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ARTICLE

High-throughput proteome analysis reveals changes of primary metabolism and energy production under artificial aging treatment in *Glycine max* seeds

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Abstract This study was conducted to obtain basic information on protein profile changes by artificial aging in soybean seeds. Seed proteins were extracted using the protamine sulfate precipitation method, which improves the detection of low-abundance proteins (LAPs) by depleting the major seed storage proteins . Isolated proteins were separated by high-resolution two-dimensional gel electrophoresis (2-DE), and differentially modulated protein spots were identified by MALDI-TOF/TOF. A total of 33 differential proteins were identified of which 31 and 2 showed decreased and increased abundances, respectively. Functional annotation of the identified proteins revealed that proteins were mainly associated with primary metabolism (55%) and response to stimulus (20.9%). Proteins with increased abundance were associated with nutrient reservoir activity (spots 5, 10), while the decreased abundance proteins were mainly involved in the primary metabolism such as carbohydrate metabolic process (spots 1–3, 11), protein folding (spots 6–9, 33), glucose metabolic process (spot 25) oxidoreductase activity (spots 19–24), UDP-glucose pyrophosphorylase activity (spots 12, 13). These results provide information about proteome changes, especially, LAPs during artificial seed aging treatment.

Keywords Artificial aging · Mass spectrometry · Protamine sulfate precipitation method · Proteomics · Seed deterioration · Two-dimensional electrophoresis

Introduction

Unfavorable environmental conditions, especially high temperature and high humidity, considerably affect biochemical and physical conditions of soybean seeds during seed growth and development (Torres et al. 2004). Temperature and moisture also influence the germination ability of soybean seeds. Moreover, these are associated with the reduction of seed germination during long-term storage in adverse environmental conditions which is well known as seed deterioration or aging (Egli et al. 2005). Seed aging is a physiological phenomenon that leads to the reduction of seed quality and loss of seed viability under high temperature and humidity conditions during their pre-and post-harvest storage (Anderson and Baker 1983; Wang et al. 2012). Especially, the post-harvest aging process is directly associated with agricultural productivity which is accompanied by unexpected yield loss, less longevity, and reduced viability of seeds.

Soybean seeds are a major source of protein and oil worldwide. They have been commonly used as food for

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humans, feeding domestic livestock, and industrial uses due to beneficial properties such as dietary and nutritional effect in their proteins (Friedman 1996; Messina 1999). However, seed aging significantly influences biochemical and physical conditions of the seeds during long-term storage in abnormal conditions. Biochemical changes during seed aging include loss of specific enzymatic activities, degradation of protein, chromosomal damage, membrane deterioration, decreased respiratory system, and lipid peroxidation which are directly associated with reduced germination of soybean seeds (Murata et al. 1984; Bailly et al. 1996; Kabinza et al. 2006; Sharam et al. 2013). Reactive oxygen species (ROS) and lipid peroxidation are strongly associated with the reduced germination and seed deterioration of soybean (Murthy et al. 2003; Parkhey et al. 2012). ROS is accumulated as a result of the imbalance between ROS generation and scavenging systems. ROS plays a critical role in cell signaling and hormone homeostasis during seed development. However, uncontrolled accumulation of ROS leads to oxidative stress towards a wide range of cellular compartments and oxidizes lipid and proteins which is one of the main factors of seed aging (Bailly et al. 2008; Yin et al. 2015). Oxidative stress is mainly associated with the accumulation of hydrogen peroxide in the seeds, and the function of ROS detoxification machinery was found to be important for seed longevity in Arabidopsis (Rajjou et al. 2008). Furthermore, it was also reported that delay of germination (DOG1) gene and heat stress responsive transcription factor were highly associated with seed longevity and aging, respectively (Bentsink et al. 2006; Prieto-Dapena et al. 2006).

Recent proteomic analysis investigated the effect of accelerated aging in *Brassica napus* seeds (Yin et al. 2015). This study identified 54 protein spots which were mainly associated with seed metabolism, protein destination, stress response, and seed development. Also, they showed that the abscisic acid (ABA) content was increased in accelerated aging-treated seeds as compared with control seeds. These results indicated that accelerated aging treatment affects not only basic seed physiology but also seed vigor (Yin et al. 2015). The biochemical and physical changes in soybean seed during their post-harvest storage affect its nutritional quality negatively. Attempts have been made previously to study the effect of natural and artificial aging, also known as seed deterioration, through the genomic and proteomic analyses (Wang et al. 2012; Yin et al. 2015; Nagel et al. 2016). In particular, denaturation of proteins and lipids has been observed during post-harvest storage which ultimately results in reduced quality of processed food (e.g., tofu, soymilk) (Mahjabin and Abidi 2015). In addition, Wang et al. reported the effect of high temperature and high humidity stress during soybean seed development using proteomic analysis (Wang et al. 2012). They identified 42 proteins spots of which 12 proteins were mainly related to amino acid metabolism, protein biosynthesis, and protein folding. These proteins were associated with ammonium recycling pathway which is affected by enhanced glutamine synthetase 1 in response to stress. Moreover, these results showed possible mechanism of seed deterioration during seed developmental stage. However, the complex mechanism associated with seed deterioration is still unclear and needs further investigations.

The advancement of omics technologies such as genomics and proteomics studies allows us to conduct humorous research in the last decade for identifying the potential biomarker in seed aging. In the present study, we performed comparative proteomic analysis of soybean seeds artificially aged using protamine sulfate precipitation (PSP) method (Kim et al. 2015) which is a high-throughput technique for the identification of the low-abundance proteins (LAPs) in artificial aging-treated soybean seeds. Therefore, we analyze the LAPs for understanding the basic mechanism of seed deterioration. These results provide a new insight of seed deterioration mechanism in soybean seeds.

Materials and methods

Plant materials

Soybean seeds (cv. Daewon) were grown in the experimental fields of National Institute of Crop Science (NICS), Rural Development Administration (RDA) at Miryang, Korea in June. The soil was supplemented with a standard RDA N–P–K fertilizer (N–P–K = 3–3–3.3 kg/10 acre). Seeds were harvested in October (average temperature 23.5 ± 3.5 °C, average day length 12 h 17 min). After harvesting, seeds were stored in a storage chamber at 4 °C for two years for further analysis (Kim et al. 2013b).

Artificial aging treatment and seed viability test

Twenty-five grams of soybean seeds were placed inside a 25 L plastic chamber, which was sealed after adding water (200 mL). The temperature and relative humidity of the chamber were maintained at 42 °C and 99%, respectively. Germination of artificially aged seeds was estimated to determine germination rates and viability of seeds. For germination and viability tests, a total of 45 seeds in 3 replicates of 15 seeds were used and checked for their germination by growing them in natural growth and soil conditions as stated above.

Protein extraction by protamine sulfate precipitation (PSP) method

Protein extraction was conducted as described previously (Kim et al. 2013a; 2015). Powdered soybean seeds (1 g) were homogenized in 10 mL of Tris–Mg/NP-40 buffer [0.5 M Tris–HCl (pH 8.3), 2% (v/v) NP-40, 20 mM $MgCl_2$) and centrifugation at 12,000×*g* for 10 min at 4 °C. The collected supernatant was incubated on ice for 30 min after adding 0.1% (final concentration) Protamine sulfate (PS) solution. The extract was again centrifuged at 12,000×*g* for 10 min at 4 °C in order to divide the PS supernatant (PSS) and PS pellet (PSP) fractions, as described earlier (Kim et al. 2015; Min et al. 2015). Pellet fraction was dissolved in Tris–Mg/NP-40 buffer, which is the equal volume with PSS fraction and then proteins were extracted from both fractions using trichloroacetic acid (TCA)/acetone precipitation method. Finally, washed pellets were dissolved in 80% acetone containing 0.07% β–mercaptoethanol and stored at −20 °C until further analysis.

Two-dimensional electrophoresis

Two-dimensional electrophoresis (2-DE) was performed as described previously (Gupta et al. 2015a). Briefly, proteins precipitated from the 80% acetone were dissolved in the rehydration buffer containing 7 M urea, 2 M thiourea, 4% v/v CHAPS, 2 M DTT, and 0.5% v/v IPG buffer pH 4–7 (GE Healthcare, Waukesha, WI, USA). Totally 600 µg proteins of each sample were loaded onto 24 cm IPG strips (pH 4–7) by rehydration loading overnight at 20 °C. Isoelectric focusing was performed following protocol: 50 V for 4 h, 100 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 2000 V for 1 h, 4000 V for 2 h, 8000 V for 5 h, 8000 V for 9 h, and 50 V for 6 h on the IPGphor II platform (GE Healthcare). Sequential equilibration of the each strips was carried out using equilibration buffer [6 M urea, 30% v/v glycerol, 2% v/v SDS, 50 mM Tris–HCl (pH 6.8), and 0.1 mg/mL bromophenol blue] containing 100 mM DTT and 55 mM iodoacetamide. The second dimensional separation was carried out on 12% SDS-PAGE at 2 W per gel, 500 V, and 300 mA for 30 min, followed by 16 W per gel, 700 V, and 300 mA. Gels were stained with colloidal Coomassie Brilliant Blue (CBB) and destained with 30% (v/v) methanol at the two times. A total of two biological replicates were performed for each dataset.

Image acquisition and data analysis

The stained gel images were acquired using a transmissive scanner (PowerLook 1129, UMAX) at 32-bit pixel depth, 300 dpi resolution. The gel spots on 2-DE gels were detected using ImageMaster 2D Platinum software 6.0 (GE Healthcare). Differential abundance of protein spots was determined by percentage volume of two biological replicates which were normalized, and protein spots showing ≥1.5-fold change were considered as differential abundance spots. Statistical analysis was conducted using Student's *t* test ($p < 0.05$) to check the significance of differential abundance.

MALDI-TOF/TOF MS and data processing and statistical analysis

The selected protein spots were excised from the gel, destained, subjected to in-gel digestion with trypsin, and identified by Matrix-Assisted Laser Desorption/Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF/TOF MS) as described previously (Kim et al. 2016). Briefly, protein spots were applied in gel reducing using 10 mM DTT in 100 mM ammonium bicarbonate at 56 °C for 30 min and 50 mM iodoacetamide in 100 mM ammonium bicarbonate for 30 min in dark for alkylating. Washing steps were applied in gel pieces with 1:1 ammonium bicarbonate and acetonitrile (ACN) solution and dehydrated using 100% ACN for 5 min. Tryptic digestion was carried out with 5 µL of trypsin solution (20 ng/µL, Gold mass spectroscopy grade, Promega, Madison, USA) in 50 mM ammonium bicarbonate at pH 7.8 for 16 h at 37 °C. MALDI-TOF/TOF MS analysis was conducted using ABI 4800 Plus TOF–TOF Mass Spectrometer (Applied Biosystems, Framingham, MA, USA). Prepared samples of tryptic-digested peptides were mixed with the same volume of matrix (10 mg/mL α-cyano hydroxycinnamic acid, 0.1% TFA, 50% ACN), loaded on a MALDI target plate, and allowed to dry at 25 °C. Calibration of spectra was performed with prepared calibration standard (Mass Standard Kit for the 4700 Proteomic Analyzer; calibration Mixture 1). The 10 most and least intense ions per MALDI spot with signal/noise ratios >25 were selected for subsequent MS/MS analysis in 1 kV mode using 800–1000 consecutive laser shots (Kim et al. 2013b). MS/MS spectra were searched against the UniProt/Swiss-Prot database (14,926,175 sequences; 5,299,740,401 residues) and soybean peptide database obtained from the soybean genome database (Phytozome ver. 8.0, http://www.phytozome.net/soybean) by Protein Pilot v.3.0 software (AB SCIEX, Framingham, MA, USA) using MASCOT search engine (ver. 2.3.0, Matrix Science, London, UK). The search parameters used for the protein identification were as follows: fixed modifications-carbamidomethylation of cysteine, variable modification-methionine oxidation, peptide, and fragment ion mass tolerances-50 ppm, maximum trypsin missed cleavage-1, and instrument type-MALDI-TOF/TOF. High confidence identifications with

statistically significant search scores (greater than 95% confidence, $p < 0.05$) were used for further analyses. Also, functional annotation of the identified proteins was carried out by Gene ontology (GO) tool using panther website (http://www.pantherdb.org/). The $\log_2$ transformation was performed to equalize the scale of abundance using spot volumes of all the spots showing differential abundance. The values of each spot were used for hierarchical clustering analysis (HCl) using R studio software to generate the heatmap.

Results and discussion

Reduced tendency of germination under high temperature and humidity

The germination rate of soybean seeds was decreased after artificial aging treatment (42 °C and 99%). Seeds were first stored for two years in a storage chamber at 4 °C and then subjected to the artificial aging treatment for 0 h (control), 24 h (aging 1 day), and 48 h (aging 2 days). Moreover, 48 h aging-treated sample was properly dried at 37 °C for 3 h (recovery). During the artificial aging conditions, the moisture content of soybean seeds was increased and germination was slightly diminished, as compared with the control sample. However, the viability of recovery sample was reduced noticeably after drying the seeds at 37 °C (Fig. 1A). This indicated that the damage occurred in seeds during dried storage conditions and seeds component was not enough to compromise the germination process.

Protein separation by SDS-PAGE

Resolution of total seed proteins on SDS-PAGE showed approximately, 30 bands from 10–170 kD mass range. However, using total protein fractions, no significant difference was observed between the control and artificially aged seeds (Fig. 1B, lanes 2 & 5 in gels 1 and 2). One of the reasons for this could be the presence of a significant proportion (approximately 40–60% of total seed protein) of seed storage proteins (SSPs), including different subunits of β-conglycinin and glycinin (Natarajan et al. 2006; Gupta et al. 2016) in soybean seeds. These SSPs are major high abundant proteins (HAPs) which obstruct the resolution and identification of LAPs (Gupta et al. 2015b). LAPs are considered as significant components of cell signaling, regulation, and diverse metabolic pathways (Min et al. 2015). Previously, it was shown that PS precipitation method is capable of depletion of high-abundance SSPs in the pellet fraction, enriching the LAPs (Kim et al. 2015). Therefore, here we employed the PS precipitation method to enrich the LAPs and to observe the differentially modulated proteins that may be potentially masked by the HAPs in the total protein fraction. After PS fractionation, a clear depletion of SSPs was observed in the pellet fractions (P), while PS supernatant fraction showed high enrichment of low-abundance proteins (S) (Fig. 1B). So, here we used PS supernatant fraction for further study.

Two-DEs analysis for detection of LAPs and functional categorization

Using ImageMaster 2D Platinum software, a total of 825 ± 50 (CV value 5.9%), 683 ± 24.5 (CV value 3.6%), 785 ± 2 (CV value 0.3%), and 660 ± 24.5 (CV value 3.7%) proteins spots were observed in the 2-DE gels of PS supernatant fractions of control, aging 1 days, aging 2 days, and recovery samples, respectively (Fig. 2A). Of these detected spots, 49 spots representatively showed significant differential abundance (≥1.5-fold change, p value < 0.05) during artificial seed aging as compared with control. Out of these 49 spots, 33 differential protein spots were identified by MALDI-TOF/TOF MS (Fig. 2A; Table 1). Among these proteins, 31 showed decreased abundance in artificially aged seeds as compared with control. To analyze the protein-accumulated profiles, hierarchical clustering was performed using R studio software. Except for two proteins which were showing increased abundance, 31 proteins were used to generate heat map and clustering analysis (Fig. 3A). Identified LAPs included different isoforms of alpha-1,4-glucan phosphorylase, urease, heat shock protein (HSP), alpha-D-phosphoglucomutase, UDP-glucose pyrophosphorylase, ATP synthase subunit beta, sucrose-binding protein, embryonic protein, oxidoreductase FAD/NAD(P)-binding protein, formate dehydrogenase, alcohol dehydrogenase (ADH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), calreticulin(CRT), triosephosphate isomerase (TPI), glutathione-S-transferase, glutaminyl-tRNA synthetase protein, superoxide dismutase, and trypsin inhibitor (Table 1).

To find out the functional groups associated with the identified proteins, GO term analysis was conducted that categorized biological process, molecular function, and cellular component. GO analysis indicated that proteins with decreased abundance were mainly associated with the primary metabolic process (55.1%) and response to stimulus (20.9%), while increased abundance proteins were related with nutrient reservoir activity in the biological process. Primary metabolic process-related proteins consisted of four major groups including carbohydrate metabolic process (41.2%), protein metabolic process (29.4%), nucleobase-containing compound metabolic process (17.6%), and cellular amino acid metabolic process (11.8%) (Fig. 3B). The molecular function category mainly

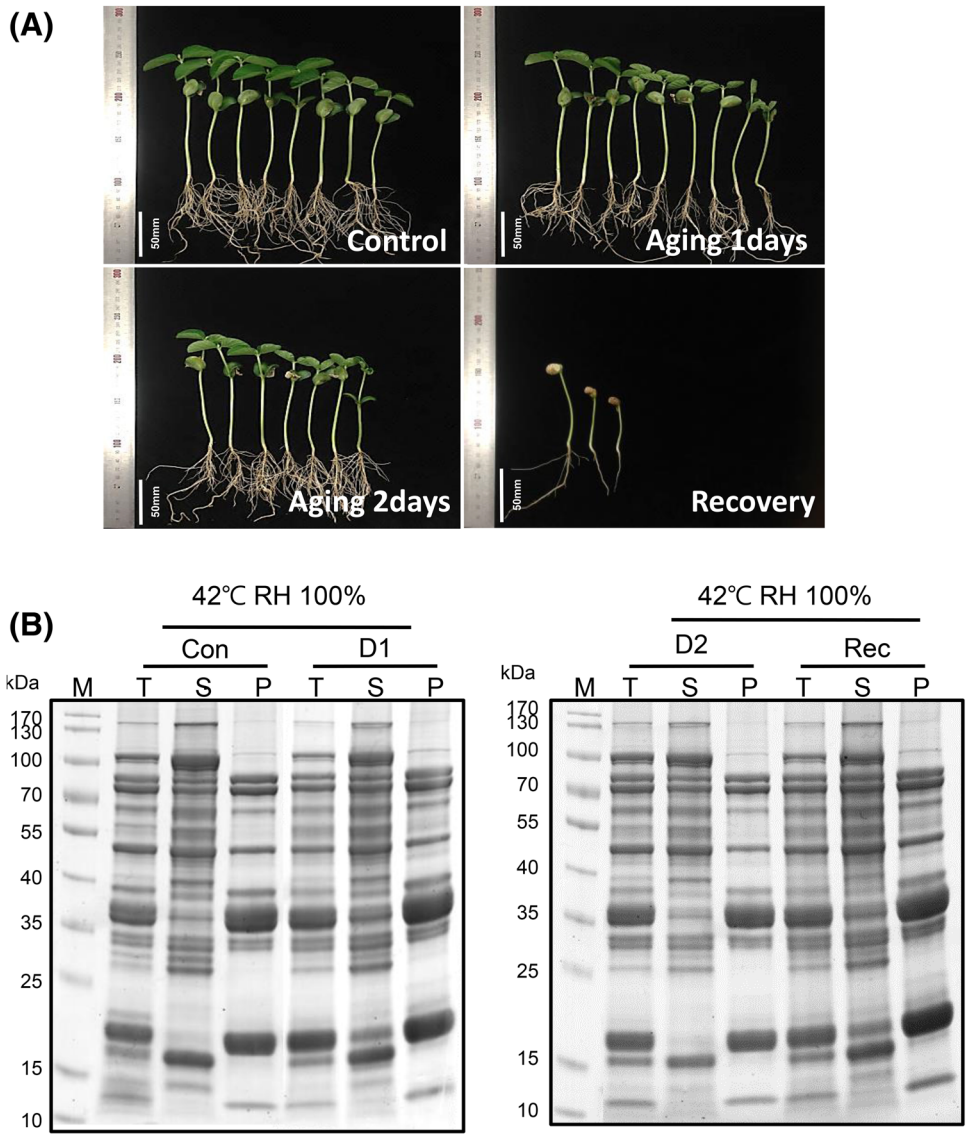
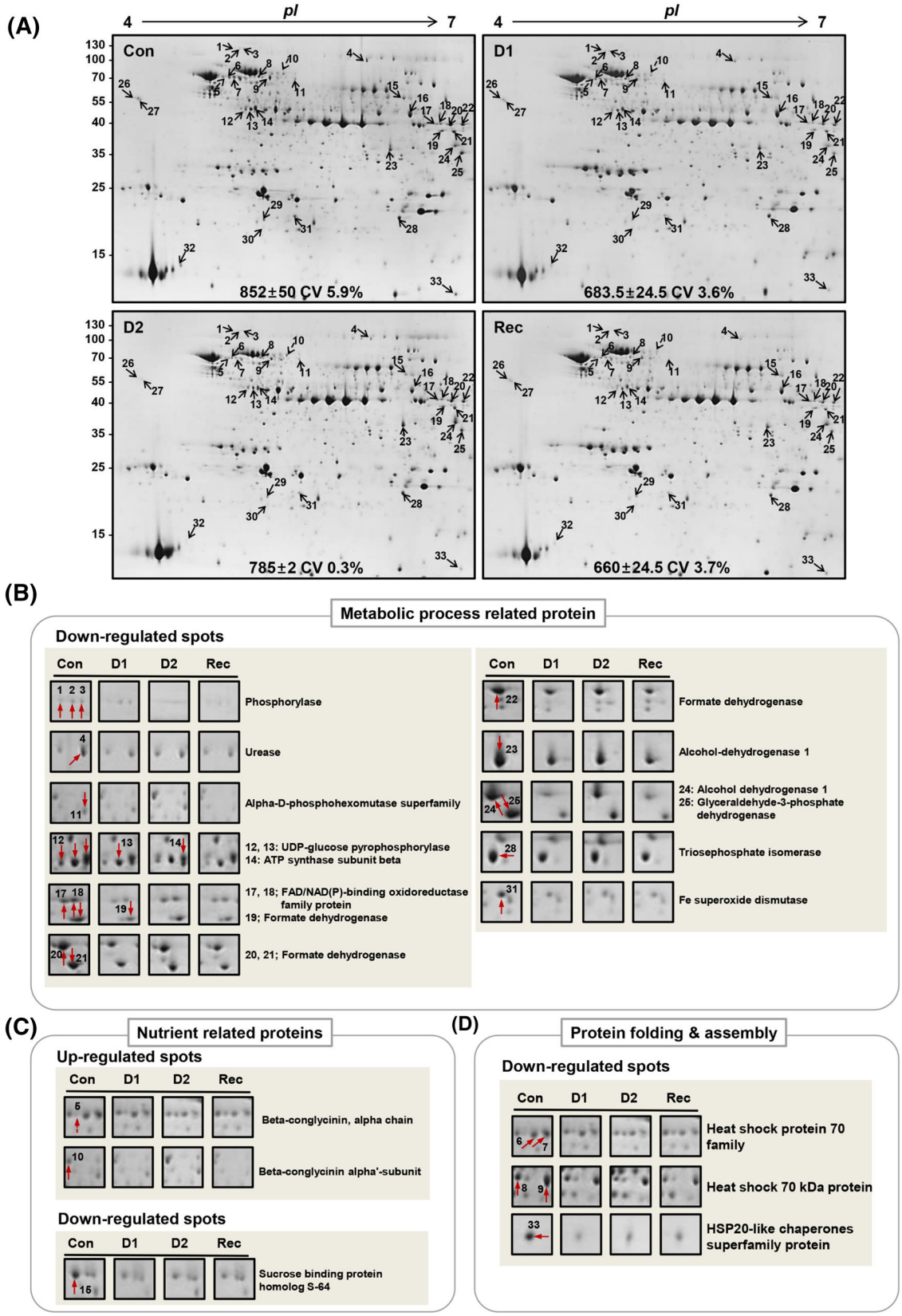


Fig. 1 Determination of germination rates using aging-treated seeds. (**A**) Representative images showing declined germination rates after artificial aging treatment (42 °C, 99% RH). (**B**) SDS-PAGE analysis of control and artificially aged seeds before and after protamine sulfate precipitation. *T* Total, *S* PS supernatant, *P* PS pellet

consisted of catalytic activity (66.5%) which is involved in oxidoreductase activity (42.9%), isomerase activity (21.4%), hydrolase and transferase activity (14.3%). The cellular component comprises cell part (50%) and organelle (33.3%) related proteins (Fig. 3C).

Recently, Wang et al. analyzed the effect of high temperature and high humidity on R7-developing stage of soybean seeds (cv. Ningzhen No.1) (Wang et al. 2012). They performed protein extraction using TCA/acetone precipitation method, and totally 42 protein spots were identified using 2-DE-MS approach. Those identified 42 proteins were majorly related to the metabolic function, signal transduction, cell defense, primary metabolism, and secondary metabolic biosynthesis. Moreover, Yin et al. 2015 reported the effect of artificial aging on *B. napus* seeds using a gel-based proteomic approach. They applied two different seed deterioration conditions at 0 and 18 h which were used for proteome analysis. They successfully identified 49 proteins using MALDI-TOF/TOF MS analysis. Among these identified proteins, most of the proteins were associated with primary metabolism, stress response, redox system, and seed development. Both of these studies commonly showed that seed deterioration affects primary metabolism which is highly associated with carbohydrate and protein metabolism of plants. In the present study, we identified 4 proteins related to carbohydrate metabolism


(A)
pI
4
7
Con
852±50 CV 5.9%
D1
683.5±24.5 CV 3.6%
D2
785±2 CV 0.3%
Rec
660±24.5 CV 3.7%
(B)
Metabolic process related protein
Down-regulated spots
Con D1 D2 Rec
Phosphorylase
Urease
Alpha-D-phosphohexomutase superfamily
12, 13: UDP-glucose pyrophosphorylase
14: ATP synthase subunit beta
17, 18; FAD/NAD(P)-binding oxidoreductase family protein
19; Formate dehydrogenase
20, 21; Formate dehydrogenase
Formate dehydrogenase
Alcohol-dehydrogenase 1
24: Alcohol dehydrogenase 1
25: Glyceraldehyde-3-phosphate dehydrogenase
Triosephosphate isomerase
Fe superoxide dismutase
(C)
Nutrient related proteins
Up-regulated spots
Beta-conglycinin, alpha chain
Beta-conglycinin alpha'-subunit
Down-regulated spots
Sucrose binding protein homolog S-64
(D)
Protein folding & assembly
Down-regulated spots
Heat shock protein 70 family
Heat shock 70 kDa protein
HSP20-like chaperones superfamily protein

◀**Fig. 2** (**A**) Comparative proteome analysis of artificially aged seeds using high-resolution 2-DE gels. Protamine sulfate precipitation method was applied and PS supernatant fraction was used for analyzing the low-abundant protein in soybean seeds. Differentially accumulated proteins were marked by *arrows*. (**B**) An enlarged view of the major proteins related to metabolic process, protein folding, and nutrient-related proteins. All of the decreased abundance proteins were mainly associated with the primary metabolic process

especially starch phosphorylation or degradation. Previously, it was reported that starch content is important for seed development which is directly or indirectly associated with early developmental stage of seeds, seed dehydration, and desiccation tolerance in late developmental stage of seeds (Da Silva et al. 1997). As enzymes related to starch degradation was downregulated during artificial aging, it can be expected that starch would not be degraded to produce sugars that are used to generate energy during seed germination. This could be one of the causes for reduced viability and lesser germinability of soybean seeds after artificial aging treatment.

Differential abundance proteins involved in primary metabolism and energy production

Proteome analysis results indicated that proteins with decreased abundance were mainly associated with primary metabolism such as carbohydrate, amino acid, and protein metabolic processes which were similar to the results reported previously on *A. thaliana* (Rajjou et al. 2008), *B. napus* (Yin et al. 2015), and *Zea mays* (Xin et al. 2011). They commonly concluded that artificially aged seeds showed decreased viability, and specific proteins which were associated with primary metabolism and seed energy metabolism showed different abundance as compared with the control.

Glycolysis is the primary metabolic pathway which is involved in the breakdown of sugars to provide ATP, reductant, pyruvate, and major components for anabolism (Plaxton 1996). Especially, glycolysis has an important role in plants because it is the initial cellular process that operates before Krebs cycle, production of the secondary metabolites, amino acids and fatty acids synthesis (Qi et al. 1995; Plaxton 1996; Agrawal et al. 2008). The major function of glycolysis during seed germination is to provide energy for growth and development of seedlings (Dong et al. 2015). In our study, 3 glycolysis-related proteins, including alpha-D-phosphopentomutase (spot 11), GAPDH (spot 25), and TPI (spot 28) were identified showing decreased abundance in artificially aged seeds. These proteins play crucial roles in glucose breakdown which is important for energy and carbon molecules supply in plants. Especially, both GAPDH and TPI are key enzymes of the glycolytic pathway that catalyzes the conversion of glyceraldehyde-3-phosphate to D-glycerate-1,3-bisphosphate and the interconversion of dihydroxyacetone phosphate with D-glyceraldehyde-3-phosphate, respectively. In our results, both proteins (spots 25, 28) showed more than 1.5-fold decrease in response to artificial aging. This indicated that artificial aging treatment affects energy metabolism which is required for seed development and plant growth. Also, alpha-D-phosphohexomutase superfamily protein showed lower abundance level in artificially aged seeds. This protein superfamily is composed of four related enzymes: phosphoglucomutase, phosphomannomutase, phosphoglucosamine mutase, and phosphoacetylglucosamine mutase in eukaryotic cells. These four enzymes play important roles in carbohydrate metabolism for various cellular processes (Shackelford et al. 2004). In this study, six proteins were found in the 2-DE analysis which showed differential abundance in response to artificial aging that attributed to decreased energy production in soybean seeds. Artificial aging treatment induced degradation of glycolytic-related proteins, which is directly associated with seed growth and development. In addition, few proteins related to both carbohydrate metabolism such as alpha-1, 4 glucan phosphorylase (spots 1–3), UDP-glucose pyrophosphorylase (spots 12, 13), and energy production such as ADH (spots 23, 24), formate dehydrogenase (spots 19–22), and ATP synthase subunit beta (spot 14) were also, respectively, detected and decreased in response to artificial aging treatment.

Differential abundance proteins associated with SSPs production, protein folding, and stress-responsive related protein

We identified 8 proteins which were related to SSPs production, protein folding, and stress response, respectively (Table 1). Previously, it was reported that urease enzyme is associated with the SSPs production in soybean seeds. Urease catalyzes the conversion of urea into carbon dioxide and ammonia. The ammonia is converted into glutamine by the function of glutamine synthetase (Min et al. 2015). In this study, urease enzyme (spot 4) showed decreased abundance after artificial aging treatment. This indicates that artificial aging causes decrease in the SSPs content of soybean seeds, thus affecting the soybean seed quality.

In addition, 5 proteins which function as molecular chaperones were also identified as major downregulated proteins. Molecular chaperones are essential for accurate synthesis, targeting, and maturation of proteins and can support protein refolding under stress conditions in cellular compartments. This is a very important biological phenomenon in a cellular system for normal functioning and

Table 1 Identification of differentially accumulated proteins by MALDI-TOF/TOF MS

	Spot No.	Acession No.	Glyma Locus	Protein name	Function	Mr/pI(T)	Mr/pI(E)	SC(%) (Sequence Coverage)
Down-regulated proteins(31)	1	I1N6A5	Glyma19g03490.1	Alpha-1,4 glucan phosphorylase	Carbohydrate metabolic process	110.8/5.35	123/5.14	19
	2	I1N6A5	Glyma19g03490.1	Alpha-1,4 glucan phosphorylase	Carbohydrate metabolic process	110.8/5.35	122/5.17	13
	3	I1N6A5	Glyma19g03490.1	Alpha-1,4 glucan phosphorylase	Carbohydrate metabolic process	110.8/5.35	123.5.20	24
	4	I1K3K3	Glyma05g27840.1	Urease	Nitrogen compound metabolic process	91.0/5.70	100/6.13	27
	6	I1JPC5	Glyma03g32850.2	Heat shock protein 70 family	Protein folding	67.9/4.98	74/5.09	28
	7	Q39804	Glyma05g36620.1	Heat shock protein 70 (Hsp 70) family protein	Protein folding	73.7/5.11	75/5.13	25
	8	P26413	Glyma17g08020.1	Heat shock 70 kDa protein	Protein folding	71.3/5.37	76/5.32	25
	9	P26413	Glyma17g08020.1	Heat shock 70 kDa protein	Protein folding	71.3/5.37	73/5.40	36
	11	I1KQ93	Glyma08g04890.1	Alpha-D-phosphohexomutase superfamily	Carbohydrate metabolic process	63.8/5.35	72/5.59	36
	12	C6T7U2	Glyma14g39140.1	UDP-glucose pyrophosphorylase	Metabolic process	51.6/5.41	55/5.22	29
	13	I1MBR7	Glyma14g39140.1	UDP-glucose pyrophosphorylase	Metabolic process	51.6/5.20	55/5.25	47
	14	I1NFS4	Glyma20g25920.1	ATP synthase subunit beta	ATP biosynthetic process	59.9/5.80	57/5.28	44
	15	Q9SP11	Glyma02g16440.1	Sucrose binding protein homolog S-64	Nutrient resevoir activity	56.1/6.32	58.8/6.42	44
	16	I1L957	Glyma10g07410.1	Embryonic protein DC-8-like	None	48.8/6.12	51.5/6.46	47
	17	K7LLY1	Glyma10g42877.1	FAD/NAD(P)-binding oxidoreductase family protein	Oxidation–reduction process	31.9/8.39	45/6.66	11
	18	K7LLY1	Glyma10g42877.1	FAD/NAD(P)-binding oxidoreductase family protein	Oxidation–reduction process	31.9/8.39	45/6.68	11
	19	I1N5S0	Glyma19g01210.1	Formate dehydrogenase	Metabolic process	43.1/6.28	40.5/6.70	52
	20	C6T9Z5	Glyma13g23790.1	Formate dehydrogenase	Metabolic process	43.1/6.28	44/6.77	53
	21	I1N5S0	Glyma19g01210.1	Formate dehydrogenase	Metabolic process	43.1/6.28	40/6.79	64
	22	C6T9Z5	Glyma13g23790.1	Formate dehydrogenase	Metabolic process	43.1/6.28	44/6.86	63
	23	Q9ZT38	Glyma04g41990.1	Alcohol dehydrogenase 1	Oxidoreductase activity	37.0/6.13	37.4/6.32	39
	24	I1LXD1	Glyma13g09530.2	Alcohol dehydrogenase 1	Oxidoreductase activity	39.4/8.19	38.1/6.81	51
	25	Q2I0H4	Glyma06g18110.1	Glyceraldehyde-3-phosphate dehydrogenase	Glucose metabolic process	36.9/6.72	37/6.85	49
	26	A0A762	Glyma10g28890.2	Calreticulin 1b	Protein folding	48.3/4.43	59/4.38	17
	27	A0A0R0EJA3	Glyma20g23080.2	Calreticulin 1b	Protein folding	48.6/4.47	58.5/4.42	23
	28	I1MDF2	Glyma15g04290.1	Triosephosphate isomerase	Metabolic process	27.4/5.87	22/6.37	72
	29	I1L8Q0	Glyma10g05480.3	Glutathione S-transferase family protein	Protein glutathionylation	27.1/5.34	21.8/5.35	71
	30	I1LEV4	Glyma10g41920.1	Glutaminyl-tRNA synthetase-like	Translation	83.0/6.10	20.8/5.35	25
	31	Q71UA1	Glyma10g33710.1	Fe superoxide dismutase 2	Oxidation–reduction process	27.5/5.45	22.6/5.58	23
	32	P01071	Glyma08g45531.1	Trypsin inhibitor B	Endopeptidase inhibitor activity	20.3/4.66	16.6/4.72	33
	33	A0A0R0L186	Glyma02g42000.1	HSP20-like chaperones superfamily protein	Protein folding	17.3/6.75	14/6.82	36
Up-regulated proteins(2)	5	P13916	Glyma20g28660.1	Beta-conglycinin, alpha chain	Nutrient resevoir activity	70.5/5.07	75/5.06	23
	10	Q0MUU5	Glyma10g39150.1	Beta-conglycinin alpha'-subunit	Nutrient resevoir activity	70.1/5.43	78/5.50	18

Table 1 continued

	Score	Expect	The two highest-scoring peptides	MSMS/ PMF	FC value Con vs 1 days	FC value Con vs 2 days	FC value Con vs Rec
Down-regulated proteins(31)	127	1.8E−006	K.AASEEFDLSAFNAGR.H (Ions score 43)/R.HIEIIEMIDEELVR.T (Ions score 16)	2/21	−1.262	−1.463	−1.601
	89	0.011	K.AASEEFDLSAFNAGR.H (Ions score 28)/R.HIEIIEMIDEELVR.T (Ions score 17)	2/18	−0.605	−0.909	−1.193
	148	1.4E−008	K.AASEEFDLSAFNAGR.H (Ions score 22)/R.FEEVKEFVR.S (Ions score 18)	3/24	−0.775	−1.293	−1.372
	223	4.4E−016	R.EIPEDLAFACSR.I (Ions score 51)/R.TIHTYHSEGAGGGHAPDIIK.V (Ions score 35)	2/29	−0.323	−0.819	−0.898
	169	1.1E−010	R.TTPSYVGFTDTER.L (Ions score 39)/R.FEELNMDLFR.K (Ions score 39)	2/25	−1.222	−1.628	−1.700
	290	8.8E−023	K.FDLSGIPPAPR.G (Ions score 48)/R.VEIESLFDGVDFSEPLTR.A (Ions score 46)	6/26	−0.524	−0.839	−0.947
	174	3.5E−011	R.TTPSYVAFTDTER.L (Ions score 43)/K.ATAGDTHLGGEDFDNR.M (Ions score 36)	3/22	−0.631	−1.378	−1.777
	344	3.5E−028	R.FEEMNMDLFR.K (Ions score 51)/K.SQVHEVVLVGGSTR.I (Ions score 41)	6/35	−0.402	−0.726	−0.963
	332	5.5E−027	K.SEPQEEPPEFGAASDGDADR.N (Ions score 39)/K.SIFDFESIR.K (Ions score 34)	7/30	−0.582	−1.753	−1.900
	144	3.5E−008	K.ATSDLLLVQSDLYTLEDGFVIR.N (Ions score 29)/R.LVAEDFLPLPSK.G (Ions score 17)	3/17	−0.421	−1.299	−1.598
	273	4.4E−021	K.ATSDLLLVQSDLYTLEDGFVIR.N (Ions score 56)/K.GGTLISYEGR.V (Ions score 44)	4/21	−0.259	−0.685	−1.039
	259	1.1E−019	K.VVDLLAPYQR.G (Ions score 39)/R.VGLTGLTVAEHFR.D (Ions score 28)	6/27	−0.333	−1.192	−1.384
	349	4.3E−028	R.KESLFFPFELPSEER.G (Ions score 59)/K.FEEFFGPGGR.D (Ions score 48)	6/29	−0.325	−0.957	−1.251
	482	2.1E−041	K.VGEYADYGSQK.A (Ions score 65)/K.AGEYTDYASQK.A (Ions score 64)	8/31	−0.108	−0.530	−0.959
	36	2.2E+003	M.VKVIIICVIDYIIR.L (Ions score 23)	1/4	−0.219	−0.666	−0.886
	34	3.4E+003	M.VKVIIICVIDYIIR.L (Ions score 22)	1/4	−0.157	−1.182	−1.553
	211	7E−015	R.LKPFSCNLLYFDR.L (Ions score 37)/K.HIPDAHVIISTPFHPAYVTAER.I (Ions score 18)	3/22	−0.186	−0.846	−1.350
	377	1.8E−031	K.GEWNVAGIAHR.A (Ions score 65)/K.LNPNFVGCVEGALGIR.E (Ions score 48)	6/24	−0.304	−0.954	−1.166
	273	4.4E−021	K.HIPDAHVIISTPFHPAYVTAER.I (Ions score 49)/K.AAAAAGLTVAEVTGSNVVSV AEDELMR.I (Ions score 43)	3/22	−0.260	−0.654	−0.801
	508	1.4E−044	R.LKPFNCNLLYFDR.L (Ions score 76)/K.LNPNFVGCVEGALGIR.E (Ions score 59)	8/29	−0.166	−0.618	−0.911
	214	3.5E−015	K.THPVNFLNER.T (Ions score 42)/K.FGVNEFVNPK.D (Ions score 33)	4/18	−0.139	−0.616	−1.296
	220	8.8E−016	R.TDVYWWDAK.G (Ions score 52)/K.SEESNLCELLR.I (Ions score 28)	3/19	−0.489	−1.331	−1.537
	246	2.2E−018	K.GILGYTEDDVVSTDFIGDSR.S (Ions score 54)/R.VPTVDVSVVDLTVR.L (Ions score 45)	4/17	−0.262	−1.395	−1.937
	101	0.0007	R.FYAISAEYPEFSNK.G (Ions score 37)/K.TLVFQFSVK.H (Ions score 19)	3/11	−0.398	−0.567	−1.035
	108	0.00054	R.FDDGWGNR.W (Ions score 30)/K.VFFEER.F (Ions score 25)	2/13	−0.315	−0.852	−1.827
	274	1.3E−020	K.WVHDNVSAEVAASVR.I (Ions score 75)/K.FFVGGNWK.C (Ions score 68)	2/17	−0.598	−0.905	−0.943
	304	1.3E−023	K.YVDENFEGTPLFPR.D (Ions score 74)/R.FQIVFAEVFK.H (Ions score 39)	4/21	−0.387	−1.220	−1.490
	103	0.0017	–	0/20	−0.949	−1.146	−1.174
	82	0.21	K.SLEEIIVTSYNK.G (Ions score 20)/K.LVSWDAVSSR.L (Ions score 18)	3/10	−0.446	−0.776	−1.144
	138	5.4E−007	K.GIGTIISSPFR.I (Ions score 31)/K.DAVDGWFR.I (Ions score 30)	3/12	−0.273	−1.728	−1.906
	90	0.033	M.SIIPNLFGGR.R (Ions score 29)/K.ETPQAHVFSVDLPGLK.K (Ions score 17)	2/11	−0.723	−1.339	−1.708
Up-regulated proteins(2)	126	2.2E−006	K.NPFLFGSNR.F (Ions score 19)/K.TISSEDKPFNLR.S (Ions score 12)	3/19	1.949	3.041	3.024
	92	0.0061	R.VPAGTTYYVVNPDNDENLR.M (Ions score 26)	1/14	0.454	0.737	0.791

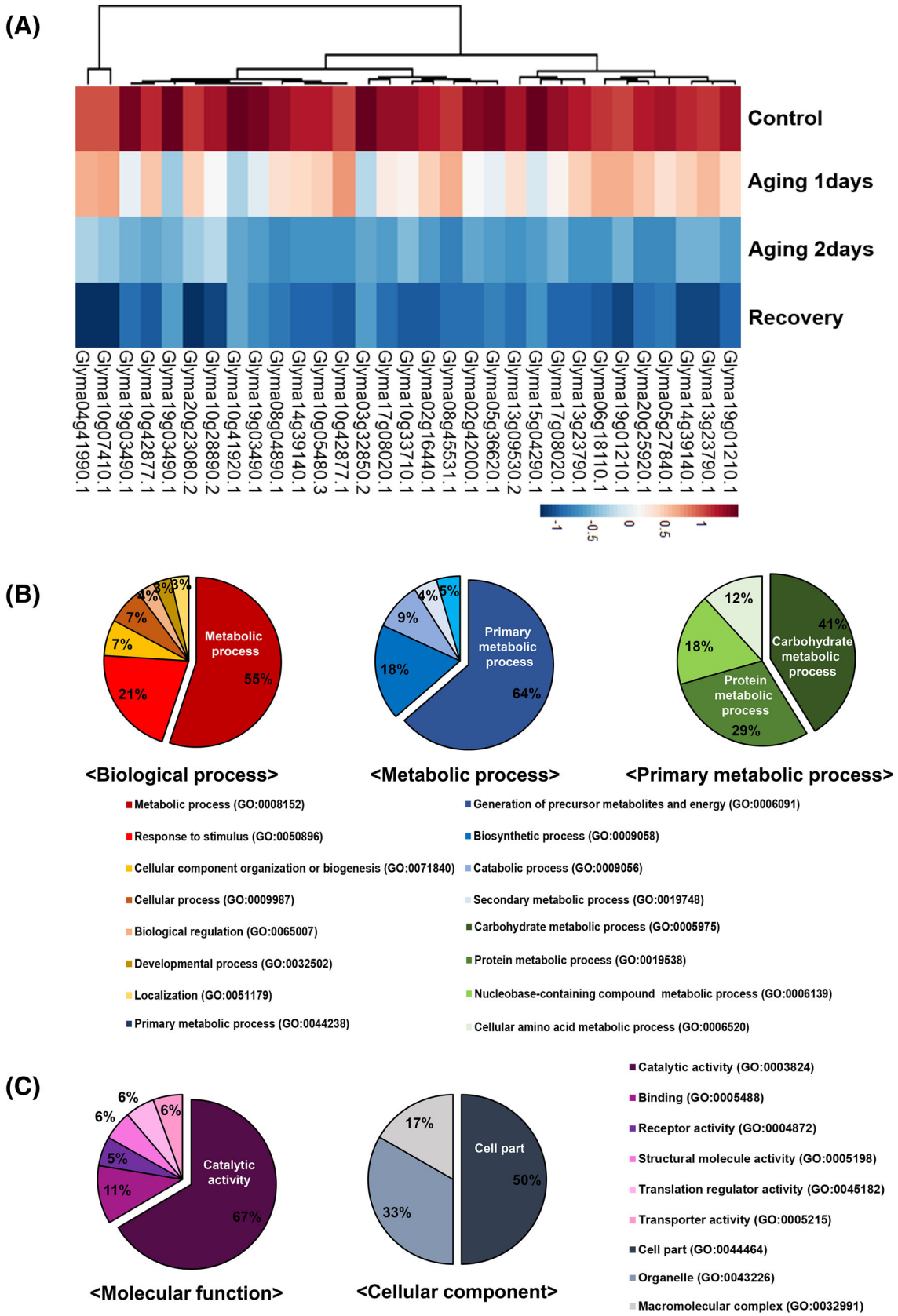


Fig. 3 (**A**) Hierarchical (HCl) clustering of decreased abundance proteins. Percentage volumes of 31 protein spots were detected by ImageMaster 2D Platinum software. R studio software was used for clustering. (**B**) GO terms analysis of the differentially accumulated proteins

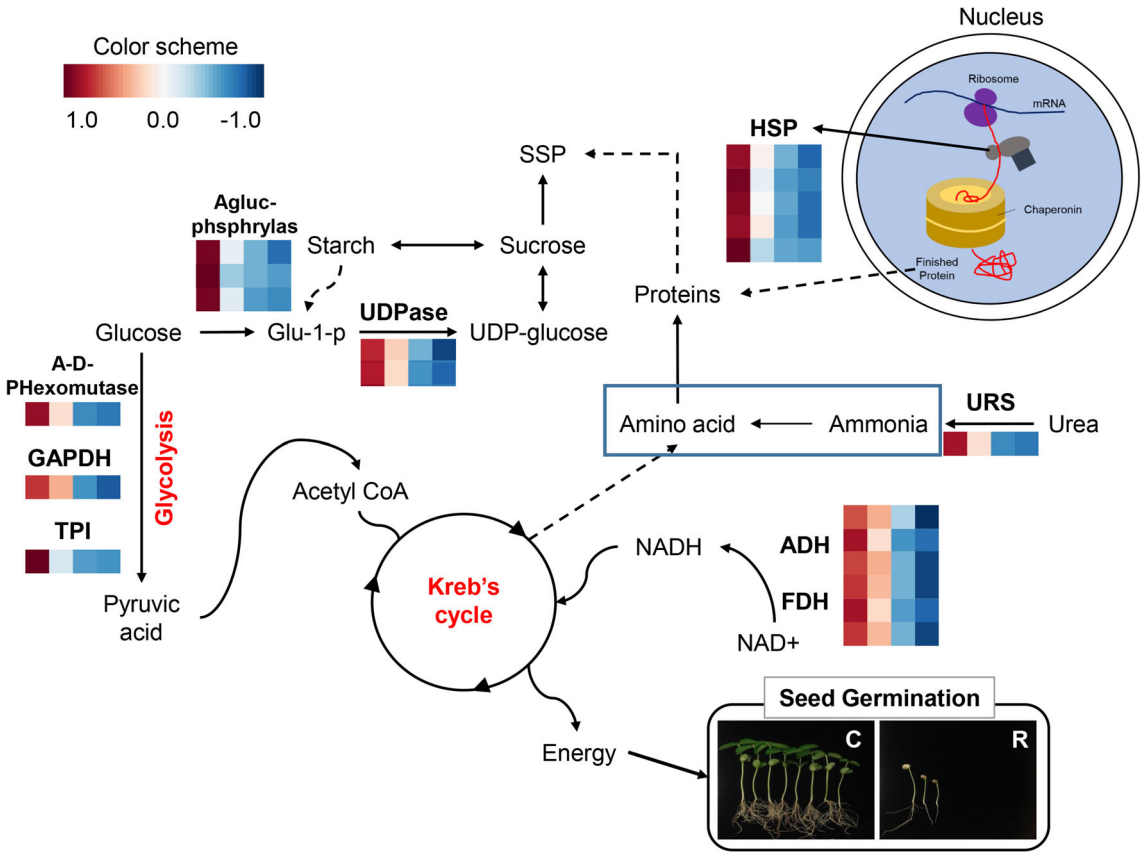


Fig. 4 Schematic representation of the decreased abundance proteins and major biological pathways related to carbohydrate, protein metabolism, and energy synthetic metabolism under high temperature and humidity

survival of cells during stress conditions (Boston et al. 1996). Most of the molecular chaperones are heat shock proteins that function in diverse kind of abiotic stresses such as drought, cold, salinity, chemical toxicity, and oxidative stress. In addition, they also have roles in membrane translocation and proteolysis of misfolded proteins by the regulatory process (Hartl 1996; Wang et al. 2004). Here, Five proteins showing decreased abundance were identified as chaperones that function as HSP (spots 6–9, 33). HSPs are encoded by a multigene family which is expressed mainly in response to temperature stress (Sung et al. 2001) as well as oxidative stress (Scarpeci et al. 2008). Downregulation of these proteins during aging indicates reduced impairment of degraded or misfolded proteins which are formed as a result of artificial aging treatment. Moreover, two antioxidant enzymes, superoxide dismutase (spot 31) and glutathione-s-transferase (spot 29), were also identified that showed decreased abundance. Taken together, our results indicate that the artificial aging condition may affect ROS scavenging system along with the protein regulatory system which remarkably influences the seed viability and growth.

In addition to the HSPs, two proteins (spots 26, 27) identified as CRT, were also associated with chaperone function. Although the primary function of CRT proteins is in calcium signaling, recent studies have indicated their role as a molecular chaperone and positive regulator in plant stress. The CRT-mediated pathway is mainly associated with Ca^{2+} signaling which is a modulator of Ca^{2+} homeostasis. Ca^{2+} homeostasis is directly associated with diverse biological phenomenon both in animal and plant cellular compartments (Gelebart et al. 2005; Jia et al. 2009). Cellular Ca^{2+} is widely established as an intracellular second messenger which transmits the wide ranges of extracellular stimulus in both animal and plant cells (Pandey et al. 2002). Recent studies have shown that CRT protein is also associated with plant immune system against biotic and abiotic stress such as biotrophic pathogen in Arabidopsis (Qiu et al. 2012) and drought in wheat (Jia et al. 2008). Our results indicated two CRT proteins which showed decreased abundance as compared with control (Table 1). Furthermore, CRT proteins are also associated with the phytohormone (e.g., gibberellin (GA), ABA)-signaling pathways (Jia et al. 2009). Especially, Khan et al.

2005 reported CRT is one of the major components in the GA-signaling pathway under cold stress. Recent studies showed that ABA content was increased under artificial aging condition (Yin et al. 2015). ABA and GA are important phytohormones; however, both of them have antagonistic effects. Based on the recent results, it can be concluded that reduced CRT accumulation during seed aging might be affecting the GA-signaling pathway that might result in increased ABA content of aging seeds which could be one of the potential factors of seed deterioration.

Schematic pathway in soybean seeds under artificial aging treatment

Based on the results obtained in this study, we proposed a possible schematic pathway which might be operating during artificial aging (Fig. 4). Our results revealed that many enzymes including enzymes of primary metabolism such as carbohydrate metabolism, protein metabolism, and energy production may play important roles in determining the seed quality during and after aging treatment. The metabolic pathway of glycolysis contributes to the degradation of glucose to convert it into pyruvate for Krebs cycle and energy production. In this study, we identified three proteins showing differential abundance which were directly or indirectly involved in glycolytic pathway all of which commonly revealed lower abundance level from the 48 h (aging 2 days) treatment. Also, ADH proteins (spots 23, 24) were decreased in abundance in response to artificial aging condition. These proteins facilitate the interconversion between alcohols and aldehydes or ketones with the reduction of NAD^+ to NADH in the plant. Moreover, Five proteins were associated with the carbohydrate metabolism. Among these, Three proteins were related to starch phosphorylation by converting starch into glucose-1-phosphate. Another two proteins were identified as UDP-glucose pyrophosphorylase (or glucose-1-phosphate uridylyltransferase) which is associated with glycogenesis and plays an important role in carbohydrate metabolism.

Taken together, our results clearly demonstrate the alteration of important cellular processes during artificial aging stress, which may be one of the factors resulting in seed deterioration. Furthermore, we identified some of the novel LAPs related to carbohydrate, protein metabolism, and energy production which were not identified previously in the aging experiments, most probably because of their masking by SSPs. These LAPs can provide further understanding about influence of seed deterioration in high temperature and high humidity stress in soybean seed and can be used to develop biomarkers for seed aging.

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