Plant Omics Journal POJ

POJ 6(6):456-463 (2013) ISSN:1836-3644

Comparative proteomics analysis of seed coat from two black colored soybean cultivars during seed development

Yu Ji Kim[1], Su-ji Lee[1], Hye Min Lee[1], Byong Won Lee[2], Tae Joung Ha[2], Dong Won Bae[3], Beom-Young Son[4], Yul Ho Kim[4], Seong-Bum Baek[4], Yong Chul Kim[1], Sang Gon Kim[4,*], Sun Tae Kim[1,*]

[1]Department of Plant Bioscience, Pusan National University, Miryang, 627-707, South Korea
[2]Department of Functional Crops, NICS, RDA, Miryang 627-803, South Korea
[3]Center for Research Facility, Gyeongsang National University, Jinju 660-701, South Korea
[4]National Institute of Crop Science, Rural Development Administration, Suwon 441-857, South Korea

***Corresponding author: stkim71@pusan.ac.kr, sen600@korea.kr**

Abstract

The seed coat plays an important role in the life cycle of soybean by controlling embryo development and determining seed dormancy and germination. In the present study, comparative proteomics analysis of the seed-coat from two black-colored soybean cultivars, Seonheuk and Geomjeong 2 which have different anthocyanin and isoflavone contents, was conducted. Two-dimensional gel electrophoresis (2-DE) coupled with matrix-assisted laser desorption/ionization-time of flight time of flight (MALDI-TOF/TOF) mass spectrometry analysis of total proteins from seed coats identified 36 proteins spots showing qualitative variation according to color changing stages of seed coat. Among them, 24 proteins were down regulated, while 11 were up regulated at stage 2 and 3 compared to stage 1. In addition, ten of the 36 protein spots may be differentially regulated by different contents of anthocyanin and isoflavone between Seonheuk and Geomjeong 2 at color changing stage of seed coat. Interestingly, the expression of trypsin inhibitors (TIs) and manganese superoxide dismutase (MnSOD) showed a greater reduction in Geomjeong 2 than Seonheuk, while isoflavone reductase homolog 1 (IFR1) showed a greater increase in Geomjeong 2 than Seouheuk, indicating that antioxidant activity of Geomjeong 2 may be higher than Seouheuk. Furthermore, their expression patterns were confirmed by Western blot analysis using rice isoflavone reductase-like protein (OsIRL), confirming the results of 2-DE. Overall, our results indicate that various proteins differentially expressed in accordance with seed-coat color may contribute to seed quality and; therefore, have a beneficial effect on human health.

Keywords: Anthocyanin, Isoflavone, MALDI-TOF/TOF, Seed coat, Soybean (*Glycine max*), Two-DE.
Abbreviations: ACN_acetonitrile; CBB_Coomassie brilliant blue; MALDI-TOF_ matrix-assisted laser desorption/ionization-time of flight time of flight; MnSOD_manganase superoxide dismutase; OsIRL_rice isoflavone reducatase like protein; SDS-PAGE_ sodium dodecyl sulphate polyacrylamide gel electrophoresis; TIs_trypsin inhibitors; 2-DE_two-dimensional electrophoresis.

Introduction

Black soybeans [*Glycine max* (L.) Merr.], a market class of soybeans with a black seed coat, are widely utilized as a dietary and health food in Asia. Black soybeans are believed to play an important role in detoxification and anti-inflammatory processes, and to improve the blood (Liao et al., 2005). Black soybeans are also used in foods such as soymilk, tofu, soy sauce, and soy sprouts in Korea because they provide protein, oil, carbohydrates, and several phytochemicals (e.g. isoflavones, saponins, and anthocyanins) (Weber et al., 2005). These components are synthesized during seed filling, which is the period when rapid metabolic and morphological (size, weight, and color) changes occur (Agrawal and Thelen, 2006). In addition to acting as a physical barrier, the seed coat plays specific roles in the metabolic control of seed development and dormancy (Weber et al., 2005), disease resistance (McClean et al., 2002), and the metabolism and transfer of nutrients from the parent plant (Weber et al., 2005). Numerous studies have revealed that the beneficial health effects of black soybean are due to several phytochemicals, isoflavones, saponins, and anthocyanins (Kim et al., 2006; Lee et al., 2009; Watanabe et al., 2002). Flavonoids, isoflavonoids, and anthocyanidins comprise the largest group of natural products in the plant kingdom (Lepiniec et al., 2006). In soybeans, four genetic loci, *I* (encoding chalcone synthase), *T* (encoding flavonoid 3'-hydroxylase), *R* (encoding a regulatory factor that controls expression of anthocyanidin synthase), and *O* (encoding anthocyanidin reductase), are largely responsible for controlling the seed coat color and distribution of phenolic compounds (Todd et al., 1993; Dao et al., 2011; Choung et al., 2001; Gillman et al., 2011). It is worth noting that, in addition to the genomic/genetic background, physiological and environmental factors can affect seed coat composition. For this reason, proteomic studies of seed coats during soybean seed development are beginning to provide a framework for identification of proteins and their dynamic metabolic processes. Proteomic technologies using two-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time-of-flight time–of-flight mass spectrometry (MALDI-TOFTOF/MS) provides a convenient method to effectively analyze protein profiles. Accordingly, these technologies have been used to evaluate various aspects of proteins in seed coats including protein expression,

modification, and stability in soybean seed coats. Recently, comparative proteomic studies of proteins involved in seed storage, coats, and development have been conducted to understand evolutionary relationships or metabolic changes among soybean accessions (Agrawal et al., 2008; Miernyk and Johnston, 2013; Xu et al., 2007). However, to the best of our knowledge, comparative analysis of black soybean seed coats during seed development has seldom been reported. In the present study, 2-DE-based proteomics and Western blot analysis were used to identify differentially expressed proteins between Seonheuk and Geomjeong 2 soybean cultivars, which have different anthocyanin and isoflavone contents, in Korea. Identified proteins were broadly related to storage proteins, protein modification, stress related proteins, and metabolic proteins. The results of the present study could provide us with a better understanding of the complex mechanism of soybean seed coat formation among different soybean accessions.

Results and Discussion

Protein separation by 2-DE analysis

In this study, we applied 2-DE combined with MALDI-TOF/TOF to study and compare the seed coat proteins in the black soybean cultivar, Seonheuk. We extracted proteins from seed coats of Seonheuk during seed coat maturation using phenol extraction with neutral IPG strips (pI 4–7) to obtain the best resolution of proteins on 2-DE gels, after which CBB staining was carried out. Two-DE maps were produced in triplicate for each of the three independent protein extraction experiments and showed a high level of reproducibility. Our results showed that the overall distribution pattern of proteins was quite similar in seed coat maturation of Seonheuk (Fig. 1). Interestingly, 36 spots were differentially expressed (Fig. 2) in Seonheuk cultivar. Among these, 24 spots decreased (Fig. 2A), whereas spots 26 to 37 increased during seed coat maturation (Fig. 2B). These results suggest that seed coat maturation caused the up- or down-regulation of many proteins with color change in soybean.

Identification of proteins involved in seed coat maturation in soybean

To better understand the mechanism of proteins involved in processes of seed coat maturation in soybean, we identified a selection of differentially expressed proteins by MALDI–TOF/TOF MS for analysis by database searches with MASCOT. The identified proteins were classified based on functional categories established by Miernyk and Hajduch (2013). These proteins were found to be associated with diverse biological processes. Specifically, down-regulated proteins included storage proteins (46%), protein modification proteins (25%), metabolic proteins (17%), and stress related proteins (4%) (Fig. 3A, Table 1), while up-regulated proteins were involved in stress related proteins (46%), metabolic proteins (36%), and storage proteins (18%) (Fig. 3B, Table 1).

Down-regulated seed coat proteins in Seonheuk cultivar

Primary storage proteins were the most abundant during seed coat maturation. Two major storage proteins, β-conglycinin and glycinin, account for about 70-80% of the total proteins, and are largely responsible for the nutritional and physicochemical properties of soybeans (Thanh and Shibasaki, 1976).

β-conglycinin is a trimeric glycoprotein consisting of α, α′, and β subunits. Among these subunits, only the α-subunit induces allergenic reactions (Thanh and Shibasaki, 1976). Glycinin consists of five subunits, G1, G2, G3, G4, and G5, in which G1 and G2 are allergens. The identified 13 proteins in our study involved in storage proteins (Table 1); 11 proteins were probable components of β-conglycinin (spots 1, 2, 3, 4, and 14) and glycinin (spots 5, 6, 7, 12, 16, and 17). Among these, five proteins (spots 1, 2, 12, 16, and 17; all down regulated during seed coat maturation) were involved in allergenic reactions (Fig. 2). Based on these findings, it is proposed that expression of storage proteins gradually decreases during seed coat maturation to enable buildup of structures of soybean seeds associated with physiological maturity such as the palisade layer, hourglass cells, and a few layers of thin-walled parenchyma. The second major group during seed coat maturation included metabolic proteins such as sucrose-binding protein (SBP, spot 14), formate dehydrogenase (FDH, spot 15), isoflavonoid reductase homolog 1 (IFR1, spot 18) and alcohol dehydrogenase (ADH, spot 20). SBP is a vehicle for sucrose transport across the plasma membrane. The accumulation pattern of SBP was nearly equivalent to the rate of sucrose uptake in soybean cotyledons (Grimes et al., 1992). FDH plays a crucial role in providing NADH to the respiratory chain. In plants, FDH catalyzes oxidation of formate ion to bicarbonate, which is coupled to the reduction of NAD^+ to NADH (Tishkov and Popov, 2004) and synthesis of ATP (Oliver, 1981). It has been reported that the amounts of FDH transcripts and proteins are positively associated with environmental stresses (Hourton-Cabassa et al., 1998), but no relationships between FDH accumulation and seed coats maturation have been reported to date. IFR is one of the key enzymes involved in isoflavonoid phytoalexin biosynthesis (Paiva et al., 1991). Phenolic compounds synthesized through the pathway of flavonoid/isoflavonoid biosynthesis, comprise the largest group of natural products and developmentally control seed coat color in plants (Lepiniec et al., 2006; van Eldik et al., 1997). ADH converts acetaldehyde to ethanol, with the concomitant regeneration of NAD^+ for glycolysis. ADH is a fermentative enzyme that is highly conserved across species. These data suggest that functions of transport sucrose, respiration, and phytoalexin biosynthesis decreased during color changing stage in seed coats. The relative abundance of six proteins known to regulate protein modification, including trypsin inhibitors (spots 22, 23, and 24), agglutinins (spots 9, 10), and a protease (spot 8), were changed during seed coat maturation. Kunitz trypsin inhibitors were identified as secreted proteins in soybean (Djordjevic et al., 2007) and were found in root hairs of soybean (Brechenmacher et al., 2009). Trypsin inhibitors were differentially accumulated in soybean seedlings in response to flooding (Hashiguchi et al., 2009). Kunitz-type trypsin inhibitors play an important role in resistance to plant pathogens (Major and Constabel, 2008). Soybean agglutinins (lectins) are carbohydrate-binding proteins found at moderately high levels in the seeds of many plants, including soybean. These proteins possess various enzymatic functions, including extension and growth regulation of cell walls, transportation of carbohydrates, and involvement in infection nodulation of leguminous plants (Han et al., 2009). In addition, protease catalyzes the hydrolysis of seed reserve proteins for seedling development during seedling growth. However, this constitutes a switch from the mode of protein accumulation during seed development (Liu et al., 2001).

Table 1. List of proteins identified by MALDI-TOF/TOF MS analysis of differentially expressed proteins during seed development.

Spot no.	AC	Protein name	Score	Expect	Mr	pI	SC	Source	Function
Down-regulated proteins									
1	gi|14245736	Beta-conglycinin alpha subunit	714	2.90E-66	70549	5.12	45	Glycine max	Storage protein
2	gi|121281	Beta-conglycinin, alpha chain	761	5.70E-71	70535	5.07	40	Glycine max	Storage protein
3	gi|9967361	Alpha' subunit of beta-conglycinin	591	5.70E-54	65160	5.23	55	Glycine max	Storage protein
4	gi|9967361	Alpha' subunit of beta-conglycinin	712	4.60E-66	65160	5.23	57	Glycine max	Storage protein
5	gi|255224	Glycinin G4 subunit	705	2.30E-65	64097	5.38	41	Glycine max	Storage protein
6	gi|33357661	Chain A, Crystal Structure Of Glycinin A3b4 Subunit Homohexamer	127	1.40E-07	55850	5.46	27	Glycine max	Storage protein
7	gi|6015515	Glycinin	564	2.90E-51	24349	4.46	72	Glycine max	Storage protein
8	gi|1199563	34 kDa maturing seed vacuolar thiol protease precursor	179	9.10E-13	43091	5.65	11	Glycine max	Protein modification
9	gi|6729836	Chain A, Soybean Agglutinin Complexed With 2,6-Pentasaccharide	240	7.20E-19	27555	5.15	41	Glycine max	Protein modification
10	gi|6729836	Chain A, Soybean Agglutinin Complexed With 2,6-Pentasaccharide	478	1.10E-42	27555	5.15	48	Glycine max	Protein modification
11	gi|126411	Seed lipoxygenase	899	9.10E-85	96871	5.78	43	Glycine max	Stress related protein
12	P04776	Glycinin	571	2.00E-53	56299	5.89	63	Glycine max	Storage protein
13	gi|548900	Sucrose-binding protein	552	4.60E-50	60884	6.42	43	Glycine max	Metabolic protein
14	gi|63852207	Beta-conglycinin beta subunit	869	9.10E-82	48358	5.67	61	Glycine max	Storage protein
15	gi|195640660	Formate dehydrogenase	173	3.60E-12	41678	6.32	32	Zea mays	Metabolic protein
16	P04776	Glycinin	413	1.30E-37	56299	5.89	36	Glycine max	Storage protein
17	P04776	Glycinin	424	1.00E-38	56299	5.89	36	Glycine max	Storage protein
18	gi|6573169	Isoflavone reductase homolog 1	454	2.90E-40	33980	5.75	62	Glycine max	Metabolic protein
19	gi|255637416	Unknown	268	1.10E-21	34373	6.19	53	Glycine max	-
20	gi|22597178	Alcohol dehydrogenase 1	387	1.40E-33	40722	6.19	54	Glycine max	Metabolic protein
21	P09756	Chlorophyll a-b binding protein 3,	193	1.30E-15	27901	5.46	28	Glycine max	Photo synthesis related protein
22	P01071	Trypsin inhibitor B	618	4.00E-58	20256	4.66	56	Glycine max	Protein modification
23	P25272	Kunitz-type trypsin inhibitor KTI1	470	2.50E-43	22817	4.97	45	Glycine max	Protein modification
24	P01071	Trypsin inhibitor B	502	1.60E-46	20256	4.66	56	Glycine max	Protein modification

Up-regulated proteins									
26	gi|255639115	Formate dehydrogenase	705	2.30E-65	42140	6.9	74	Glycine max	Metabolic protein
27	gi|14970841	Beta-galactosidase	130	7.20E-08	92316	7.2	11	Fragaria x ananassa	Metabolic protein
28	P41916	GTP-binding nuclear protein Ran-1	517	5.10E-48	25602	6.38	47	Arabidopsis thaliana	Storage protein
29	gi|147945633	MnSOD	354	2.90E-30	26690	8.56	59	Glycine max	Stress related protein
31	Q96452	14-3-3-like protein	227	5.10E-19	29304	4.72	48	Glycine max	Metabolic protein
32	gi|170010	Late embryongenesis abundant protein	144	2.90E-09	50613	6.33	20	Glycine max	Stress related protein
33	gi|4102190	35 kDa seed maturation protein	267	1.40E-21	35320	5.96	49	Glycine max	Storage protein
34	gi|33303618	LEA protein (dehydrine)	62	4.30E-01	25354	6.19	24	Glycine max	Stress related protein
35	gi|5733686	Maturation protein pPM32	81	0.0055	18871	5.49	45	Glycine max	Metabolic protein
36	gi|1762955	Late embryogenesis-abundant protein	82	0.0051	11485	5.52	40	Glycine max	Stress related protein
37	gi|11385463	Glutathione S-transferase GST 24	404	2.90E-35	24928	5.74	62	Glycine max	Stress related protein

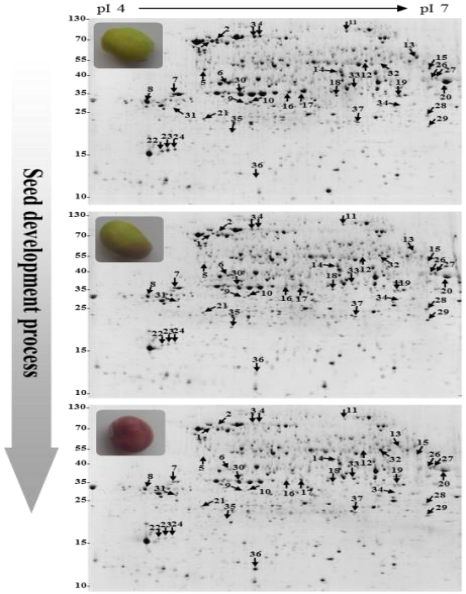

Fig 1. 2-DE analysis of seed coat protein during seed maturation of Seonheuk cultivar. Distinct protein spots detected on the 2-DE gel are marked by arrows. A total of 500 μg protein was used for each 2-DE gel. Proteins were resolved using a linear gradient pI 4–7 in the first dimension and 13% SDS-polyacrylamide gels in the second dimension. Gels were stained with CBB. Positions and numbers of the 36 identified protein spots were indicated by arrows.

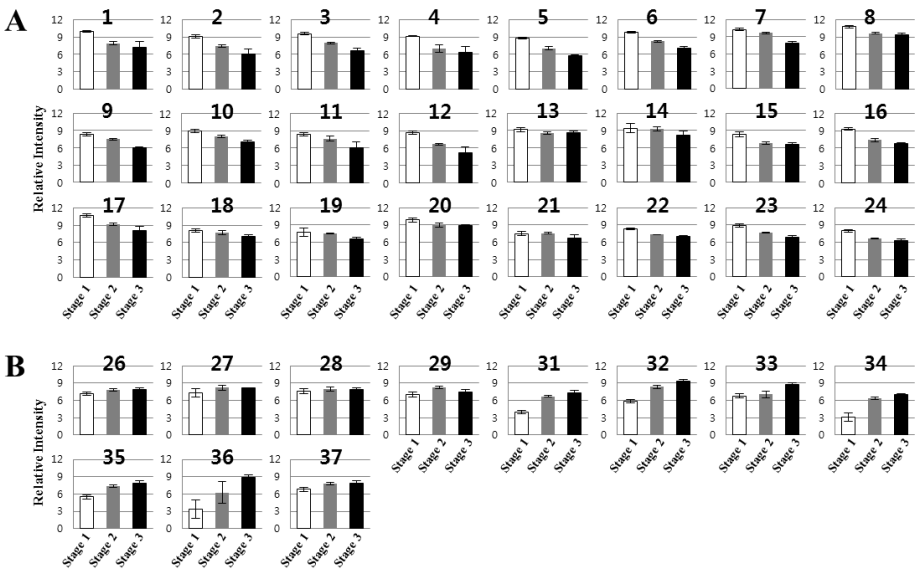

Fig 2. Proteins modulated during soybean seed coat maturation. Stage 1: color was not changed, Stage 2: color was half changed, Stage 3: color was changed.

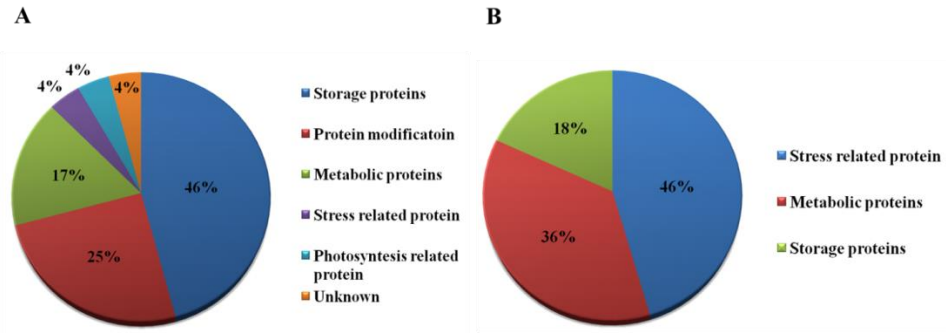

Fig 3. Classification of soybean seed coat proteins identified by 2-DE coupled with MALDI-TOF/TOF MS. Down-regulated proteins (A) and up-regulated proteins (B) were classified from the NCBI database according to their predicted function.

The decrease in the abundance of these proteins is probably caused by miniature metabolic processes during color changing stage in seed coats.

Up-regulated seed coat proteins in Seonheuk cultivar

The following stress related proteins were highly expressed during seed coat maturation: manganese-superoxide dismutase (MnSOD, spot 29), late embryogenesis abundant protein (LEA protein, spots 32, 34, 36), and glutathione S-transferase GST 24 (GST, spot 37). MnSOD and GST have antioxidant functions and may be associated with seed dormancy. Interestingly, three LEA proteins were identified. LEA proteins are hydrophilic proteins that accumulate in desiccation-tolerant tissues and are thus prominent in seeds (Grelet et al., 2005). These proteins are expected to play a protective role during dehydration and oxidative stresses. Several metabolic proteins were up-regulated during seed coat maturation. One such protein, beta-galactosidase (spot 27), is involved in the glycosyl hydrolase family and has been detected in a wide range of plants and plant tissues (Dey and Campillo, 1984). Beta-galactosidase activity releases terminal galactose residues from these complex carbohydrates and plays a role in senescence and fruit ripening. Additionally, 14-3-3-like proteins (spot 31) play important roles in various aspects of plant and metabolic processes (Roberts, 2003). Proteomic analysis of nitrogen mobilization in pea leaves during seed filling identified three 14-3-3-like proteins, two of which increased and one that decreased in abundance (Schiltz et al., 2004). Seed maturation proteins (SMPs, spot 33) were also found to be synthesized during the later stages of seed development.

Comparison of protein expression patterns in Seonheuk and Geomjeong 2 during seed coat maturation

Among the 36 identified proteins, ten were differentially expressed in Seonheuk and Geomjeong 2 cultivars. Protein expression of three trypsin inhibitors (spots 22, 23, 24), SBP (spot 13), FDH (spot 15), and IFR1 (spot 18) decreased in Seonheuk, while it increased in Geomjeong 2 (Fig. 4). In addition, expression of MnSOD (spot 29), GST (spot 37), beta-galactosidase (spot 27), and LEA (spot 32) increased in Seonheuk, while it decreased in Geomjeong 2 (Fig. 4). It was recently reported that the antioxidant effects of black soybeans are related to their phenolic pigments in the seed coats (Takahashi et al., 2005). Ha et al. (2009) reported that contents of anthocyanin and isoflavone in Geomjeong 2 were much higher than those of Seonheuk cultivar (Ha et al., 2009). Moreover, cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, pelargonidin-3-*O*-glucoside, 6"-*O*-malonyldaidzin, 6"-*O*-malonylgenistin, linoleic acid (C18:2), and linolenic acid (C18:3) composition increased significantly

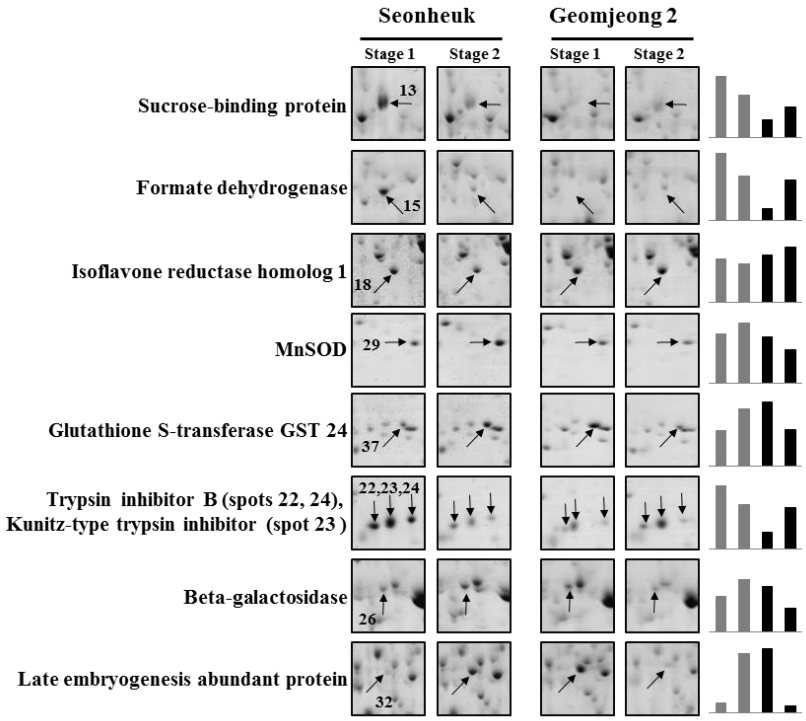

Fig 4. Seed coat proteins differentially expressed between Seonheuk and Geomjeong 2 cultivars. Differentially expressed proteins were marked by numbers and arrows during stage 1 and 2, but stage 3 showed little difference between Seonheuk and Geomjeong 2 cultivars.

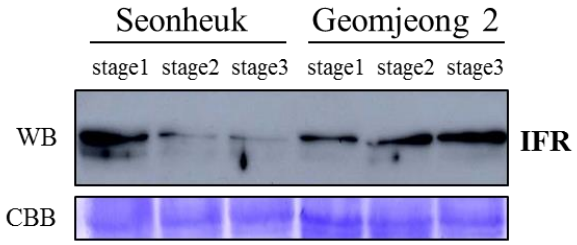

Fig 5. Detection of IFR expression in Seonheuk and Geomjeong 2 cultivars using Western blotting approach. The colloidal Coomassie brilliant blue (CBB)-stained PVDF area for IFR is shown.

in Geomjeong 2 cultivar (Ha et al., 2009). Taken together, these findings indicate that several proteins may be differentially expressed between Seonheuk and Geomjeong 2 cultivars; therefore, we conducted Western blot analysis using rice IFR for checking antioxidant activity. IFR was increased in Geomjeong cultivar, while it was decreased in Seonheuk during seed coat maturation (Fig. 5). These results suggest that content of anthocyanin and isoflavone in seed coats may cause different modulation of metabolic proteins and different antioxidant activity between Seonheuk and Geomjeong 2 cultivars.

Materials and Methods

Plant material and growth

The black seed coated soybean cultivars, Seonheuk and Geomjeong 2, developed by the National Institute of Crop Science (NICS) were selected for this study. Cultivars were grown at the experimental field of the Department of Functional Crops Institute, NICS, RDA at Miryang, South Korea (latitude 35°N) in 2011.

Protein extraction

Extraction of proteins was conducted according to Kim et al. (2001). Briefly, Soybean seed coats were separated from sequential developing seeds in liquid nitrogen using tweezers, after which they were powdered in liquid nitrogen using a pestle. Powdered tissues of soybean seed coats were subsequently homogenized with 5 mL of Mg/NP-40 buffer containing 0.5 M Tris-HCI (pH 8.3), 2% (v/v) NP-40, 20 mM $MgCl_2$ and 2% β-mercaptoethanol, after which they were centrifuged at 12,000 × *g* for 10 min at 4°C. Next, the supernatant was mixed thoroughly with an equal volume of water-saturated phenol, and then centrifuged at 12,000 × *g* for 10 min at 4°C. The phenol phase was mixed with four volumes of methanol containing 0.1 M ammonium acetate, after which protein was precipitated at -20°C for 1 hr and then centrifuged at 12,000 × *g* for 10 min at 4°C. The pellet was then washed 2-3 times with 5 ml of methanol containing 0.1 M ammonium acetate, after which it was centrifuged at 12,000 × *g* for 5 min at 4°C. Finally, the pellet was rinsed with 5 ml of ice-cold acetone repeatedly until a white pellet was obtained, which was

stored in 80% acetone at -20°C until the protein content was measured using a 2-D quant kit (GE Healthcare, Waukesha, WI, USA).

2-DE analysis

2-DE analysis was performed as previously described (Kim et al., 2008). Briefly, the rehydration solution for IPG gel consisted of 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). The IPG (24 cm) strips were rehydrated in rehydration solution containing equivalent samples (500 μg). IPG focusing was then performed at 50 V for 4 hr, 100 V for 1 hr, 500 V for 1 hr, 1000 V for 1 hr, 2000 V for 1 hr, 4000 V for 2 hr, 8000 V for 5 hr, 8000 V for 9 hr, and 50 V for 6 hr using the IPGphore II platform (GE Healthcare, Waukesha, WI, USA). Each focused IPG strip was then placed into a 20 ml screw-cap tube with 5 ml of equilibration buffer that contained 30% (v/v) glycerol, 2% (w/v) SDS, 50 mM Tris-HCl (pH 6.8), 100 mM DTT, 6 M urea, and 0.1 mg/ml bromophenol blue. Strips were then agitated gently at room temperature for 20 min, after which a second equilibration was performed with 55 mM iodoacetamide solution (without DTT) in equilibration buffer under dark conditions for 20 min with gentle agitation. The second dimension analysis was carried out on 13% SDS-polyacrylamide gels, after which 2-DE gels were stained by colloidal Coomassie Brilliant Blue (CBB) (Kim et al., 2008). Images were acquired using a transmissive scanner (PowerLook 1120, UMAX) with a 32 bit pixel depth, 300 dpi resolution, and brightness and contrast set to default. Gel spots were automatically detected using the Image Master 2D Platinum software 6.0 (GE Healthcare, Waukesha, WI, USA). The volume of each spot was then normalized as an average of the volume of spots on the gel.

In-gel digestion

In-gel digestion was carried out according to the method described by Kim et al. (2008). Briefly, CBB-stained protein spots were excised, washed with 50% (v/v) acetonitrile (ACN) in 0.1 M NH_4HCO_3 and vacuum-dried. Dried gels were then treated with 10 mM DTT in 0.1 M NH_4HCO_3 for 45 min at 55°C, after which DTT solution was immediately replaced with 55 mM iodoacetamide in 0.1 M NH_4HCO_3 and samples were incubated for 30 min at room temperature in the dark. Gel pieces were then washed with 50% ACN in 0.1 M NH_4HCO_3 and digested with 12.5 ng/μl trypsin and 25 mM NH_4HCO_3 in 10 μl of digesting solution overnight at 37°C and air-dried.

MALDI-TOF/TOF MS

MALDI-TOF/TOF MS analysis was conducted as previously described (Kwon et al., 2010). Briefly, MS and MS/MS analysis were performed on an ABI 4800 Plus TOF-TOF Mass Spectrometer (Applied Biosystems, Framingham, MA, USA), which uses a 200 Hz ND:YAG laser operating at 355 nm. The ten 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 800–1,000 consecutive laser shots. During MS/MS analysis, air was used as the collision gas. Data were subjected to a Mass Standard Kit for the 4700 Proteomics Analyzer (calibration Mixture 1). MS/MS spectra were searched against the NCBI database by Protein Pilot v.3.0 (with MASCOT as the database search engine) with peptide and fragment ion mass tolerances of 50 ppm. Carbamidomethylation of cysteines and oxidation of methionines were allowed during the peptide search. In addition, one missing trypsin cleavage was allowed. The peptide mass tolerance and fragment mass tolerance of the selected 95 proteins were set to 50 ppm. High confidence identifications had statistically significant search scores (> 95% confidence), were equivalent to MASCOT expected value ($p < 0.05$), were consistent with the protein's experimental p*I* and MW, and accounted for the majority of ions present in the mass spectra.

Statistical analysis

Spot volumes were used to determine the relative concentration of proteins in the experimental samples. The spot volumes from different gels but the same experimental variables can be combined to give a refined estimate of the optical volume for the matched spots by treatment. Statistical analyses of volumes values were performed using the analysis of variance (ANOVA) to determine statistically different values at a significance of $p \leq 0.05$.

Western blot analysis

Total protein (20 μg) was extracted with Mg/NP-40 extraction buffer as previously described (Kim et al., 2004). The total proteins were separated by 12% SDS-PAGE, then transferred to a PVDF membrane (Millipore, Massachusetts, USA) using a semidry electrophoretic apparatus (Hoefer, Holliston, MA). The blotted membrane was blocked for 4 h at room temperature in 1 × TTBS buffer (50 mM Tris-HCl, pH 8.2, 0.1% v/v Tween 20, and 150 mM NaCl) with 7% (w/v) skim milk (BD Bio, USA), then incubated for 2 h after the addition of primary antibody (diluted to 1:1,000). Next, the membranes were sequentially incubated with purified rice isoflavone reductase-like protein (OsIRL), after which they were washed 3 times for 20 min each with 1 × TTBS. A secondary anti-rabbit IgG antibody conjugated with horseradish peroxidase diluted 1:10,000 in 1 × TTBS was used for immunodetection. Signals were detected via ECL (Perkin Elmer Life Sciences, Boston, MA).

Conclusions

In this work, the black soybean (Seonheuk and Geomjeong 2 cultivars) seed coat protein composition was deciphered during seed coat maturation based on identification of the most abundant protein components. Among proteins differentially expressed during seed coat production, 36 were storage proteins, while a number of other proteins involved in seed storage, metabolism, and stress were observed. Ten of these proteins were involved in metabolic processes, protein modification or were stress related proteins. These ten proteins may be responsible for the biochemical differences between Seonheuk and Geomjeong 2 cultivars.

Acknowledgements

This study was supported by a grant from the Next-Generation BioGreen 21 Program (SSAC, grant#: PJ009571) and National Agenda Programs for Agricultural R&D (PJ007155) of Rural Development Administration (RDA), Republic of Korea and supported by 2013 Postdoctoral Fellowship of National Institute of Crop Science, Rural Development Administration (RDA), Republic of Korea.

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