JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Identification and Roles of Proteins for Seed Development in Mungbean (*Vigna radiata* L.) Seed Proteomes

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Supporting Information

ABSTRACT: Proteomic analysis of developing mungbean (*Vigna radiata* L.) seeds has not yet been investigated in detail. Fifty-seven proteins were separated by 2-DE, identified by nanoelectrospray mass spectrometry from the present protein databases, and categorized according to their functions. Many of the identified enzymes were involved in central carbon metabolism; thus, a pathway illustrating starch synthesis/breakdown, sugar conversion for glycolysis, and tricarboxylic acid (TCA) cycle was proposed. Quantitative comparison of the protein expression revealed that during developmental process (11−21 days after flowering, DAF), proteins involved in glycolysis, TCA cycle, and alcoholic fermentation showed a trend to be down-regulated, whereas storage proteins were generally up-regulated. The downward tendency of central carbon metabolic proteins suggests a reduction in ATP and oxygen consumption associated with accumulation of storage compounds. UDP-glucose-1-pyrophosphorylase, an upstream enzyme in the starch ADP-Glc pathway, was found as a stably expressed protein throughout the growth stage, demonstrating its importance in mungbean starch biosynthesis. The temporal expression of metabolic enzymes suggests the coordination of an acclimation mechanism and cellular processes associated with accumulation of storage compounds in seed development.

KEYWORDS: *mungbean seed development, Vigna radiata L., metabolism, storage protein, proteomic, nanoelectrospray mass spectrometry*

INTRODUCTION

Fabaceae (Leguminosae) are important crops and the staple food for part of the world's population. Seeds of mungbean (*Vigna radiata* L.), a grain legume, are rich in protein sources for human nutrition and are widely cultivated in South and Southeast Asia as well as dry regions of southern Europe and the southern United States. Because of its relatively small genome and the accessibility to a large number of legume-expressed sequences within public databases, mungbean is also a suitable model for analysis in protein interactions regulating seed development, which made proteomic research on mungbean much more practical.[1]

Legume development has been described in many studies.[2] The fertilized egg undergoes cell division, producing two daughter cells, one of which develops into a highly specialized suspensor tissue, whereas the other cell forms the embryo. The first division of the zygote initiates the development of the embryo. Embryogenesis is a complex process that requires regulation of cell-specific and housekeeping genes within the embryo and neighboring seed tissues (e.g., endosperm) surrounding the embryo. For legumes, embryogenesis then is divided into three main phases.[3] In the first or cell division phase, the cotyledon cells actively divide. In the second or maturation phase, the embryo stops growing, mitosis is less intense, storage compounds start to accumulate, and the seed develops tolerance to desiccation (third phase). The transition between early and late seed development is accomplished by gene expression in large-scale patterns and metabolic changes.

The accumulation of reserve compounds in the seeds is meant to feed the embryo during development and guarantee seed germination. Although seeds from different species are diverse in form, they have one common characteristic during development: accumulation of reserves. Legume seeds usually contain 15.5−47.0% protein, 0.6−21% oil, and 26−78% carbohydrates.[4,5] Mungbean seeds contain 22.9−23.6% protein, 1.2% oil, and 58.2−61.8% carbohydrates. Starch is the major storage carbohydrate for mungbean seeds; however, it also contains verbascose, stachyose, rafinose, sucrose, and fibers (this category includes other soluble and insoluble carbohydrates).[4] The reserved materials are not only essential for postembryonic growth and development by nourishing germinated embryos before the seedlings start photosynthesis but also make seeds an important food source for humans and livestock. Therefore, the mechanism describing the protein interactions regulating seed development has become significant for the molecular control of yield and quality of crops; however, this mechanism remains largely unknown.[6,7]

Received: March 15, 2013
Revised: June 7, 2013
Accepted: June 11, 2013
Published: June 11, 2013

In an effort to understand the molecular regulation and metabolic processes as well as synthesis and accumulation of the reserve materials during seed development, we analyzed the protein expression profiles in mungbean (*V. radiata* L.) during three sequential developmental stages associated with grain filling from 11 to 21 days after flowering (DAF). These differentially expressed proteins were identified and categorized. The dynamic change of seed proteomes was used to evaluate their functional features in the developmental processes. The results presented here provide information that the down-regulation of proteins involved in glycolysis, TCA cycle, and fermentation allows decreasing the consumption of ATP and oxygen. This adaptation mechanism prevents the tissue from becoming anoxic and may be associated with the accumulation of reserves in the developing mungbean (*V. radiata* L.) seeds.

MATERIALS AND METHODS

Plant Material and Sampling. Mungbean cultivar Khampang Saen 1 (KPS1), having a high starch content of 53.5%, was selected from the germplasm library at Asia Vegetable Research and Development Center (AVRDC) in Shanhua, Taiwan. This variety is distributed worldwide under the names KPS1 in Europe, Taiwan, and Thailand, Zhong Lu 1 in mainland China, Seonhwa-nogdu in Korea, Silangan in The Philippines, and Tex-Sprout in the United States and Canada.[8] A batch of field growth was arranged during August to November. On-site labeling of mungbean samples started from 1 DAF. Mungbean pods of different developing stages were picked and stored at −80 °C for further analysis. A growth curve monitoring pod length was constructed to be used as sampling standard. The value of each point on the growth curve was the average length of 10 pods.

Extraction of Mungbean Cytosolic Proteins. Mungbeans were picked from the bean pods and pulverized in a liquid nitrogen bath using a pestle and mortar. Three individuals were mixed for proteome extraction. The extraction experiment was performed three times for each developing stage. All procedures were performed at 4 °C unless specified. The fine powder was suspended in three (w/v) ratios of homogenizing buffer [0.7 M sucrose, 0.5 M Tris, pH 7.5, 30 mM HCl, 50 mM EDTA, 0.1 M KCl, 2% β-mercaptoethanol (2-ME)] and centrifuged at 15000*g* for 30 min (all chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA). The solubilized supernatant was extracted with equal volumes of Tris-HCl (pH 7.5)-saturated phenol for 3 min by vortex, followed by centrifugation at 15000*g* for 20 min. The upper phenol phase was withdrawn and extracted twice with equal volumes of homogenizing buffer for 3 min by vortex, followed by centrifugation at 15000*g* for 10 min each time. Proteins were precipitated from the final phenol phase with 5 volumes of ice-cold methanol solution (containing 0.1 M ammonium acetate and 10 mM β-ME) by standing overnight at −20 °C and pelleted by centrifugation at 15000*g* for 20 min. The protein pellet was washed with the ice-cold methanol solution (three times) and ice-cold acetone containing 10 mM β-ME (twice). Finally, phenol-extracted soluble proteins were resuspended in 9.5 M urea containing 4% CHAPS. Protein content was quantified using a Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Bovine serum albumin was used as standard to construct calibration curves within 10 μg/mL range.[9]

2-DE and Image Analysis. A sample aliquot containing 100 μg of protein was diluted with a final volume of 350 μL of rehydration buffer (8 M urea, 4% CHAPS, 65 mM DTE, 0.5% ampholytes, and a trace of bromophenol blue), followed by hydration into an immobilized pH gradient (IPG) strip holder containing a 17 cm, pH 3−10 or pH 4−7 linear gradient-immobilized strip (ReadyStrip IPG strip, Bio-Rad) overnight. The hydrated strip was focused for a total of 60 kVh (PROTEAN IEF cell, Bio-Rad) at 20 °C and then stored at −80 °C until use. For the second-dimension analysis, the strip was first equilibrated with 3 mL of solution consisting of 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS), a trace amount of bromophenol blue, and DTE (1% w/v) for 20 min and then equilibrated for another 20 min in the same buffer containing iodoacetamide (2.5% w/v) instead of DTE. Then the strip was transferred to the top of a 12% polyacrylamide gel and held in position with molten 0.5% agarose in running buffer (25 mM Tris, 0.192 M glycine, 0.1% SDS). The gel was run at 16 mA for 30 min followed by 50 mA for 4−5 h. Three 2-DE runs were performed for each experimental condition. The gel image was analyzed by Proteomweaver software (Bio-Rad Laboratories). After spot detection, quantification, and background subtraction, the spot groups were determined. For spot profile analysis, the two-dimensional gel of 11 DAF seed samples was selected as the reference gel. All analyzed gels were matched individually to the reference gel, and matched spots from the different gels were assigned to a spot group. Normalized volume (NV) of the matched protein spots found to vary in expression between the developmental stages were analyzed for statistical significance using Student's *t* test, $P < 0.05$. Induction and repression factors (IF and RF) correspond to the ratio of NV, 14 DAF/11 DAF, 18 DAF/14 DAF, and 21 DAF/18 DAF for developmental stages 11−14, 14−18, and 18−21 DAF, respectively.

In-Gel Digestion. Each protein gel spot was cut out and placed in an eppendorf tube. The gel pieces were washed twice with 50 μL of acetonitrile (ACN) and 50 μL of 200 mM ammonium bicarbonate for 5 min, shrunk with 100% ACN, and then dried for 5 min in a speed vacuum system at room temperature. The gel pieces were rehydrated in 15 μL of 50 mM ammonium bicarbonate (37 °C, 4 min), treated with 15 μL of trypsin (Promega Corp., Madison, WI, USA) solution (20 ng/μL in 50 mM ammonium bicarbonate), and incubated at 37 °C for 4 h or at 30 °C for at least 16 h. After digestion, the mixture was sonicated, and the gel pieces were spun down to recover the peptide supernatant. The supernatants were stored at −20 °C until LC-MS/MS analysis.[10,11]

Nanoelectrospray Mass Spectrometry. Nanoscale capillary liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis was performed using an Ultimate capillary LC system (LC Packings, Amsterdam, The Netherlands) coupled to a $QSTAR_{XL}$ quadrupole-time-of-flight (Q-TOF) mass spectrometer (Applied Biosystem/MDS Sciex, Foster City, CA, USA). Nanoscale capillary LC separation was performed on a RP C18 column (15 cm × 75 μm i.d.) with a flow rate of 200 nL/min and 70 min linear gradient of 5−50% buffer B. Buffer A contained 0.1% formic acid in 2% aqueous ACN; buffer B contained 0.1% formic acid in 98% aqueous ACN.[12] The nanoLC tip for online LC-MS used was a PicoTip (FS360-20-10-D-20; New Objective, Cambridge, MA, USA). Data acquisition was performed by automatic Information Dependent Acquisition (IDA; Applied Biosystem/MDS Sciex). The IDA automatically finds the most intense ions in a TOF MS spectrum and then performs an optimized MS/MS analysis on the selected ions. The product ion spectra generated by nanoLC-MS/MS were searched against National Center for Biotechnology Information (NCBI) databases for exact matches using the MASCOT search program (http://www.matrixscience.com/search_form_select.html). A Viridiplantae (Green Plants) taxonomy restriction was used, and the mass tolerance of both precursor ions and fragment ions was set to ±0.3 Da. Carbamidomethyl cysteine was set as a fixed modification, whereas serine, threonine, or tyrosine phosphorylation was set as variable modification. All phosphopeptides identified were confirmed by manual interpretation of the spectra.

RESULTS AND DISCUSSION

Morphology and Protein Functional Categories. To obtain basic information about mungbean seed development, we observed morphological features in the developing seeds (Figure 1). The color of the seeds appeared to change slightly from 11 to 18 DAF but quickly darkened thereafter until 21 DAF (Figure 1A). The developing seeds greatly increased in size from 1 to 11 DAF and then had a slight increase and appeared to reach the size of mature seeds at 18 DAF (Figure 1B). These results suggest that developing seeds until 11 DAF mainly undergo active cell division and differentiation and likely

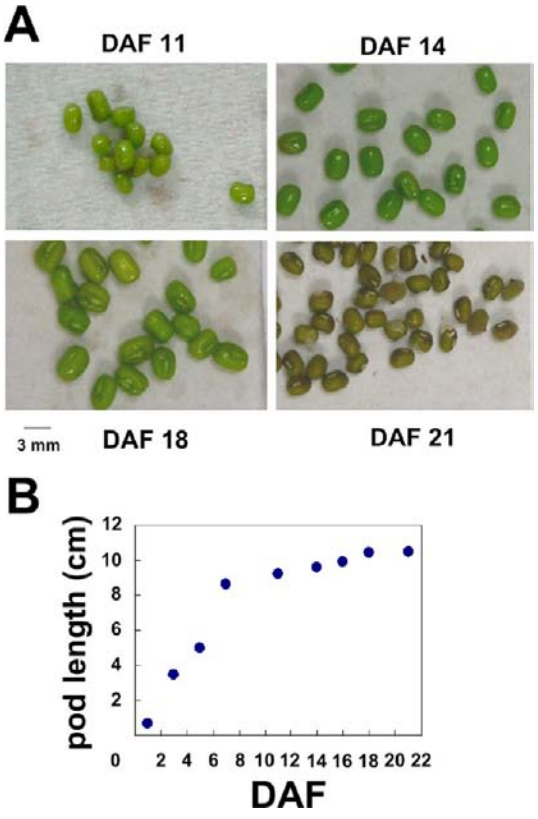

Figure 1. Mungbean seed morphology (A) and pod size (B) in different growth stages.

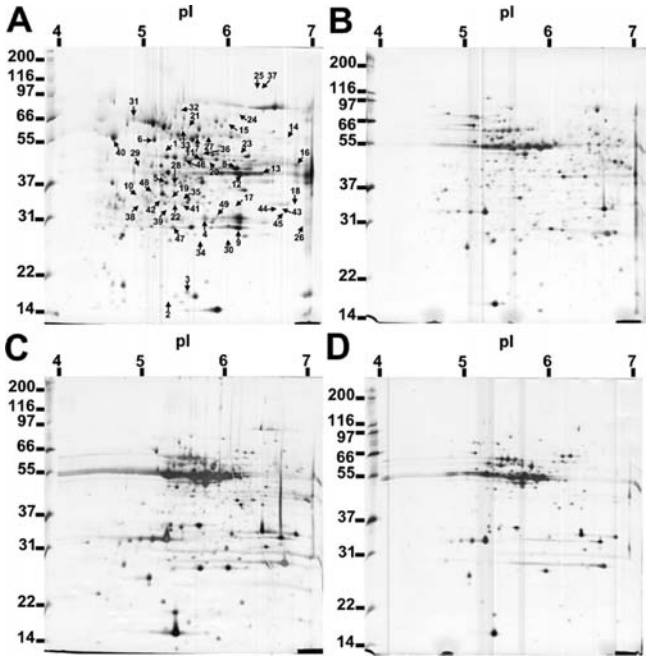

Figure 2. 2-DE image of proteins associated with different functional categories at 11 (A), 14 (B), 18 (C), and 21 (D) DAF. Labeled spots indicate identified functional proteins (A).

begin grain filling at 11 DAF, continuing until 21 DAF. Therefore, we divided the development process from 11 to 21 DAF into early (11−14 DAF), middle (14−18 DAF), and late (18−21 DAF) stages; thus, we used the developing seeds at 11, 14, 18, and 21 DAF for further study (Supplemental Table S1 in the Supporting Information).

A total of 300 protein spots were isolated for in-gel trypsin digestion, subsequent nanoscale capillary LC-MS/MS analysis, and database searching. Fifty-seven spots were identified (Figure 2) and are summarized in Table 1. We classified these proteins into eight functional categories according to Protein Knowledgebase (UniProtKB) gene ontology: (i) metabolism pathways including central carbon metabolism (sugar conversion, glycolysis, TCA cycle, ATP synthesis and binding, fermentation, gluconeogenesis), other carbohydrate metabolism, amino acid metabolism, cell wall structure formation, and chlorophyll metabolism; (ii) transcription regulation; (iii) redox regulation, stress response, and signaling; (iv) defense response; (v) storage; and (vi) proteolysis. Also, we identified multidrug resistance protein 1 P-glycoprotein 1 (spot number 50; no. 50) and meiotic recombination protein DMC1 homologue (no. 51) belonging to transport (vii) and cell cycle (viii) categories, respectively. Because only one member of each category (transport, cell cycle) was identified, these two proteins are not described in detail. Proteins without gene ontology terms in UniProtKB and those that could not be classified into the above categories were assigned as unknown (Table 1).

Metabolism. Harris reported starch degradation in mungbean cotyledons by a cytoplasmic enzyme for the first time.[13] In the present study, two patterns of the starch degradation seem to be found (Figure 3). First, the starch was attacked by α-amylase (no. 1). Oligosaccharides released during starch degradation, such as maltose, maltotriose, and maltotetraose, would be hydrolyzed by unidentified α-glucosidase to glucose during 11−21 DAF. The results showed the expression of α-amylase was highest at 14−21 DAF (Figure 4). Second, breakdown of starch by α-1,4-glucan phosphorylase (no. 2−4) into glucose-1-phosphate and then by UDP-glucose pyrophosphorylase (no. 6) finally provided sucrose, which was hydrolyzed by invertase to produce reducing sugars such as glucose and fructose (Figure 3). UDP-glucose-pyrophosphorylase (Figure 4, no. 6), an upstream enzyme in the starch biosynthesis pathway for UDP-Glc conversion into G-1-P, was found as a stably expressed protein throughout the growth stage, demonstrating its important roles in mungbean starch biosynthesis. The expression of α-1,4-glucan phosphorylases was most active at 11 and 18 DAF, whereas the UDP-glucose pyrophosphorylase was down-regulated from 14 to 21 DAF (Figure 4). Through the synthesis of sucrose, the glucose could be transferred into glucose phosphate and fructose phosphate to glyceraldehyde 3-P (putative fructokinase I, no. 5; the enzyme was down-regulated from 11 to 18 DAF; Figures 3 and 4). Both amylase- and phosphorylase-mediated starch degradation was reported in previous publications.[1,14,15]

In this study enolase (no. 7), phosphoglycerate kinase (no. 8), and triosephosphate isomerase (no. 9) enzymes that catalyze steps in the glycolysis pathway[15] were identified (Figure 3). The glycolysis enzymes identified in this study were visibly expressed at 11 DAF, decreased at 14 DAF, increased at 18 DAF again, and thereafter decreased to a lowest level at 21 DAF (Figure 5D). The final product of glycolysis is pyruvate, which could be transferred into mitochondria and used as the substrate for TCA cycle.[1,15] For the TCA cycle, four enzymes were identified such as fumarate hydratases (no. 10 and 11) and malate dehydrogenases (no. 12 and 13) (Figure 3). The enzymes involved in the TCA cycle were generally down-regulated from 11 to 21 DAF (Figure 5E). The enzymes

Table 1. Proteins Involved in Different Functional Categories

spot no.	description	accession no.	M_r; pI[g]	score[h]
	Metabolism			
	Central Carbon Metabolism			
sugar conversion				
1	α-amylase type B isozyme	P04747	40761; 5.79	27
2	α-1,4-glucan phosphorylase	P04045	109437; 5.45	22
3	α-1,4-glucan phosphorylase	P53535	110631; 5.20	24
4	α-1,4-glucan phosphorylase	P53535	110631; 5.20	17
5	putative fructokinase I	gil34907582	34698; 5.07	73
6[a]	UDP-glucose pyrophosphorylase	Q9LKG7	51518; 5.92	319
glycolysis				
7[b]	enolase	P42896	47883; 5.56	157
8[b]	phosphoglycerate kinase	gil21272	45544; 5.83	124
9[b]	triosephosphate isomerase	gil48773765	27213; 5.87	107
TCA cycle				
10	fumarate hydratase 1	P93033	52966; 8.01	27
11	fumarate hydratase 1	P93033	52966; 8.01	36
12	cytosolic malate dehydrogenase	gil10334493	35475; 5.92	103
13[c]	malate dehydrogenase	Q08062	35567; 5.77	46
ATP synthesis and binding				
14	mitochondrial membrane ATP synthase subunit α	P18260	55452; 6.02	56
15[d]	serine−threonine kinase	gil38194923	40834; 5.46	50
fermentation				
16	alcohol dehydrogenase	Q96533	40673; 6.51	48
	Other Carbohydrate Metabolism			
17	glucose and ribitol dehydrogenase	gil7431022	31627; 6.54	42
18	glucose and ribitol dehydrogenase	gil7431022	31627; 6.54	48
	Amino Acid Metabolism			
19[e]	adenosylhomocysteinase	P35007	53199; 5.60	18
	Cell Wall Structure Formation			
20	type IIIa membrane protein cp-wap13	gil2218152	39396; 6.24	64
21	pectin methylesterase	gil29539387	60251; 7.63	34
	Chlorophyll Metabolism			
22	chloroplastic ATP synthase subunit α	P56757	55294; 5.19	28
23	chlorophyllase type 0	Q9LE89	38682; 8.97	21
24	chlorophyllase type 0	Q9LE89	38682; 8.97	22
25	chlorophyllase type 0	Q9LE89	38682; 8.97	36
26	glutamyl-tRNA reductase	O48674	58602; 8.97	43
	Transcription Regulation			
27	DNA-directed RNA polymerase β chain	Q9TL05	98103; 9.30	48
28	homeobox−leucine zipper	P46601	31708; 7.67	28
	Redox Regulation, Stress Response, and Signaling			
29	probable phospholipid hydroperoxide glutathione peroxidase	Q9LEF0	18906; 6.59	31
30	RAB24	gil1710078	24127; 5.87	79
31	protein disulfide isomerase precursor	Q9XF61	57054; 4.84	42
32	heat shock protein 70 kDa	P26413	70835; 5.37	208
33	heat shock protein 60 kDa	gil24637539	57727; 5.26	84
34	ferritin-2	gil2970654	28023; 5.90	57
	Defense Response			
35	disease resistance protein RPP8	Q8W4J9	106615; 6.27	28
36	disease resistance protein RPS5	O64973	101737; 6.83	46
37	powdery mildew resistance protein PM3b	gil53791619	119619; 7.83	37
38[f]	canavalin	gil17977	50296; 5.44	34
39[f]	canavalin	gil17977	50296; 5.44	74
40[f]	convicilin	gil1297070	62772; 6.09	40
41[f]	convicilin	gil1297070	62772; 6.09	34
42[f]	legumin	gil403336	64463; 5.49	36
43[f]	mung bean seed albumin	gil1000708	30214; 6.89	131
44[f]	mung bean seed albumin	gil1000708	30214; 6.89	92
45[f]	albumin-2	PO8688	26222; 5.16	54

Table 1. continued

spot no.	description	accession no.	M_r; pI^g	score^h
	Storage			
46	α-amylase/trypsine inhibitor CM16 precursor	P16159	15772; 5.31	39
	Proteolysis			
47	ATP-dependent Cip protease	P56317	22423; 5.78	19
48	putative α 6 proteasome subunit	gil14594923	17715; 8.65	65
49	proteasome IOTA subunit	gil3377794	27375; 5.83	131
	Transport			
50	multidrug resistance protein 1 P-glycoprotein 1	gil9294573	135976; 8.60	36
	Cell Cycle			
51	meiotic recombination protein DMC1 homologue	Q39009	37489; 5.54	41
	Unknown			
52	plant disease resistance polyprotein	gil53791870	21788; 11.50	34
53	hypothetical protein	gil50935791	17823; 11.88	51
54	rubber elongation factor protein	P15252	14582; 5.04	118
55	rubber elongation factor	gil31747338	14713; 5.04	57
56	seed maturation protein PM34	gil9622153	31747; 6.60	77
57	B1097D05.21	gil50901852	56814; 5.92	34

[a]Protein could be involved in two groups of central carbon metabolism (sugar conversion and gluconeogenesis). [b]Proteins could be involved in two groups of central carbon metabolism (glycolysis and gluconeogenesis). [c]Protein could be involved in two groups of central carbon metabolism (TCA cycle and gluconeogenesis). [d]Protein could be involved in two groups of metabolism (amino acid metabolism and chlorophyll metabolism) and storage. [e]Protein could be involved in central carbon metabolism and redox, stress response, and signaling. [f]Proteins could be involved into defense response and storage. [g]Molecular mass (M_r), isoelectric point (pI). [h]Matched protein data need to contain one confident peptide ($P < 0.05$), and peptide ions score <15 was excluded.

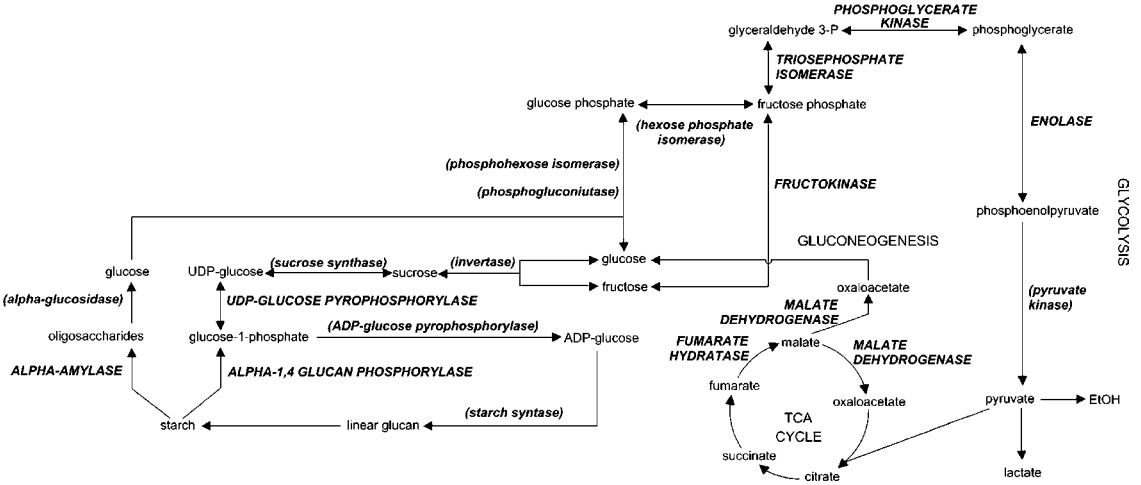

Figure 3. Diagram of identified enzymes involved in sugar conversion, TCA cycle, glycolysis, and gluconeogenesis in mungbean seed. Enzymes in parentheses were not found during our experiment from 11 to 21 DAF. Adapted from Ghosh and Pal.[1]

involved in different functional groups of central carbon metabolism, UDP-glucose phosphorylase (no. 6), enolase (no. 7), phosphoglycerate kinase (no. 8), triosephosphate isomerase (no. 9), malate dehydrogenase (no. 13), all work in the gluconeogenesis. This indicated that the de novo biosynthesis of carbohydrates might happen in mungbean seeds.[16]

We also identified two enzymes for ATP synthesis and binding: mitochondrial membrane ATP synthase subunit α (no. 14), which produces ATP from ADP in the presence of a proton gradient across the membrane, which is generated by electron transport complexes of the respiratory chain;[17] and serine−threonine kinase (no. 15), which interacts selectively and noncovalently with ATP.[18] The ATP synthase subunit α and the serine−threonine kinase were low-level expressed at 14−18 and 18−21 DAF, respectively (Figure 4). The existence of a fermentation pathway was suggested by identification of alcohol dehydrogenase (no. 16), which catalyzes the conversion of acetaldehyde to ethanol.[15] The alcohol dehydrogenase peaked at 14 DAF and then decreased to a lower level, which remained until 21 DAF (Figure 4).

Besides the above-mentioned enzymes involved in the central carbon metabolism, glucose and ribitol dehydrogenases (no. 17 and 18) are involved in other carbohydrate metabolism and the acquisition of desiccation tolerance.[19] The glucose and ribitol dehydrogenases were distinctly active at 18 and 21 DAF (Figures 4 and 5I). One amino acid metabolism-related protein was identified in our experiments. The adenosylhomocysteine hydrolase (no. 19) enzyme (up-regulated at 14 and 18 DAF; Figure 4) is involved in a multistep process that breaks down the protein building block methionine. Specifically, *S*-adenosylhomocysteine hydrolase controls the step that converts the compound *S*-adenosylhomocysteine to the compounds adenosine and homocysteine. This reaction also plays an

dx.doi.org/10.1021/jf401170g | J. Agric. Food Chem. 2013, 61, 6650−6659

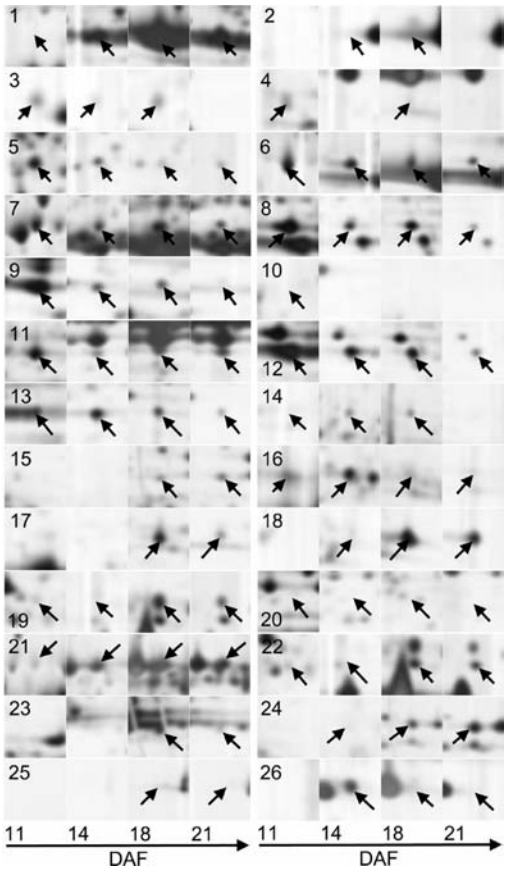

Figure 4. Close-up image of differentially expressed protein spots associated with metabolism category at 11, 14, 18, and 21 DAF.

important role in regulating the addition of methyl groups.[20] For the rest of the amino acids, the request for them during 11−21 DAF might be satisfied by the degradation of stocking and other proteins.[16] Two identified enzymes, type IIIa membrane protein cp-wap13 (no. 20) and pectin methylesterase (no. 21), are involved in cell wall structure formation. The type IIIa membrane protein cp-wap13 that takes part in cell wall metabolism[21] was expressed at low level during 14−18 DAF (Figure 4). Pectin methylesterase, a ubiquitous cell-wall-associated enzyme presenting several isoforms that facilitate plant cell wall modification and subsequent breakdown,[22] was visibly expressed at 14−21 DAF (Figure 4).

For chlorophyll metabolism, chloroplastic ATP synthase subunit α (no. 22), chlorophyllases type 0 (no. 23−25), and glutamyl-tRNA reductase (GluTR) (no. 26) were identified. The chloroplastic ATP synthase subunit α that produces ATP from ADP in the presence of a proton gradient across the membrane[23] as well as the chlorophyllases that are the catalysts for the hydrolysis of the ester bond in chlorophyll to yield chlorophyllide and phytol[24] were generally up-regulated at 14−18 DAF (Figure 4). The GluTR reduces glutamyl-tRNA to glutamate-1-semialdehyde during the first stage of tetrapyrrole biosynthesis by the C5 pathway in chloroplast.[25] Goslings et al. demonstrated that failure to repress GluTR activities in darkness led to increased accumulation of protochlorophyllide in the *flu* mutant of *Arabidopsis thaliana*; therefore, the glutamyl-tRNA reduction is the limiting step of chlorophyll biosynthesis.[26] The expression of GluTR peaked at 14 DAF and thereafter decreased to a lower level (Figure 4). The role of chlorophyll in seeds has been a matter of controversy. Whereas maturing canola (*Brassica napus* L.) seeds have been shown to refix respired CO_2 for their metabolic needs,[27] other studies have indicated that photosynthetic processes contribute little to developing seeds. Seeds appear to be dependent on cytosolic processes for ATP, reducing power, and carbon precursors that are required for development and maturation.[28]

Transcription, Redox, Stress Response, Signaling, Defense Response, Storage, and Proteolysis. Two RNA-associated proteins were detected in this study: DNA-directed RNA polymerase β chain (no. 27), which catalyzes RNA synthesis and can initiate a chain de novo,[29] and homeobox-leucine zipper (HD-Zip) protein (no. 28), which plays a role in auxin-mediated morphogenesis.[30] The HD-Zip proteins are transcription factors as yet found only in plants. Previously, one HD-Zip gene, ATHB6, from *A. thaliana* was characterized that was expressed constitutively in seedlings, but significantly up-regulated in seedlings subjected to water deficit or osmotic stress.[31] The DNA-directed RNA polymerase β chain was distinctly expressed at 18−21 DAF, whereas the HD-Zip protein peaked at 11 DAF and thereafter decreased to a lower level, which remained until 21 DAF (Figure 6). Previously, Walling et al. found that the soybean seed protein mRNA concentrations increased and decreased during embryogenesis and germination and reached maximum values at 70 DAF.[32]

Reactive oxygen species (ROS) can be produced in all living organisms as signal molecules or byproducts. Overaccumulation of ROS can result in oxidative stress, especially the oxidization of some functionally important proteins.[33,34] To keep a balance of the redox homeostasis and protect themselves from oxidative damages, plants have evolved highly efficient antioxidant systems, among which antioxidant enzymes might be the most efficient one.[16] In this study three redox regulation proteins, phospholipid hydroperoxide glutathione peroxidase (no. 29), RAB24 (no. 30), and protein disulfide isomerase precursor (no. 31), were identified. Both the phospholipid hydroperoxide glutathione peroxidase and the RAB24 possess peroxidase activity.[35] The phospholipid hydroperoxide glutathione peroxidase that protects cells and enzymes from oxidative damage by catalyzing the reduction of hydrogen peroxide, lipid peroxides, and organic hydroperoxide and glutathione was slightly expressed at 14 DAF (Figure 6). The RAB24 peroxidase activity described previously as contributing to the inhibition of germination during stress was highly expressed at 18−21 DAF (Figure 6). Besides the scavenging by antioxidant enzymes, another critical way of keeping redox homeostasis is reducing the oxidized proteins.[16] The protein disulfide isomerase precursor involved in this process[36] was expressed at 11 DAF (Figure 6).

In this study, five proteins for stress response and signaling were identified. Two heat shock proteins, 70 kDa (no. 32) and 60 kDa (no. 33), were responding to the abiotic stress[37] and were expressed at 11 DAF and thereafter increased throughout 14−18 DAF (Figure 6). Adenosylhomocysteinase (no. 19) may play a key role in the control of methylations via regulation of the intracellular concentration of adenosylhomocysteine.[20] Ferritin-2 (no. 34) stores iron in a soluble, nontoxic, readily available form, which is important for iron homeostasis. Ferritin-2 has ferroxidase activity, takes up the iron in the ferrous form, and deposits it as ferric hydroxides after oxidation.[38] Ferritin-2 was highly expressed at 18 DAF (Figure 6). In addition, the serine−threonine kinase (no. 15) is involved in the MAPK signaling pathway. This signaling protein

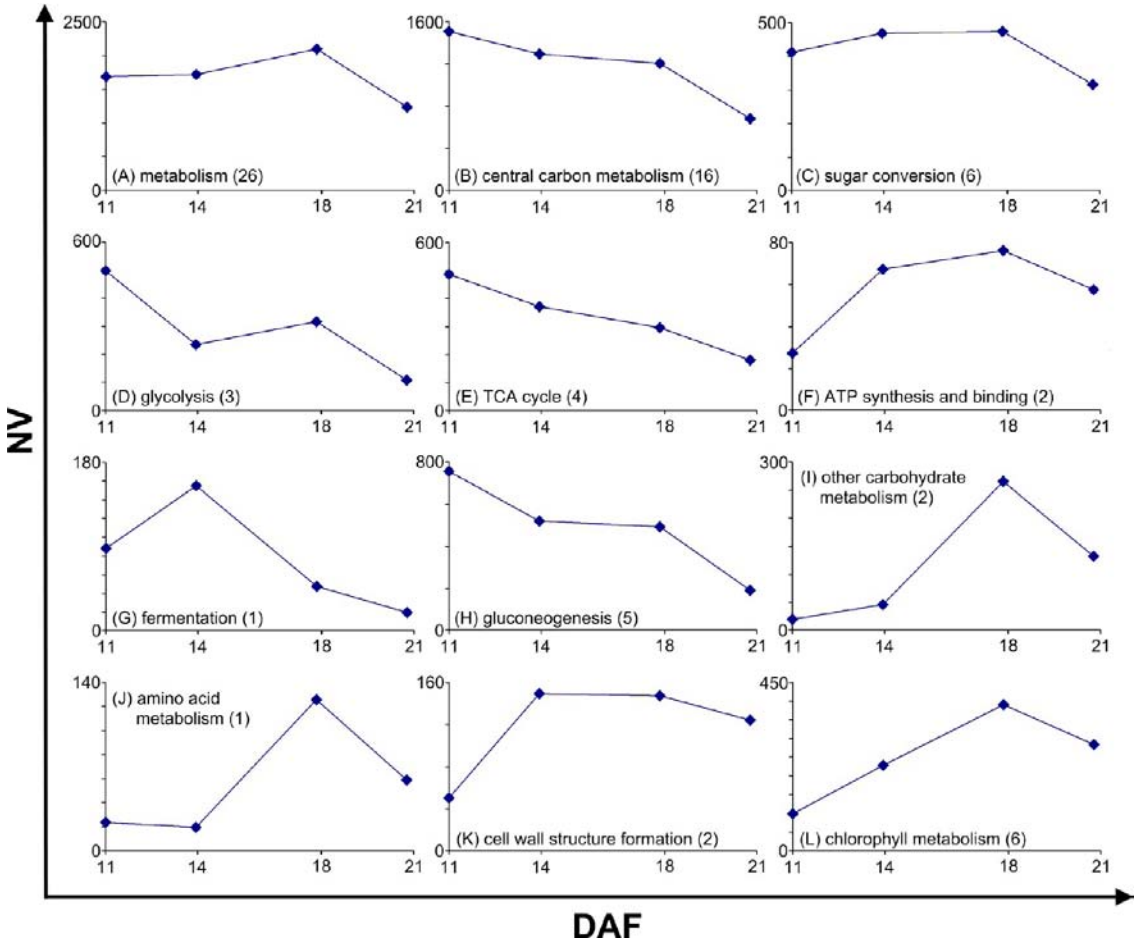

Figure 5. Combined expression profile of proteins associated with metabolism category (A) and selected metabolism subcategories (B, I–L) and groups (C–H). The profiles were established by the sum of all NV for protein components in a given functional group/category/subcategory (vertical axis) at each developmental stage (DAF; horizontal axis). The total spot numbers used to draw the composite profiles are indicated in parentheses.

contributes to the germination; however, the mechanism details are still unspecified.

During our experiments we identified plant defense proteins such as disease resistance proteins RPP8 (no. 35) and RPS5 (no. 36) as well as powdery mildew resistance protein PM3b (no. 37); these play potent defensive roles against pathogens.[39−41] The disease resistance protein RPP8 was expressed at 18−21 DAF, whereas the disease resistance protein RPS5 and the putative powdery mildew resistance protein PM3b were mainly expressed at 11−21 and 18−21 DAF, respectively (Figure 6). Increasing expression of these defense proteins (Figure 7C) is in agreement with previous publications. Hajduch et al. demonstrated high expression of proteins involved in disease and defense mechanisms in soybean seed at 14−21 DAF,[42] and as well Xu et al. demonstrated expression of defense response-related proteins in rice grain increased to 20 DAF.[43] Also, in our experiment we identified plant disease resistance polyprotein (no. 52) expressed at detectable level at 11−18 DAF (Figure 6); however, gene ontology of this protein is not assigned as a plant defense.

Many of the reserve proteins accumulated in seeds and fruits are considered to have a constitutive defense function against microbial pathogens and invertebrate pests in addition to their storage function. These inducible or constitutive defense mechanisms of higher plants are relatively conserved during the course of evolution. Accordingly, most plants produce or accumulate structurally and functionally similar protective proteins under certain situations, irrespective of their morphological differences. In mungbean, we found eight reserve proteins, canavalins (no. 38 and 39), convicilins (no. 40 and 41), legumin (no. 42), and albumins (no. 43−45), that seem to fulfill these functions.[44−46] The canavalins and albumins were expressed at 14−21 DAF, whereas the convicilins and legumin were predominantly expressed at 11 and 18 DAF, respectively (Figure 6). Also, we identified α-amylase/trypsine inhibitor CM16 precursor (no. 46), gradually down-regulated (Figure 6), which is a member of family I6 for seed storage proteins that is bifunctional in being inhibitors not only of peptidases but also of α-amylases.[47] Also, Kawasaki et al. described serine−threonine kinase (no. 15) as being involved in ATP synthesis and binding, signaling, and the MAPK signaling pathway and may participate in the synthesis of seed storage compounds during seed development.[48] Overall, during the developmental stages probably associated with grain filling from 11 to 21 DAF, the enzymes involved in storage were up-regulated from 11 to 18 DAF and maintained high expression until 21 DAF (Figure 7D).

In this study, we also identified three proteins participating in proteolysis of misfolded proteins and cytosolic proteins no longer needed by a cell.[49−51] ATP-dependent Cip protease (no.

dx.doi.org/10.1021/jf401170g | J. Agric. Food Chem. 2013, 61, 6650−6659

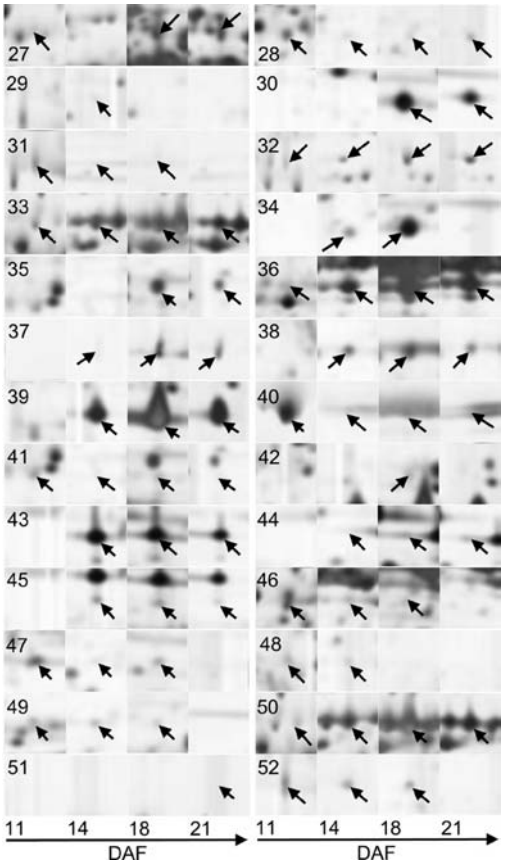

Figure 6. Close-up image of differentially expressed protein spots associated with different functional categories: transcription regulation, redox regulation, stress response and signaling, defense response, storage, proteolysis, transport, cell cycle, and unknown at 11, 14, 18, and 21 DAF.

47), putative α 6 proteasome subunit (no. 48), and proteasome IOTA subunit (no. 49) decreased their expression during 11–21 DAF (Figure 7E). This result is in agreement with a previous publication, which demonstrated that the expression of proteolysis proteins in rice grain was decreased throughout 6–20 DAF.[43]

Relationship between Acclimation Mechanism and Synthesis of Storage Compounds. Glycolysis and TCA cycle provide energy, cofactor regeneration, and building blocks for interconversions and synthesis of metabolites, with metabolite concentration gradients usually acting as signals for the regulation of diverse processes.[7] Analyses of intermetabolites suggest the central importance of the glycolysis and the TCA cycle in fruit/seed development and the accumulation of reserves in different species,[52−54] but relatively little is known about their regulation.[53] A typical feature of developing seeds and bulky organs such as potato (*Solanum tuberosum*) tubers is greatly decreased internal oxygen concentration at ambient oxygen levels (21%).[52,55−57] Low oxygen concentration-mediated decreases in carbon flux through the glycolysis and the TCA cycle in energy production have been observed in bulky organs/seeds from several species.[54,56−58] This pattern appeared to be confirmed by the results of studies demonstrating a great decrease in TCA cycle-related protein expression and TCA cycle activity, as developing seeds enter the stage of reserve accumulation in maize and *Arabidopsis*, respectively.[54,59] Similarly to previous publications, the down-regulation of the enzyme proteins related to glycolysis and TCA cycle as well as the low expression of enzyme proteins related to ATP synthesis and binding was observed when storage materials were accumulating in mungbean seeds in our experiments (Figures 5 and 7). Thus, these results indicate the importance of the regulation of glycolysis and TCA cycle in mungbean seed development.

Alcoholic fermentation is a two-step reaction branching of the glycolytic pathway at pyruvate with concomitant oxidization of NADH to NAD^+, finally generating ATP without the consumption of oxygen.[56,60] The down-regulation of enzyme proteins related to glycolysis and TCA cycle as well as low expression of the enzyme proteins related to the ATP synthesis described above, together with down-regulation of alcohol dehydrogenase, may indicate that low oxygen tension involves a temporal decrease in glycolysis, TCA, and alcoholic fermentation activities. This adaptation mechanism, presented in previous publications,[55,61] suggests that the metabolic acclimation allowing a decrease in the consumption of ATP and oxygen, which prevents the tissue from becoming anoxic and stops the fermentation, also occurs in mungbean seeds.

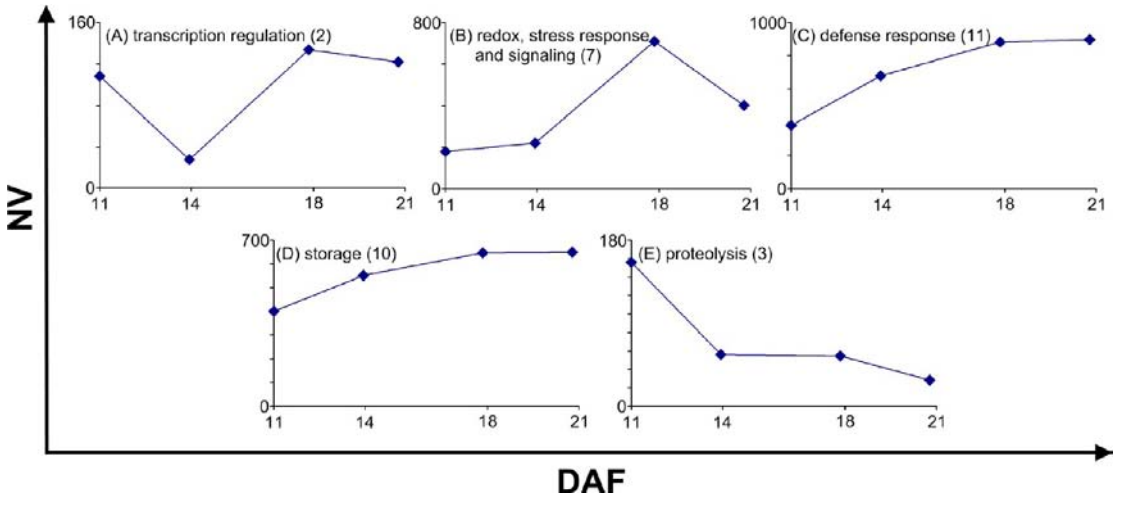

Figure 7. Combined expression profile of proteins associated with different functional categories: transcription regulation (A); redox regulation, stress response, and signaling (B); defense response (C); storage (D); proteolysis (E). The profiles were established in the same way as in Figure 5.

In summary, we analyzed the dynamic changes in mungbean seed protein expression profiles during sequential developmental stages from 11 to 21 DAF. Our results indicate that during the developmental process, proteins involved in glycolysis, TCA cycle, and alcoholic fermentation show a tendency to decrease in expression, whereas storage proteins are generally up-regulated. These suggest that the coordination of acclimation mechanism and cellular processes associated with accumulation of storage compounds in seed development proposed in previous publications[55,61] seems to appear in mungbean (*V. radiata* L.) seeds. Also, this work provides some information for better understanding of the metabolic processes in developing mungbean seeds.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplemental Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Funding

This work was supported by National Science Council, Republic of China, Grants NSC95-2313-B-019-023, NSC97-2313-B-019-011-MY3, and NSC101-2811-B-019-007.

Notes

The authors declare no competing financial interest.

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