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Proteomic Approach of the Protein Profiles during Seed Maturation in Common Buckwheat (*Fagopyrum esculentum* Moench.)

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Abstract - Single seeds of common buckwheat cultivar Suwon No. 1 when subjected to SDS-PAGE revealed very high polymorphism. High variation existed for protein or protein subunits with molecular weight 54-47kDa, 45-25kDa and 16-11kDa. The electrophoregram showed variation for globulin as well as other protein fractions. About 300 proteins were separated by two-dimensional electrophoresis in common buckwheat (*Fagopyrum esculentum* Moench.) seed. Seed maturation is a dynamic and temporally regulated phase of seed development that determines the composition of storage proteins reserves in mature seeds. Buckwheat seeds from 5, 10, 15, 20, and 25 days after pollination and matured stage were used for the analysis. This led to the establishment of high-resolution proteome reference maps, expression profiles of 48 spots. It was identified 48 proteins from MALDI-TOF/MS analysis of wild buckwheat seed storage proteins. The 48 proteins were found identical or similar to those of proteins reported in buckwheat and other plants; it is belonging to 9 major functional categories including seed storage proteins, stress/defense response, protein synthesis, photosynthesis, allergy proteins, amino acid, enzyme, metabolism, and miscellaneous. It appears that the major allergenic storage protein separated played the important role in buckwheat breeding and biochemical characterization.

Key words - common buckwheat, seed storage protein, proteomics, MALDI-TOF/MS

Introduction

Buckwheat (*Fagopyrum* sp.) belongs to the family Polygonaceae and is believed to have originated from China. *Fagopyrum* has distributed in different parts of the world near about 15 species (Tahir and Farook, 1988). Common buckwheat is essentially self-infertile and posses mainly two types of flowers; pin and thrum. The pin type has long style and short stamens, the thrum type has long stamens and short style. Other irregular and intermediate forms are also visible. Due to these complexities, the seed storage proteins and other constituents show high polymorphism. Electrophoretic studies have been attempted by different authors to study protein polymorphism (Shevchuk, 1986; Nishiyama et al, 1991). However, Shevchuk (1994) has been able to attribute cultivar identification despite the protein polymorphism.

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Proteome analysis is a systematic functional analysis of the genes uncovered by genome sequences (Pandy and Mann, 2000). In this analysis, a number of proteins are mainly separated by two-dimensional electrophoresis (Woo et al., 2002). Based on the peptide-mass fingerprint obtained by mass-spectrometry (MS) and partial amino acid sequence by MS or gas-phase sequencing, then the proteins are identified using protein database, and simultaneously the genes were encoded them to identified in the gene library available in the genome project (Velculescu et al., 1995; Williams and Hochstrasser 1997). The proteins without the known function are characterized in amount, localization, structure, post-translational modification, enzyme activity, protein-protein interaction and so on. The functions of a number of proteins and genes are determined based on the data obtained from both protein and gene analyses.In order to know various biological functions, it is essential to analyze the proteome of

common buckwheat (*Fagopyrum esculentum* Moench.), one of the important food-crops in Asia.

Recently, a number of plant proteome studies have been published, some of them more descriptive, providing an overview of proteins present in a given tissue, organelle, or stage of development (Kristoffersen and Flengsrud, 2000; Skylas et al., 2000; haebel and Kehr 2001; Schiltz, 2004), whereas some have been more directed, using the techniques of proteome analysis to address a specific biological question such as the role of jasmonate in defense signaling (Rakwal and Komatsu, 2000) or to identify proteins phosphorylated in response to bacterial or fungal elicitors (Peck et al., 2001). The plant species studied include barley, maize (*Zea mays*), and rice (*Oryza sativa*), as well as the model plant *Arabidopsis*. In fact, two-dimensional electrophoresis techniques were established early on for buckwheat (Woo et al., 2001; Woo et al., 2006).Two-dimensional gel analysis has also been used previously to analyze differences in common buckwheat and self-pollinating buckwheat cultivars (Woo et al., 2006), however, these analyses focused mainly on the globulins, which are the major storage proteins in buckwheat. Identification of proteins from two-dimensional gels using mass spectrometry (MS) relies on sequence information in the databases. This is clearly a limitation for buckwheat, for which the genome has not been sequenced, although closely related proteins can often be identified from the corresponding rice sequence. Despite the limited amount of buckwheat sequence information available, we decided to use two-dimensional electrophoresis and MS techniques to conduct a time-resolved analysis of the seed development process in buckwheat, with emphasis on the water-soluble protein fraction. By observing the way in which different proteins show changing patterns of appearance, a clearer overview is obtained of the events of seed development than by a comparison of specific developmental stages.

In the present study, as the first step of the proteome analysis, we separated the buckwheat seed proteins by 2-DE. Also, we present a detailed account of protein subunit variation in common buckwheat (*Fagopyrum esculentum* Moench.) through the analysis of single seed protein. Finally, we defined and characterized the expressed proteome of the grain filling and different developmental stages of seed maturation in buckwheat, in order to use the knowledge of proteins are expressed to gain insights into the mechanisms of seed maturation.

Materials and Methods

Growing Conditions

Common buckwheat (*F. esculentum* Moench.) plants were grown in a climate-controlled greenhouse that had an average daily maximum day temperature at 24℃ and night temperature at 17℃. Water and fertilizer were supplied by drip irrigation. In this study materials used in this investigation cultivated species, self-incompatible, common buckwheat (*Fagopyrum esculentum* Moench. 2n=16). Common buckwheat is a short-styled (thrum-type) and long-styled (pin-type) plants are found in approximately equal frequencies in a population plants were grown in pots in a green house conditions. The whole influrescence was bagged and flowers were anthesis. Hand-pollination was conducted by rubbing the anther against the fresh stigma of the female parent under pollinator-free conditions between *F. esculentum* (pin) and *F. esculentum* (thrum) in the morning when flowers were in full blossom. Grain was harvested from developing heads at 5, 10, 15, 20, and 25 days (post anthesis). Samples were stored at －80℃ until needed.

SDS-PAGE analysis

Electrophoresis was carried out on an SDS-PAGE (sodium dodecyl sulphate-poly acrylamide gel electrophoresis) discontinuous system as described by Laemmli (1970) with few modifications. The proteins were separated on 12% acrylamide (30% T, 2.5% C) and 10% SDS. The electrophoretic buffer contained 0.025M Tris, 0.192M glycine, pH 8.3, 0.1% w/v SDS. The electrophoresis was carried out for about 3h at constant current of 25 mA. The electrophoresis system used Nihon aido, Japan. The gels were stained in 0.25% Coomassie Brilliant Blue R-250 in methanol : water : acetic acid (4:5:1) for 10mts, and then destained using methanol 25% and acetic acid 7.5%. The gels were dried on a gel drying processor (Atto, Japan) at constant temperature of 60℃ for 3h. The molecular weights of sample proteins were calculated from the calibration curve by plotting the R_f values (distance of protein migration/distance of tracking dye migration) against known molecular weights on semi-logarithmic paper. The standard markers were obtained from

Sigma Chemical Company, USA.

Two-dimensional gel electrophoresis (2-DE)

Soluble proteins of whole seed storage were examined by two-dimensional gel electrophoresis according to the protocol of O'Farrell (1975). The resultant supernatant (50 μl) was subjected to 2-DE (Hirano 1982). The isoelectric-focusing (IEF) gel solution contained 48.6g urea, 2% ampholytes (pH 3.5-10), 11.8 ml acrylamide/bis solution (29.2%(w/v) acrylamide, 0.8% (w/v) N'-bis-methylene-acrylamide), 20.3ml 10%(v/v) Triton X-100, 4.5 ml Bio-Lyte 5/7, 0.5 ml Bio-Lyte 3/10 and 28.8 ml distilled water. For preparation of the IEF gel, 1.0 ml IEF gel solution was degassed for 15 min, and 1 μl of N, N, N', N'-tetramethylethylenediamine and 1.3 μl of freshly prepared ammonium persulfate were added. Then, the gel solution was loaded into the gel tube (3.5 mm Ø X 13~27 cm). Solid urea was added to the protein sample (100 to 150 μg protein in about 30 μl) until saturated. To a 5 μl sample was added 1 μl IEF solution containing 0.1 ml 10% (w/v) SDS, 0.02 ml Bio-Lyte 3/10, 0.1 ml 2-mercaptoethanol, and 0.2 ml Triton X-100. The lower and upper reservoirs were filled with 0.1N NaOH and 0.06% phosphoric acid, respectively. After the sample solution was loaded, the gels were run at 200 V for 1 h, 400 V for 30 min and 600V for 16 h, respectively. Then the gels were removed from the gel tubes, and equilibrated in 10 ml of reducing SDS buffer (62.5 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, and 0.0125 %(w/v) bromophenol blue by shaking for 30 min at room temperature. The second dimension of gel electrophoresis was carried out using a separation gel of 15% acrylamide and a stacking gel of 5% acrylamide according to the procedure of Laemmli (1970). The gels were run with a constant current at 15mM/gel and 4℃ until the BPB reached the bottom of the gel. Preparative gels were stained with Silver staining. The isoelectric point and relative molecular weight of each protein were determinated using molecular weight marker protein kits (Pharmacia LKB, Uppsala, Sweden). The new instrument used in this study is simple and allows to easily separate with IEF tube gels in adoption 27cm length. Using this instrument, the highly reproducible IEF can be formed in polyacrilamide gels. Actually, seed storage proteins were separate by 2-DE in the first dimension using IEF tube gels and detected by silver staining.

Image analysis of the electrophoresis patterns

Following staining, the 2-DE gel patterns were scanned using a flatbed scanner, and analyzed using PDQuest software version 6.2 (Bio-Rad discovery series, Bio-Rad Laboratories, USA). After scanning, spots in gels were detected using same parameters and quantified by 2-dimensional Gaussian modelling. Gels were normalized and data were exported to Ex-cell (Microsoft). A specific spot present in all the gels was selected as internal standard and the intensity of all other spots was expressed as the ratio of the internal standard (Islam et al., 2002).

In-gel digestion

Individual spots were excised from the gel and destained by washing twice with 200 μl of acetonitrile/water 1:1 for 15 min. The gels were washed with acetonitrile and subjected to complete dryness using a centrifugal vacuum concentrator. Using iodeacetamide, a reduction and alkylation process of cysteine residues was performed on several samples before adding trypsin solution. Each spot was rehydrated in 10 mM dithiothreitol (DTT) in 100mM ammonium bicarbonate for 30 min at 56℃ The supernatant was removed and the gels were washed with acetonitrile, and dried completely with a vacuum centrifugation. After drying, the gels were incubated in darkness for 1 hr with 55 mM iodoacetamide in 100 mM ammonium bicarbonate. The supernatant was removed, and the gels were washed with 100mM ammonium bicarbonate followed by washing with acetonitrile and evaporating to complete dryness. For tryptic digestion, the dried gel was rehydrated in 12.5 ng/μl trypsin (Boehringer Mannheim, Germany, sequencing grade) solution containing 50mM ammonium bicarbonate and 5mM $CaCl_2$. After 20min of incubation, excess trypsin solution was removed, and the digestion buffer containing 50mM ammonium bicarbonate was added. The gel pieces were then incubated at 37℃ for 15 hr.

MALDI-TOF/MS analysis

The improved Cleveland peptide mapping/sequencing was compared in efficiency of identification of proteins to the peptide mass fingerprinting by MALDI-TOF/MS (TofSpec II, Micromass, Manchester, UK). In MALDI-TOF/MS analysis,

proteins separated by 2-DE were digested in gels according to the method described by Fukuda et al. 2003. The digests were desalted with ZipTip (Millipore, Boston) and subjected to the analysis by MALDI-TOF/MS. Based on the peptide mass fingerprints obtained; the proteins homologous to each protein which were compiled in the SWISS-PROT database were searched using ProteinLynx software (Micromass).

All MS/MS experiments for peptide sequencing were conducted using a nano-LC/MS system, consisting of an Ultimate HPLC system (LC Packings, Netherlands) and a QTOF2 mass spectrometer (Micromass, UK) equipped with a nano-ESI source. Each of the samples, corresponding 1 to 10, was loaded with an autosampler (FAMOS, LC Packings, Netherlands) onto a C18 trap column (inner diameter 300 mm, length 150 mm, particle size 5 μm; LC Packings) for desalting and concentration at a flow rate of 20 μl/min. The trapped peptides were then back-flushed and separated on a C18 nano-column (i.d. 7.5 mm, length 150 mm, particle size 5 μm; LC Packings). The gradient used was as follows: 0% acetonitrile for 10 minutes, followed by 0−50% acetonitrile for 80 minutes, and 50% acetonitrile for 10 minutes, at a flow rate of 150 nl/min. In the nano-ESI source, the end of the capillary tubing from the nano-LC column was connected to pico-tip silica tubing (i.d. 8 (m; New Objectives, USA). A 2-2.5 kV voltage was applied to the liquid junction to generate an electrospray, and the cone voltage was 30 V. Argon was introduced as a collision gas at a pressure of 10 psi. The MS/MS spectra were acquired in data-dependent MS/MS mode, in which the collision energy was increased stepwise to 25, 30, and 35 eV. For protein identification, the MS/MS spectra were analyzed via the MASCOT program (Matrix Science, www.matrixscience.com, UK) in the green plant protein database downloaded from NCBI (Perkins et al., 1999). Using the MASCOT program, the matched peptide list with a significant level over the observed peptide match can be a random sequence with the probability of less than 5%, which were selected from database search for protein identification. MS/MS spectra with a Mascot score higher than the significant score, using a confidence interval greater than 95%, were assumed to be correct calls. When more than one peptide sequence was assigned to a spectrum with a significant score, the spectra were examined manually. The cases where the top-scoring peptide sequences had equal score were discarded.

Results and Discussion

The proteins from single seeds revealed distinct variation among the total seeds investigated. The differences in banding pattern can be characterized into three areas on the electrophoregram (1a-4a - 54-47kDa); (1b-7b - 45-24kDa); (1c-3c - 16-11kDa) - Fig. 1. A variation of 29.68% was found among the protein subunits between 47 to 54kDa. While the 45kDa showed variation among 32.5% of the total seeds tested, 17.8% showed variation for 40-42kDa protein subunits. The protein subunits of 38kDa and 32kDa showed variation to the extent of 23.43%. The 28kDa protein subunit showed variation of 11.25%, while the protein subunit 24kDa was not present only in 8 seeds out of 160 seeds tested. The 16kDa and 13kDa protein subunits showed 6.25% variation and the 11kDa protein

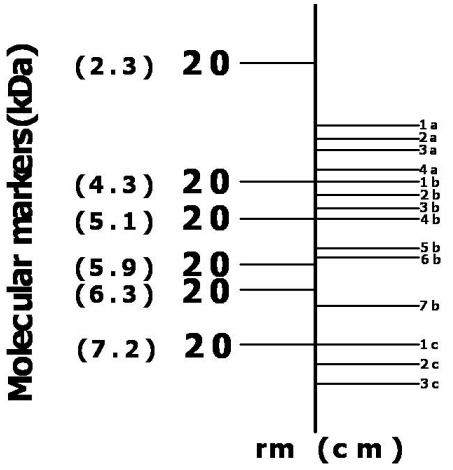

Fig. 1. Schematic presentation of the protein bands which shows polymorphism as by single seeds of buckwheat (*Fagopyrum esculentum* Moench.) rm=relative mobility. The values in parantheses represents rm values

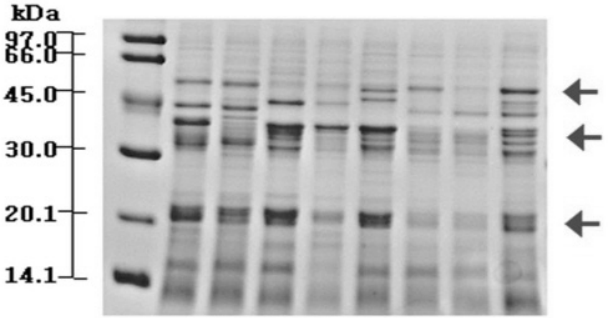

Fig. 2. SDS-PAGE of buckwheat water soluble common buckwheat seed storage protein. Arrowheads show polymorphism.

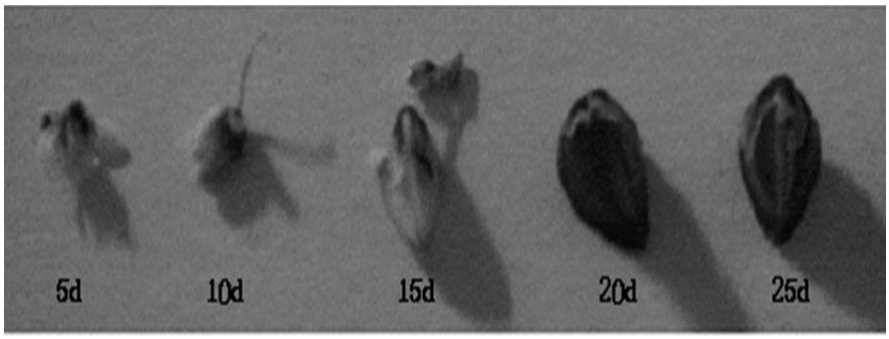

Fig. 3. Development of buckwheat seeds during the experimental period. Whole seed at the five stages of seed maturation. Experimental sampling began exactly at 5 days after pollination and continued at precisely 5 day intervals until 25 days after pollination.

subunit showed 13.12% variation (Fig. 2). In our attempt to find single seeds without the 24 kDa subunit, interesting results were obtained on the spectrum of variation among the seeds of common buckwheat cultivar Suwon No. 1. The 24 kDa subunit was reported as allergic protein subunit by Urisu et al, 1995. The intravarietal heterogeneity for different subunits wa s also reported by Svetek, 1994. The major class of buckwheat protein was reported as 13s globulin by Svetlana et al, 1996.

The 13s globulin on SDS-PAGE revealed protein subunits from 43 to 68 kDa and another fraction between 14 to 23 kDa. The maximum polymorphism was revealed among these globulin subunits. Variation among 11 to 16 kDa indicates that polymorphism also exists amongest other protein fractions of buckwheat.

The primary objective of this study was to characterize global protein expression during the seed maturation stage of buckwheat seed development. For the best coverage of this period, whole seeds were analyzed at precisely 5, 10, 15, 20, and 25 days after pollination. The characteristics of the developing seeds used in this experiment (Fig. 3). Whole seed proteins from stages developing buckwheat seed were resolved and detected using high-resolution two-dimensional electrophoresis followed by Silver staining. Initial analyses were performed with isoelectric-focusing (IEF) that ranged from pH 3.5 to 10 (Fig. 4). The progress of buckwheat cv Suwon 1. seed development was accompanied by a change in the pattern of soluble proteins as visualized by two-dimensional gel electrophoresis (Fig. 4). Approximately 300 well-defined spots could be resolved on the stage 20 gel, and about 300 spots could be resolved on the stage 25 gel in the *pI* 4 to 7 region. Conventional standard size gel are capable of separating about 250 to 300 protein spots (Woo et al., 2001).

Approximately 50 μl of protein extracts were prepared from 0.02 g of buckwheat seed. Each 2 g of protein extracts were separated by 2-DE using pH 3.5 -10 IEF and 12% polyacrylamide SDS gel. After 2-DE and silver staining, approximately 100 proteins were detected on the 2-D gels and 2-D gel images of buckwheat seeds from five stages were comparatively analyzed (Fig. 4). Especially, protein variations from five regions on the 2-D gel were noticeable. The 48 proteins were found identical or similar to those of proteins reported in buckwheat and other plants; the proteins included granule bound starch synthase (gi56407503), seed oleosin isoform 1 (gi9858857), major allergenic storage protein (two spots, gi4895075), protein R=thaumatin homologue (gi237804), ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (gi4038721), hypothetical protein (gi6723746, gi50253135), cytochrome (gi117988), and unknown protein (gi50940739, gi30678420) (Table 1). Therefore, it is belonging to 9 major functional categories including seed storage proteins, stress/defense response, protein synthesis, photosynthesis, allergy proteins, amino acid, enzyme, metabolism, and miscellaneous (Fig. 5).

The protein accumulation in seeds of common buckwheat was found to start from 10 days after pollination (DAP). Up to 15 DAP, the major band was that of 69 kDa protein. The seeds on maturing showed a shift in banding pattern with high intensity bands at 56-57 kDa, 32-34 kDa and 23 kDa. As the maximum and most consistent response was found from the 22 kDa protein band, this band was electro eluted and subjected to N-terminal micro sequencing. The extracted 22 kDa protein on SDS-PAGE gave a single band. The N-terminal amino acid

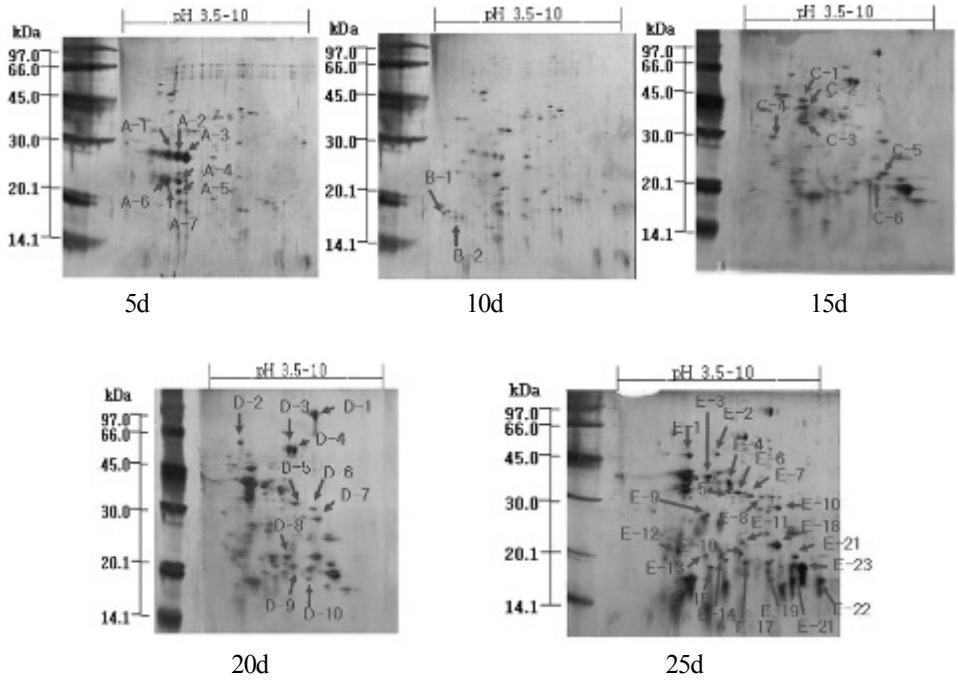

Fig. 4. Analysis of proteins isolated from immature buckwheat seeds (*Fagopyrum esculentum* (thrum) × *Fagopyrum esculentum* (pin)) of 5. 10, 15, 20, and 25 days after pollination periods by two-dimensional gel electrophoresis (2-DE). First dimension (left to right), isoelectric focusing; second dimension (up to down), SDS-PAGE. The proteins were automatically identified on 2D-PAGE gels with PDquest based on relative molecular weights and isoelectric points after detection by Silver staining

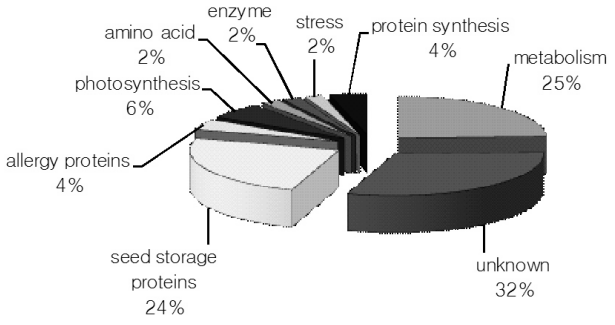

Fig. 5. Functional distribution of identified proteins expressed in common buckwheat during development seed protein.

sequence of the 22 kDa protein had significant similarity to BW24 kDa reported by Urisu et al., 1994. The results presented here lead to the proposal that patients with hypersensitivity to buckwheat flour should use only fine flour from buckwheat endosperm, as this fraction contains no or very few low M.W. seed proteins. Homology search of protein database with BLAST and SWISS-PROT showed maximum alignment with storage proteins of both dicot and monocot species. Buckwheat allergenic protein showed 60-75% identify in the alignment with 11S globulins from peanut, cashew, and soybean (Yoshioka et al., 2004). The sequence also showed similarity with proteins from leguminous and non-leguminous plants whose seed meal contains sulphur rich amino acids.

The fact that of the different proteins identified, several are involved in oxidative stress responses and nine are associated with storage protein deposition can be taken as a measure of the importance of these processes during seed development.

The use of MALDI-TOF MS and ESI MS/MS to identify proteins has enabled us to distinguish between varying forms of proteins that may have consequences for their function. We have also demonstrated the potential for identification of post-translational modification of proteins on two-dimensional gels (e.g. Rubisco small subunit). It is also possible, based on the available information in EST databases, to identify previously unknown proteins (e.g. adenylcyclase-like protein in spot E-12). We have identified variations in spot pattern in alpha subunit of beta-conglycinin / Alpha-glucan phosphorylase that apparently do not arise directly due to differences in gene expression or obvious post-translational modification but by a mechanism affecting the accessibility or extract ability of the proteins.

Table 1. Identiified of protein spots from two-dimensional gel analysis of development after pollination proteins(see Figure 5).

Spot no.	p*I*value	Mr	Functions	Sequence	G.I.
A-1	8.83	18533	ribulose-1,5-bisphosphate carboxylase/ oxygenase small subunit	R.SSASLGSVSNGGR.I	gi4038721
A-2	11.2	10619	hypothetical protein	R.IELERPSQIANTNLLR.I	gi6723746
A-3	6.56	41800	unknown	R.AVCTAWRAAIPAASPSLLVR.L	gi50940739
A-4	5.67	130825	unknown	K.KLAFDLIR.S	gi30678420
A-5	9.85	11890	Cytochrome	K.TKCAQCHTVEK.G	gi117989
A-6	6.63	109065	Os11g0258500	R.QCNNSFKGVNEEILTMK.L	gi115484993
A-7	6.63	109065	Beta-conglycinin, alpha chain precursorprsor	R.VPSGTTYYVVNPDNNENLR.L	gi121281
B-1	7.88	96088	putative chloroplast RNA processing protein	R.RMTELGCIPDVFSYNNLLK.G	gi22128712
B-2	8.04	38128	ATPG_CHLREATP synthase gamma(ISS)	K.KNLSLVYNR.A	gi116059564
C-1	5.68	64479	major allergenic storage protein	R.NFFLAGQSK.Q	gi4895075
C-2	10.60	61127	major allerge nicstorage protein	R.LTASEPSR.R	gi4895075
C-3	8.82	4450	protein R=thaumatin homologue	-.YESPRPTLGIHR.F	gi237804
C-4	6.15	55472	unnamed protein product	R.FYLAGNQEQEFLK.Y	Gi18615
C-5	5.51	50301	13S globulin	R.FRHNLGPR.T	gi30144561
C-6	10.85	43710	OSJNBa0019K04.17	R.REGVVAEPR.R	Gi38605956
D-1	6.95	128065	structura lmaintenance of chromosomes 1 protein(ISS)	K.CVSLDGTLVDK.S	gi116056735
D-2	9.07	29584	kinase	R.KGSNPRPLEASLVQR.F	gi42569741
D-3	4.76	6529	hypothetical protein	R.DDAREDAGDGSGDK.R	gi50253135
D-4	5.92	15941	granule bound starch synthase	K.YTDVKYDITTVMDAKPLLK.E	gi56407503
D-5	6.18	163076	PWWP domain containing protein	K.TTDCLLVSSEVGNDDCGK.G	gi108708256
D-6	5.07	70250	Beta-conglycinin	R.QFPFPRPPHQK.E	gi121281
D-7	5.89	24315	1-Cys peroxiredoxin	K.LSFLYPATTGR.N	gi6466096
D-8	8.29	42823	unnamed protein product	R.DYLLLPR.E	gi8885597
D-9	12.11	6420	hypothetical protein	M.SSLPPLPASPSMTR.H	gi54290237
D-10	4.92	63127	alpha subunit of beta conglycinin	R.VPSGTTYYVVNPDNNENLR.L	gi9967357
E-1	5.68	64479	13S globulin seed storage protein 1 precursor	R.VVIQPGGLLLPSYSNAPYITFVEQGR.G	gi29839254
E-2	6.00	61127	13S globulin seed storage protein 3 precursor	R.SEAGVTEIWDHDTPEFR.C	gi29839419
E-3	5.21	25786	induced immature seed protein	K.VGSGQTTEHGYNTSGVGSGDMGDFGR.R	gi2317672
E-4	8.88	38131	hypothetical protein MtrDRAFT_AC140774g15v1	R.LEGWFLGNVSRFLGDGR.N	gi92870362
E-5	5.78	55657	Glycinin A1aBx	-.MAKLVFSLCFLLFSGCCFAFSSR.E	gi225651
E-6	4.59	131056	putative GTP-binding protein	R.CLDPKPNFLVR.A	gi40714690
E-7	6.15	55472	unnamed protein product	R.RPSYTNGPQEIYIQQGK.G	gi18615
E-8	9.92	25421	hypothetical protein	R.CRPSSSLPLEVMRTLAR.Q	gi53793334
E-9	10.34	11366	Os01g0299400	R.LLAGAGTPAR.S	gi115436110
E-10	5.89	24315	1-Cys peroxiredoxin	K.LSFLYPATTGR.N	gi6466096
E-11	9.8	10262	hypothetical protein LOC_Os11g16720	R.AHCGGGDGKDGGSGHHGAR.G	gi62734345

E-12	11.82	46066	adenyl cyclase-like protein	R.ALRADGPAR.A	gi23617189
E-13	5.38	47208	Transferase	M.EGSPVTSVR.L	gi15233851
E-14	5.42	137858	OSJNBa0053K19.19	-.MAHSPLCSR.S	gi38344180
E-15	9.52	15635	Maturase	K.NPSEAFLTKR.N	gi56675672
E-16	6.13	133301	kinesin motor protein-related	K.RMQMKPSPR.Q	gi15218418
E-17	7.06	175551	protein synthesis initiation factor-like	K.CQEEFER.G	gi7576200
E-18	5.51	50301	13S globulin	K.LPILEFIDMSAEK.G	gi30144561
E-19	8.94	36382	putative pod-specific dehydrogenase SAC25 (ISS)	R.VCEFADSFIASARNR.S	gi116057865
E-20	6.29	66949	Convicilin precursor	R.SDLFENLQNYR.L	gi117655
E-21	5.58	15250	class I heat shock protein	K.VEVEEGRILQISGER.R	gi32401095
E-22	5.64	95864	Alpha-glucan phosphorylase, H isozyme	K.WSQPVMWKLLPR.H	gi1730560
E-23	0.61	18233	seed oleosin isoform 1	K.DHTSQAQQHGQQALSNMAGYLQEK.T	gi9858857

In conclusion, the techniques of two-dimensional gel electrophoresis and protein identification by MS can be used to visualize and describe the complex metabolic processes occurring during seed development. Here, we have concentrated on the proteins within the *pI* range 4 to 7. Addition of the high-*pI* proteins will provide a more comprehensive analysis.

Our results will be useful for the future examination of the complex nature of protein expression and seed maturation in buckwheat. It appears that the major allergenic storage protein separated played the important role in buckwheat breeding and biochemical characterization.

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