent binding of aminoacyl tRNA to ribosomes during the elongation phase of translation and participating in the proofreading of codon–anticodon accuracy (Song et al. 1989). EF-I α is one of the important proteins, which is directly required for synthesizing of all cellular proteins, and at least one active encoding EF-I α gene is necessary for cell viability (Cottrelle et al. 1985).

Histone protein is a core component of the nucleosome. This organ wraps and compacts DNA into chromatin, limiting DNA accessibility to the cellular machineries that require DNA as a template and also playing a central role in transcription regulation, DNA repair and replication, and chromosomal stability. It is induced by wounding and a methyl jasmonate elicitor, and is initiated in the anthers, floral buds, pollen, petals, and fruits (Kim et al. 1998).

Conclusion

We have characterized the enriched seed storage proteins of common buckwheat. By separating grains into their embryo and endosperm components, we were able to identify 67 proteins, using LC-ESI-Q/TOF-MS/MS to examine metabolism in those grains. Out of the total identified proteins, 20 proteins were identified in the endosperm, 29 in the embryo, and 18 were shared in both. We accomplished that 11S globulin seed storage protein is in embryo, whereas 13S globulin seed storage protein subunits are in both of embryo and endosperm, which is responsible for

allergen. In addition to the knowledge gained through functional annotation, these studies provided fruitful findings for understanding seed physiology and quality in the protein level of common buckwheat.

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