



Workshop Report

Utility of proteomics techniques for assessing protein expression

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ABSTRACT

Proteomic technologies are currently used as an effective analytical tool for examining modifications in protein profiles. Understanding the natural variation of soybean seed proteins is necessary to evaluate potential unintended (collateral) effects due to transgenic modifications in genetically modified (GMO) soybeans. We used two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry, and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) to separate, identify and quantify the different classes of soybean seed proteins. Sixteen soybean genotypes, including four wild and twelve cultivated genotypes, belonging to four different subgroups were used as models for protein profile evaluation. Significant variations of allergen and anti-nutritional protein profiles were observed between two different groups, cultivated and wild soybean genotypes. However, only minor variations in protein profiles were observed within the soybean samples from the same group (cultivated or wild). These results may be useful to scientists needing to compare GMO and non-GMO soybeans once additional data are generated on additional soybean varieties and the same varieties grown at different geographical locations.

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1. Introduction

Soybean [*Glycine max* L. (Merr.)], is one of the most important commercial crops grown mainly for supply of edible vegetable oil, high-protein feed supplement and other phytonutrients (Boulter et al., 1980). This has generated significant interest by major agricultural research institutes and biotech industries to enhance the quality of nutrients in soybeans as well as increase resistance against various pests. This has resulted in introduction of several new germplasms in US and other countries around the globe. Therefore it is essential to examine the changes in protein profiles utilizing new analytical tools.

At present, proteomics tools such as two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry, and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) provide accurate and sensitive methods to effectively examine protein profiles. These tools can be used to evaluate protein expression and potential protein modifications and their relative abundance that occurred in GMO soybean (Chassy et al., 2004; Cellini et al., 2004). However, protein extrac-

tion remains a challenge in the accurate analysis of proteins because of the presence of contaminants that could affect the performance of the 2D-PAGE, MALDI-TOF and LC-MS/MS analyses. We have previously carried out a detailed comparison of three commonly used protein extraction methods described in the literature (Hurkman and Tanaka, 1986; Berkelman et al., 1998; Herman et al., 2003) for 2D-PAGE analysis. We have further optimized the previously published method and the modified TCA/acetone developed in our laboratory was used for current analysis (Natarajan et al., 2005).

Soybean is a rich source of proteins for humans and animals. However, some consumers have severe allergic reactions to soy proteins (Bardare et al., 1988). About 16 soybean seed proteins have been reported as allergens in literature (www.AllergenOnline.com; Besler et al., 2000; Weber et al., 2007). Gly m Bd 60K (α subunit β -conglycinin), Gly m Bd 30K (P34) and Gly m Bd 28K are the main allergens in soybean seeds (Besler et al., 2000; Ogawa et al., 2000; Weber et al., 2007; Holzhauser et al., 2008). Recently, Mooney et al. (2004) studied the proteome of a cultivated soybean genotype, *G. max* cv. Jefferson by 2D-PAGE analysis and reported the presence of Gly m Bd 60K and glycinin subunits. Herman et al. (2003) have also analyzed allergen proteins by 2D-PAGE in cultivated soybean cv. Jack, a soybean cultivar in which expression of P34 protein was suppressed. In addition, Kunitz trypsin inhibitor

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(KTI) was reported as one of the anti-nutritional proteins as it acts as a proteinase inhibitor (Liener, 1979; Laskowski and Kato, 1980). KTI is involved in respiratory hypersensitivity reactions, and can reduce the growth and development of herbivorous insects (Jansen et al., 1986; Besler et al., 2000). The other anti-nutritional groups of proteins, Soybean Agglutinin, are carbohydrate-binding proteins and are abundantly present in soybeans.

This report summarizes some of the results obtained in our laboratory over the past few years. In this report, four groups consisting of wild soybean (*Glycine soja*), the cultivated soybean (*G. max*) ancestors of modern N. American soybean cultivars (old), modern N. American soybean (elite), and Asian cultivated soybean landraces that were the immediate results of domestication from the wild soybean, were selected as models for carrying out protein analysis. We have reported the profiles of major allergen and anti-nutritional proteins in 16 soybean genotypes, including wild and cultivated.

2. Materials and methods

2.1. Plant materials

Soybean seeds of wild and cultivated genotypes were obtained from the USDA soybean germplasm collection (Urbana, Illinois): wild soybean (*G. soja*), cultivated soybean (*G. max*) ancestor of modern N American soybean cultivars (old), modern N American soybean cultivars (elite), and Asian cultivated soybean landraces. All the soybean seeds used in the current study were planted in the Beltsville greenhouse and their controls were set using computer program for photoperiod and temperature similar to field condition to minimize protein variations due to environmental conditions. Seeds were stored at -80°C until used.

2.2. Chemicals

Chemicals needed for 2D-PAGE including acrylamide, bis-acrylamide, SDS, TEMED, ammonium persulfate, thiourea, dithiothreitol (DTT), CHAPS and IPG strips were purchased from GE Healthcare (Piscataway, NJ). Tris-HCl (pH 8.8), 2-mercaptoethanol (2-ME), trichloroacetic acid (TCA) and glycerol were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Urea and ampholytes (pH 4.0–7.0) were purchased from Bio-Rad Laboratories (Hercules, CA). Alpha-cyanohydroxycinnamic acid (CHCA) matrix was purchased from Bruker Daltonics (Billerica, MA). Water from a Millipore Milli-RO4 reverse osmosis system was used for making all solutions.

2.3. Protein extraction and 2D-PAGE analysis

Protein extraction from soybean seeds and 2D-PAGE separation were performed according to Natarajan et al. (2005). Proteins were extracted by the modified TCA/acetone method and the proteins concentration was determined by the Bradford method (Bradford, 1976). The first dimension IEF was performed using 13 cm pH 3.0–10.0 or 4.0–7.0 linear IPG strips in an IPGphor apparatus (GE Healthcare, Piscataway, NJ), according to the manufacturer's recommendations. For the second dimension, the IPG strips were incubated with 50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, and 1% DTT for 15 min, acetylated with iodoacetamide 15 min two times, and subsequently placed onto 12% SDS-polyacrylamide gels prepared as described by Laemmli (1970) with a protein molecular weight standard lane in each gel. Electrophoresis was performed using a Hoefer SE 600 Ruby electrophoresis unit (GE Healthcare) according to the manufacturer's recommendations. The 2D-PAGE gels were stained with

Colloidal Coomassie Blue G-250 as described by Newsholme et al. (2000). The gels were stored in 20% ammonium sulfate solution and scanned using laser densitometry (GE Healthcare). Triplicate samples were used for soybean seed protein extraction and 2D-PAGE analysis.

2.4. In-gel digestion of protein spots

Protein spots were isolated and excised carefully from the stained gel. The protein spots were washed first with distilled water to remove ammonium sulfate and then with 50% acetonitrile containing 25 mM ammonium bicarbonate to destain the gel plug. Then the gel plug was dehydrated with 100% acetonitrile, dried under vacuum, and re-swollen with 20 μL of 10 $\mu\text{g}/\text{mL}$ trypsin (modified porcine trypsin, sequencing grade, Promega, Madison, WI) in 25 mM ammonium bicarbonate. Overnight digestion was performed at 37 $^{\circ}\text{C}$. The resulting tryptic fragments were extracted with 50% acetonitrile and 5% trifluoroacetic acid with sonication. The extract was dried and dissolved in 50% acetonitrile and 0.1% trifluoroacetic acid.

2.5. Protein identification

Protein spots were analyzed by MALDI-TOF-MS and LC-MS/MS using the protocols developed in our laboratory (Natarajan et al., 2005). To analyze tryptic peptides, a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, Framingham, MA) operated in positive ion reflector mode was used. Samples were co-crystallized with CHCA matrix, and spectra were acquired with 50 shots of a 337 nm nitrogen laser operating at 20 Hz. Spectra were calibrated using the trypsin autolysis peaks as internal standards at m/z 842.51 and 2211.10. The raw data were processed by Sequest to generate DTA files for database searching. The *merge.pl* script from Matrix Science (Boston, MA) was used to convert multiple Sequest DTA files into a single Mascot generic file suitable for searching in Mascot. For MALDI-TOF-MS data to qualify as a positive identification, a protein score had to equal or exceed the minimum significant score of 64. For LC-MS/MS, the positive identification required a minimum of two unique peptides with at least one peptide having a significant ion score.

3. Results and discussion

In this study, we have compared the composition of major allergen proteins and anti-nutritional proteins in wild *G. soja* and cultivated *G. max* seeds by 2D-PAGE separation technique with two pH ranges (3.0–10.0 and 4.0–7.0) by combined proteomic tools, MALDI-TOF-MS and LC-MS/MS. We focused on the characterization of three major allergen proteins, Gly m Bd 60K, Gly m Bd 30K, and Gly m Bd 28K and two anti-nutritional proteins, KTI and soybean agglutinin from 16 soybean genotypes. Representative gel images obtained in this study are presented in Fig. 1. In Table 1, spot numbers of allergen and anti-nutritional proteins observed in each genotype are listed. Our results showed that the overall distribution pattern of allergen proteins identified in this study were similar in both wild and cultivated genotypes (Fig. 1A, C and B, D). However, major variation was observed in terms of the number of spots and their intensities between genotypes (Table 1 and Fig. 1).

3.1. Proteomic analysis of soybean allergen proteins

Soybean Gly m Bd 60K are described as storage seed proteins. It includes β -conglycinin and glycinin. Only the α subunit of β -conglycinin is allergenic for some consumers (Ogawa et al., 1995). In

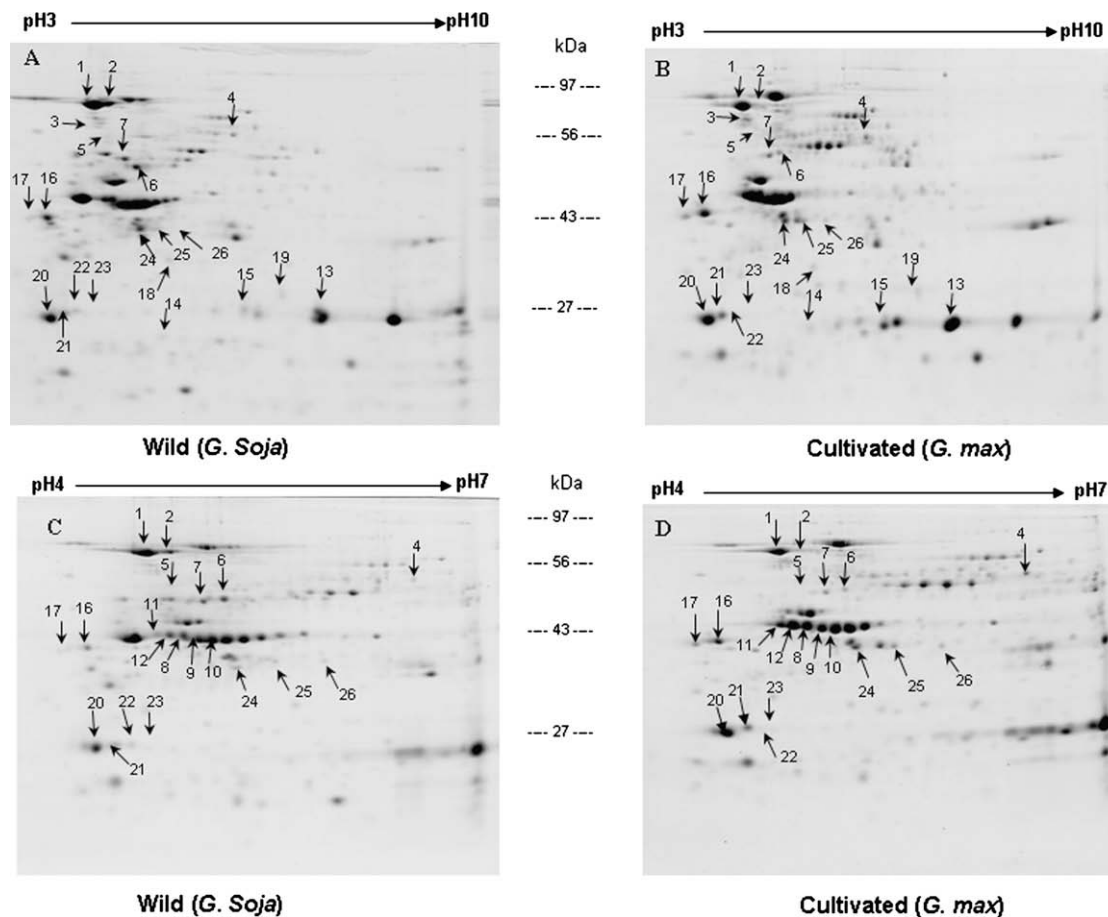


Fig. 1. Two-dimensional electrophoresis profile of allergen proteins of wild, *G. soja* (A and C) and cultivated, *G. max* (B and D). The first dimension IEF was performed using pH 3.0–10.0 (A and B) or 4.0–7.0 (C and D) linear IPG strips. Numbered arrows indicate the polypeptides referred to in the text and table.

Table 1
Soybean allergen and anti-nutritional proteins composition in 16 soybean genotypes.

Soybean genotypes	Accession number	Allergenic protein				Anti-nutritional Proteins	
		Gly m Bd 60K (storage proteins)				KTI	Soybean agglutinin
		α -Subunit of β conglycinin	Glycinin G2	Gly m Bd 30K	Gly m Bd 28K		
Wild (<i>G. soja</i>)	PI 366120	1, 3, 4, 5, 6, 7	8, 9, 10, 11, 12, 13, 14, 15	16	18, 19	20,21,22,23	24, 25
	PI 393551	1, 2, 3, 4, 5, 6, 7	8, 9, 10, 11, 12, 13, 14, 15	16	18, 19	20,21,22	24
	PI 407027	1, 2, 3, 4, 6, 7	8, 9, 10, 11, 12, 13, 14, 15	16, 17	18, 19	20,21,22	24,25
	PI 407282	1, 3, 4, 5, 6, 7	8, 9, 10, 11, 12, 13, 14, 15	16, 17	18, 19	20,21,22	24
Landrace (<i>G. max</i>)	PI 423954	1, 3, 4, 6, 7	8, 9, 10, 11, 12, 13, 14, 15	16, 17	18, 19	20,21	24,25,26
	PI 89138	1, 2, 3, 4, 5, 6, 7	8, 9, 10, 11, 12, 13, 14, 15	16, 17	18, 19	20,21	24,25,26
	PI 59477	1, 3, 4, 6, 7	8, 9, 10, 11, 12, 13, 14, 15	16, 17	18, 19	20,21	24,25,26
	PI 59845	1, 3, 4, 6, 7	8, 9, 10, 11, 12, 13, 14, 15	16, 17	18, 19	20,21	24,25,26
Ancestor (old)	PI 548445	1, 3, 4	8, 9, 10, 11, 12, 13, 14	16, 17	18, 19	20,21	24,25,26
	PI 548298	1, 3, 4	8, 9, 10, 11, 12, 13, 14, 15	16, 17	18, 19	20,21	24,25,26
	PI 548318	1, 3, 4, 6, 7	8, 9, 10, 11, 12, 13, 14, 15	16, 17	18, 19	20,21	24,25
	PI 548362	1, 3, 4, 6, 7	8, 9, 10, 11, 12, 13, 14, 15	16, 17	18, 19	20,21	24,25
Modern (elite)	PI 536635	1, 3, 4	8, 9, 10, 11, 12, 13, 14, 15	16, 17	18, 19	20,21	24,25,26
	PI 533655	1, 3, 4, 6, 7	8, 9, 10, 11, 12, 13, 14, 15	16, 17	18, 19	20,21	24,25,26
	PI 525453	1, 3, 4, 6, 7	8, 9, 10, 11, 12, 13, 14, 15	16, 17	18, 19	20,21	24,25,26
	PI 513382	1, 3, 4	8, 9, 10, 11, 12, 13, 14, 15	16, 17	18, 19	20,21	24,25,26

addition, the acidic polypeptides of G1 and G2 subunits of glycinin are also reported to be allergenic (Beardslee et al., 2000; Xiang et al., 2004). Beardslee et al. (2000) reported that soybean glycinin G1 acidic polypeptide shares IgE epitopes with peanut allergen Ara h 3. The allergenic potential of all soybean allergen has been demonstrated by elevated serum IgE binding ability in soy sensitive patients. The acidic polypeptides of G1 subunit even though known

for allergenic reactions were not observed in our study, probably because these proteins are too low in abundance to be detected by the current 2D-PAGE method. The accurate localization on 2D-PAGE profile of α subunit of β -conglycinin and glycinin subunits in cultivated soybeans has been previously reported (Mooney et al., 2004; Herman et al., 2003). However, limited information is available about the variation of these allergens among different

soybean genotypes. In another study, the expression of α and α subunits of β -conglycinin was suppressed by sequence-mediated gene silencing in transgenic soybean seed (Kinney et al., 2001).

In our study, we observed considerable variation in protein spot number in the α subunit of β -conglycinin. The soybeans from all wild and landrace genotypes showed five spots (spots 1, 3, 4, 6, and 7) of the α subunit. One wild (PI 393551) and one landrace (PI 89138) genotype showed two additional spots (spots 2 and 5), and two wild genotypes (PI 366120 and PI 407282) had only one additional protein spot (spot 5). In all ancestral and modern genotypes, we observed spots 1, 3, and 4 of the α subunit, while two ancestors (PI 548318 and PI 548362) and two modern cultivars (PI 533655 and PI 525453) showed two additional spots (spots 6 and 7).

The glycinin allergens showed eight spots (spots 8–15) and are identified as G2 subunits (Tables 1 and 2). The distribution of glycinin G2 subunits was very similar among these genotypes. Fifteen genotypes showed eight spots (spots 8–15), but only one ancestral genotype (PI 548445) showed seven spots of G2 subunits (spots 8–14). The presence of multiple spots of β -conglycinin and glycinin (Fig. 1) could be the result of proteolysis, or posttranslational modifications or products of a multi-gene family.

Gly m Bd 30K is another protein of soybean designated as a major allergen. This protein was previously described in soybean seed as 34-kD vacuolar protein, P34 (Ogawa et al., 1993). The P34 protein can be detected in a wide variety of soybean processed foods (Tsuji et al., 1995). The elimination of P34 from soybean seeds would enhance food safety and make the use of soybean products available to sensitive individuals. Using a monoclonal antibody against P34, Yaklich et al. (1999) found that P34 was present in the genetic diversity of soybean with protein intensity variation. In our study P34 is present in all these 16 soybean genotypes. However, two wild soybean genotypes (PI 366120 and PI 393551) showed only one spot (spot 16) while the other 14 soybean genotypes, two spots (spots 16 and 17) of P34 were detected. Joseph et al. (2006) screened 16266 accessions of USDA soybean germplasm, and identified 12 genotypes as P34 null lines.

Gly m Bd 28K is a less abundant and major allergen protein of soybean (Xiang et al., 2004) that was originally isolated from soybean meal as a 28 kDa glycosylated protein (Tsuji et al., 1997). In the present study, two spots (spots 18 and 19) of Gly m Bd 28K were identified by MALDI-TOF and LC-MS/MS in all 16 genotypes (Tables 1 and 2). No major variation of this protein is observed in these genotypes. A mutant line of soybean *G. max*, cv Tohoku 124, with absence of β -conglycinin also lacks Gly m Bd 28K (Samoto et al., 1997). Tsuji et al. (2001) reported that Gly m Bd 28K was about twice as large as anticipated from its initial discovery as a 28 kDa polypeptide by one-dimensional SDS-PAGE. The Gly m Bd 28K allergen is probably processed into two smaller polypeptides of 240 and 212 amino acids in the soybean seed, and both portions are expected to be present in soybean-derived foods (Xiang et al., 2004; Tsuji et al., 2001). These two spots of Gly m Bd 28K probably come from the post-translational processing of the same gene.

3.2. Proteomic analysis of anti-nutritional proteins

Kunitz trypsin inhibitor is one of the abundant anti-nutritional proteins, which can inhibit trypsin, an important animal digestive enzyme (Baur et al., 1996). In addition, KTIs have been characterized as food allergens in humans (Burks et al., 1994; Quirce et al., 2002) and have 32% sequence homology with a rye grass pollen allergen (Vanvan Ree et al., 1995). Our results showed that although the overall distribution patterns of KTI protein spots are quite similar in all 16 genotypes we have tested, the number of protein spots and their intensities varied among these genotypes. Three protein spots (spots 20, 21, and 22) of KTI were identified by MALDI-TOF-MS and database searches in three wild soybean varieties, PI 393551, PI 407027, and PI 407282. The wild genotype PI 366120 also showed three protein spots (spots 20, 21, and 22) as KTI and one very close protein spot (spot 23). We were unable to identify the spot 23 because of low MOWSE score and low protein intensity. This spot might be the product of post-translational modification of

Table 2
Soybean allergen/anti-nutritional proteins identified by MALDI-TOF/LC-MS/MS.

S. No.	Theoretical pI/Mr	Protein identity	Peptides matched	Sequence coverage (%)	MOWSE score	Expect value	NCBI Accession No.
1	4.92/63127	α Subunit of β conglycinin	25	39	217	3.00E-17	gi 9967357
2	4.92/63127	α Subunit of β conglycinin	25	41	167	4.00E-12	gi 9967357
3	4.92/63184	α Subunit of β conglycinin	27	43	250	1.50E-20	gi 9967357
4	4.92/63184	α Subunit of β conglycinin	20	34	204	6.00E-16	gi 9967357
5	5.32/72717	α Subunit of β conglycinin	9	18	73	7.20E-03	gi 15425633
6	5.32/72717	α Subunit of β conglycinin	14	24	112	1.10E-03	gi 15425633
7*	5.32/72717	α Subunit of β conglycinin	9	23	238		gi 51247837
8	5.46/54927	Glycinin G2	9	19	91	1.20E-04	gi 121277
9	5.46/54927	Glycinin G2	9	19	73	7.20E-03	gi 121277
10	5.46/54927	Glycinin G2	8	15	71	1.30E-02	gi 121277
11	5.46/54927	Glycinin G2	9	14	74	6.70E-03	gi 121277
12	5.46/54927	Glycinin G2	12	21	104	6.10E-06	gi 121277
13	5.78/54047	Glycinin G2	6	37	67	3.20E-02	gi 169967
14*	5.46/54961	Glycinin G2	3	9	175		gi 121277
15*	5.46/54961	Glycinin G2	9	14	313		gi 121277
16*	5.74/43136	P34	4	9	102		gi 129353
17*	5.74/43136	P34	2	9	60		gi 129353
18*	5.73/52813	Allergen Gly m Bd 28K	3	6	187		gi 12697782
19	5.73/52780	Allergen Gly m Bd 28K	10	19	97	2.90E-05	gi 12697782
20	4.61/20310	Kunitz trypsin inhibitor	15	53	147	3.00E-10	gi 3318877
21	4.97/22817	Kunitz trypsin inhibitor	8	42	72	1.10E-02	gi 125722
22	4.61/20310	Kunitz trypsin inhibitor	9	44	60	1.40E-01	gi 3318877
24*	5.65/30909	Soybean agglutinin	7	10	244		gi 282898
25	5.15/27555	Soybean agglutinin	9	41	90	1.50E-04	gi 3114258
26*	5.15/27555	Soybean agglutinin	7	30	358		gi 3114258

The symbol * indicates protein spots identified by LC/MS/MS.

one of the other KTI proteins, or an unidentified KTI or an unrelated protein. The ancestor, modern and Asian landraces genotypes showed only two protein spots (spots 20 and 21). Our results were similar to the previous reports, in which polymorphism had been observed among the KTIs of wild soybeans by Western blotting and PAGE analysis (Hymowitz, 1973; Stahlhut and Hymowitz, 1983; Becker-Ritt et al., 2004; Wang et al., 2005).

Soybean agglutinins are anti-nutritional proteins present abundantly in soybeans. The presence of this protein accounts for about 10% of total protein in some legumes. Two wild-type soybeans (PI 366120 and PI 407027) showed two spots (spots 24 and 25, Fig. 1A) of soybean agglutinins, whereas other two wild (PI 393551 and PI 407282) soybean genotype showed one spot (spot 24). All genotypes of landrace and modern soybean genotypes showed no variation with presence of three spots, 24, 25, and 26. Two ancestors (PI 548445 and PI 548298) also showed three spots (spots 24, 25, and 26) and other two genotypes (PI 548318 and PI 548362) showed presence of two spots (spots 24 and 25). We observed minor variation in the distribution of soybean agglutinins between these genotypes. As limited number of genotypes we analyzed for protein profile in this study, additional genotypes with different maturity groups grown at different environments and locations would be needed to understand the natural variation of such proteins.

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References

- Bardare, M., Magnolfi, C., Zani, G., 1988. *Allergy Immunol.* 20, 63–66.
- Baur, X., Pau, M., Czuppon, A., Fruhmman, G., 1996. *Allergy* 51, 326–330.
- Beardslee, T.A., Zeece, M.G., Sarath, G., Markwell, J.P., 2000. *Int. Arch. Allergy Immunol.* 123, 299–307.
- Becker-Ritt, A.B., Mulinari, F., Vasconcelos, I.M., Carlini, C.R., 2004. *J. Sci. Food Agric.* 84, 263–270.
- Berkelman, T., Stenstedt, T., Bjellqvist, B., Laird, N., McDowell, M., Olsson, I., Westermeier, R., 1998. *Handbooks from Amersham Biosciences*, p. 26.
- Besler, M., Helm, R.M., Ogawa, T., 2000. *Int. Sympos. Food Allergens* 2, 1–35.
- Boulter, D., Summerfield, R.J., Bunting, A.H. (Eds.), 1980. *Advanced Legume Science, Proceedings of the International conference*, Royal Botany Garden Publishing, Kew, UK, pp. 127–134.
- Bradford, M.M., 1976. *Anal. Biochem.* 72, 248–254.
- Burks, A.W., Cockrell, G., Connaughton, C., Guin, J., Allen, W., Helm, R.M., 1994. *Int. Arch. Allergy Immunol.* 105, 143–149.
- Cellini, F., Chesson, A., Colquhoun, I., Constable, A., Davies, H.V., Engel, K.H., Gatehouse, A.M., Karenlampi, S., Kok, E.J., Leguay, J.J., Lehesranta, S., Noteborn, H.P., Pedersen, J., Smith, M., 2004. *Food Chem. Toxicol.* 42, 1089–1125.
- Chassy, B., Hlywka, J.J., Kleter, G.A., Kok, E.J., Kuiper, H.A., McGloughlin, M., Munro, I.C., Phipps, R.H., Reid, J.E., 2004. *Compr. Rev. Food Sci. Food Saf.* 3, 38–104.
- Herman, E.M., Helm, R.M., Jung, R., Kinney, A.J., 2003. *Plant Physiol.* 132, 36–43.
- Holzhauser, T., Wackermann, O., Barbara, K., Weber, B., Jensen, C.B., Scibilia, J., Garoffo, L.P., Utsumi, S., Poulsen, L.K., Vieths, S., 2008. *J. Allergy Clin. Immunol.* 18996574.
- Hurkman, W.J., Tanaka, C.K., 1986. *Plant Physiol.* 81, 802–806.
- Hymowitz, T., 1973. *Crop Sci.* 13, 420–421.
- Jansen, D.H., Ryan, C.A., Liener, I.E., Pearce, G., 1986. *J. Chem. Ecol.* 12, 1469–1480.
- Joseph, L.M., Hymowitz, T., Schmidt, M.A., Herman, E.M., 2006. *Corp Sci.* 46, 1755–1763.
- Kinney, A.J., Jung, R., Herman, E.M., 2001. *Plant Cell* 13, 1165–1178.
- Laemmli, U., 1970. *Nature* 227, 680–685.
- Laskowski Jr., M., Kato, I., 1980. *Annu. Rev. Biochem.* 49, 593–626.
- Liener, I.E., 1979. *J. Am. Oil Chem. Soc.* 56, 121–129.
- Mooney, B.P., Krishnan, H.B., Thelen, J.J., 2004. *Phytochemistry* 65, 1733–1744.
- Natarajan, S.S., Xu, C., Caperna, T.J., Garrett, W.M., 2005. *Anal. Biochem.* 342, 214–220.
- Newsholme, S.J., Maleeff, B.F., Steiner, S., Anderson, N.L., Schwartz, L.W., 2000. *Electrophoresis* 21, 2122–2128.
- Ogawa, T., Tsuji, H., Bando, N., Kitamura, K., Zhu, Y.L., Hirano, H., Nishikawa, K., 1993. *Biosci. Biotechnol. Biochem.* 57, 1030–1033.
- Ogawa, T., Bando, N., Tsuji, H., Nishikawa, K., Kitamura, K., 1995. *Biosci. Biotechnol. Biochem.* 59, 831–833.
- Ogawa, T., Samoto, M., Takahashi, K., 2000. *J. Nutr. Sci. Vitaminol.* 46, 271–279.
- Quirce, S., Fernandez-Nieto, M., Polo, F., Sastre, J., 2002. *J. Allergy Clin. Immunol.* 109, 178.
- Samoto, M., Fukuda, Y., Takahashi, K., Tabuchi, K., Hiemori, M., Tsuji, H., Ogawa, T., Kawamura, Y., 1997. *Biosci. Biotechnol. Biochem.* 61, 2148–2150.
- Stahlhut, R.W., Hymowitz, T., 1983. *Crop Sci.* 23, 766–769.
- Tsuji, H., Okada, N., Yamanishi, R., Bando, N., Kimoto, M., Ogawa, T., 1995. *Biosci. Biotechnol. Biochem.* 59, 150–151.
- Tsuji, H., Bando, N., Hiemori, M., Yamanishi, R., Kimoto, M., Nishikawa, K., Ogawa, T., 1997. *Biosci. Biotechnol. Biochem.* 61, 942–947.
- Tsuji, H., Hiemori, M., Kimoto, M., Yamashita, H., Kobatake, R., Adachi, M., Fukuda, T., Bando, N., Okita, M., Utsumi, S., 2001. *Biochem. Biophys. Acta* 1518, 178–182.
- Vanvan Ree, R., Hoffman, D.R., van Dijk, W., Brodard, V., Mahieu, K., Koelman, C.A., Grande, M., van Leeuwen, W.A., Aalberse, R.C., 1995. *J. Allergy Clin. Immunol.* 95, 970–978.
- Wang, K.J., Takahata, Y., Kono, Y., Kaizuma, N., 2005. *Theor. Appl. Genet.* 112, 66–71.
- Weber, B., Barbara, K., Vieths, S., 2007. *J. Allergy Clin. Immunol.* 119, 1489–1496.
- Xiang, P., Haas, E.J., Zeece, M.G., Markwell, J., Sarath, G., 2004. *Planta* 220, 56–63.
- Yaklich, R.W., Helm, R.M., Cockrell, G., Herman, E.M., 1999. *Crop Sci.* 39, 1444–1447.