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Application of Proteomic Tools in Assessment of Soybean Protein Variance



Proteomics & Bioinformatics, August 4-6, 2014, Chicago

Savi.natarajan@ars.usda.gov

Agricultural Research Service (ARS) Mission

- ARS is the U.S. Department of Agriculture's chief scientific in-house research agency. ARS conducts research to develop solutions to agricultural problems of high national priority and provide information access and dissemination in order to:

  - Ensure high-quality safe food and other agricultural products
  - Assess the nutritional needs of Americans
  - Sustain a competitive agricultural economy
  - Enhance the natural resource base and the environment
  - Provide economic opportunities for rural citizens, communities, and society as a whole

Source: USDA

Soybean Genomics and Improvement Laboratory (SG&IL)







Mission: To improve the quality of Soybean and to reduce the costs of production

Research Goals:

1) Define the function and interaction of genes and metabolites controlling soybean resistance to disease

2) Develop genetic markers and genome maps to expedite the deployment of genes

3) Determine and define any unintended effects that may be associated with transgenic soybean

Soybeans

- Second most valuable crop in the US (Annual value ~$41 Billion)
- Inexpensive source of proteins
- More than 90 % of soybean planted in the US is genetically modified (GMO)
- Health issues include allergens and anti-nutrients
- Transgenic technology has emerged as a solution to improve deficiencies
- Critical to feeding future world populations

Source: Council for Biotech. Inf.







GMO Soybeans

Roundup Ready® Soybean (1996)

- Glyphosate herbicide tolerance
- Improved weed control

Genuity® Roundup Ready 2 Yield® (2008)

- Offers the same effective Glyphosate herbicide tolerance
- Same Improved weed control
- Higher yields by producing more beans per pod

Plenish® High Oleic Soybean (2010)

- Low in saturated fat with no trans-fatty acids
- Highest amount of heart-healthy monounsaturated fat (~75% oleic), similar to olive oil
- Enhanced thermostability of the oil for cooking purposes

The increase in income benefits for farmers growing biotech soybean during the 15-year period (1996 to 2010) was US $28.4 billion and for 2010 alone, US $3.3 billion (Brookes and Barfoot, 2012)

Other GMO varieties include…

- Allergen suppression (P34)
- Milk protein (Bovine casein)
- Virus resistance (Bean pod mottle)
- Production of Monoclonal antibody
- White mold resistance (oxalate oxidase)
- High Phytase content
- High Methionine

Source: http://www.plenish.com/Libraries/Resources/Plenish_Grower_Overview.sflb.ashx; http://www.monsanto.com/products/Pages/genuity-roundup-ready-2-yield-soybeans.aspx;James, Clive. 2011. Global Status of Commercialized Biotech/GM Crops: 2011. ISAAA Brief No. 43. ISAAA: Ithaca, New York.

Proteomics

- To improve the probability of detecting any changes in protein profiles, profiling techniques such as proteomics are being evaluated.
- The evaluation of substantial equivalence requires the availability of a database on the natural variation of proteins.
- Such a database is important for determining if a new biotech product falls within or outside the range of natural variation.
- Proteomics allows us to build such a database for protein variation.
- Proteomics can be a useful tool for the identification of precursors, post-translational modifications, and degradation products of proteins.

3 Kinds of Proteomics

- Structural Proteomics
  - High throughput X-ray Crystallography/Modeling
  - High throughput NMR Spectroscopy/Modeling
- Expressional or Analytical Proteomics
  - Electrophoresis, MudPIT, Protein Chips, DNA Chips, 2D-HPLC
  - Mass Spectrometry, Microsequencing
- Functional or Interaction Proteomics
  - HT Functional Assays, Ligand Chips
  - Yeast 2-hybrid, Deletion Analysis, Motif Analysis

Source: Dr. David Wishart, University of Alberta

Research Challenges

- Analytical challenges for better separation and accurate identification of proteins
- Limitations in detecting very large/small, acidic/ basic proteins
- The sensitivity of current staining procedures sets limits on the amount of protein required for loading, identification and quantification
- The comparisons of datasets between different laboratories requires a standardized method to be developed for sample isolation and electrophoresis
- More background data on natural variation are needed, because the environment greatly influences protein abundance

Cellini et al. 2004. Food & Chem. Toxicol.42: 1089-1125

Research Objectives



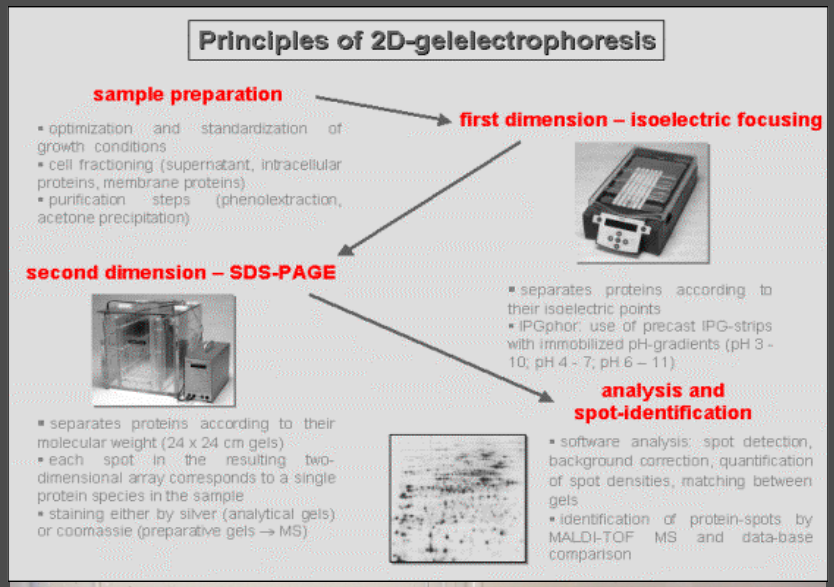




- Develop and standardize effective proteomic methodologies and establish a database of soybean seed proteins
- Determine the natural variation of seed proteins of a wide range of soybeans to establish a baseline
- Determine expression of new protein/changes in proteins/quantity of the protein in transgenic soybean by comparing with the natural variation of seed proteins

Proteomic Tools

2-D gel electrophoresis







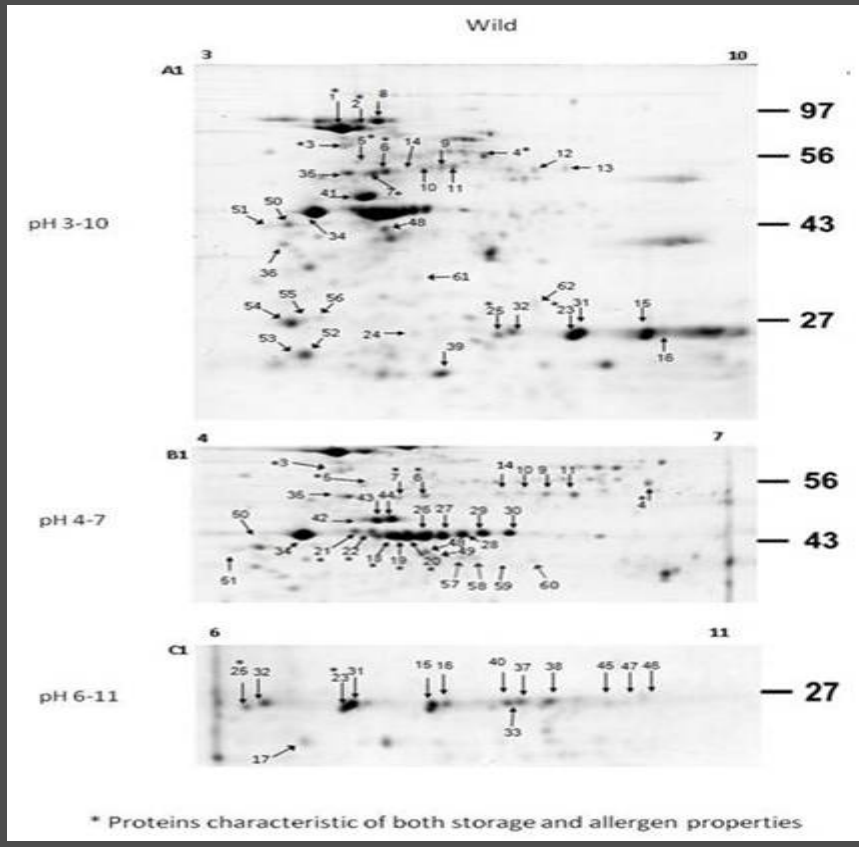
MALDI-TOF-MS



LC-MS/MS

Source: Waters Corporation

2D-PAGE of Soybean Protein



Spot ID	Protein	Class
1-7	α-subunit of β-conglycinin	Storage / Allergen
8	α′-subunit of β-conglycinin	Storage
9-14	β-conglycinin β-subunit	Storage
15-17	glycinin G1/A1aBx subunit	Storage
18-25	glycinin G2/A2B1 precursor	Storage / Allergen
26-33	glycinin subunit G3/A1ab1B	Storage
34-40	glycinin G4/A5A4B3 precursor	Storage
41-47	glycinin G5/A3B4 subunit	Storage
50-51	P34 probable thiol protease	Allergen
48-49, 57-60	Soybean agglutinin	Antinutrient
52-53	SAM22 (Allergen Gly m 4)	Allergen
54-56	Kunitz trypsin inhibitor	Antinutrient
61-62	Allergen Gly m Bd 28K	Allergen

- Developed an efficient protein extraction method for soybean seed proteins
- The modified TCA/acetone extraction method was more efficient in solubilizing abundant and low abundant proteins as compared to the thiourea, urea and phenol extraction methods

Natarajan SS. 2014. J. of Data Mining in Genomics and Proteomics 5:1-3; Natarajan et al. 2005. Anal. Biochem. 342: 214-220

Study the Natural Protein Variation of a Wide Range of Soybeans

- Man made gene transfer (GMO) has been a major issue in crop production.
- Gene transfer by conventional methods is acceptable.
- Our initial research focus is to:

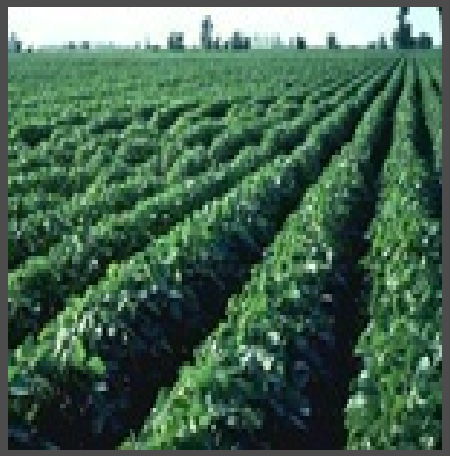
1. Examine the range of variability of proteins produced in a wide range of soybean genotypes that could readily be transferred using conventional breeding.
2. Compare the range of protein variation in transgenic versus normal soybean using proteomics.

Plant Materials to Study Naturally Occurring Variation in Seed Proteins

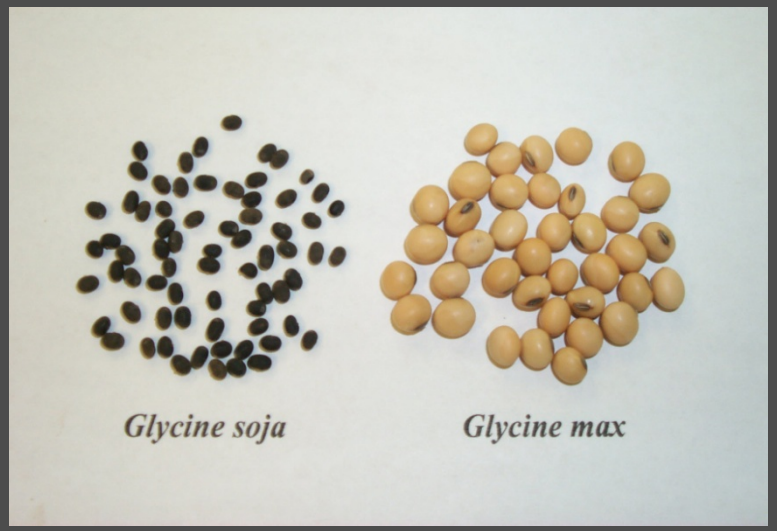
- "Ancestral cultivars" (Old or first progeny) of N. Am. cultivated soybean estimated to have contributed approx. 81.5 % of the genes in current cultivars.
- Asian *G. max* (Landrace and Elite) accessions collected from Chinese Provinces, Korean provinces, Japanese prefectures. Collected from 22-50 degrees N, 104-140 degrees E.
- *G. soja* (Wild) Plant Introductions from China, Korea, and Japan. Collected from 23-50.2 degrees N, 106-140 degrees E.
- Selected 16 soybean genotypes from 4 groups-
  wild, Asian landrace, Ancestors and elite



Wild-*G. soja*



Cultivated-*G. max*



Natural Variation of Soybean Seed Proteins

- Storage proteins
- Allergen proteins
- Anti-nutritional proteins

Storage Proteins

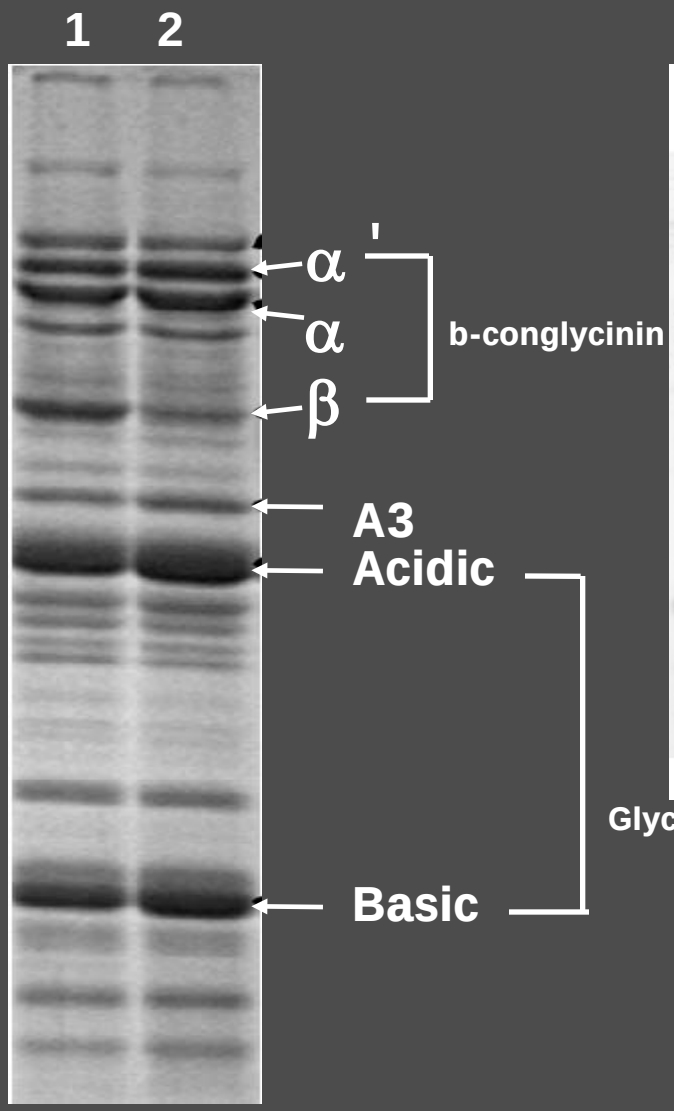
- **β-conglycinin**
  - α subunit of β-conglycinin
  - α’ subunit of β-conglycinin
  - β subunit of β-conglycinin

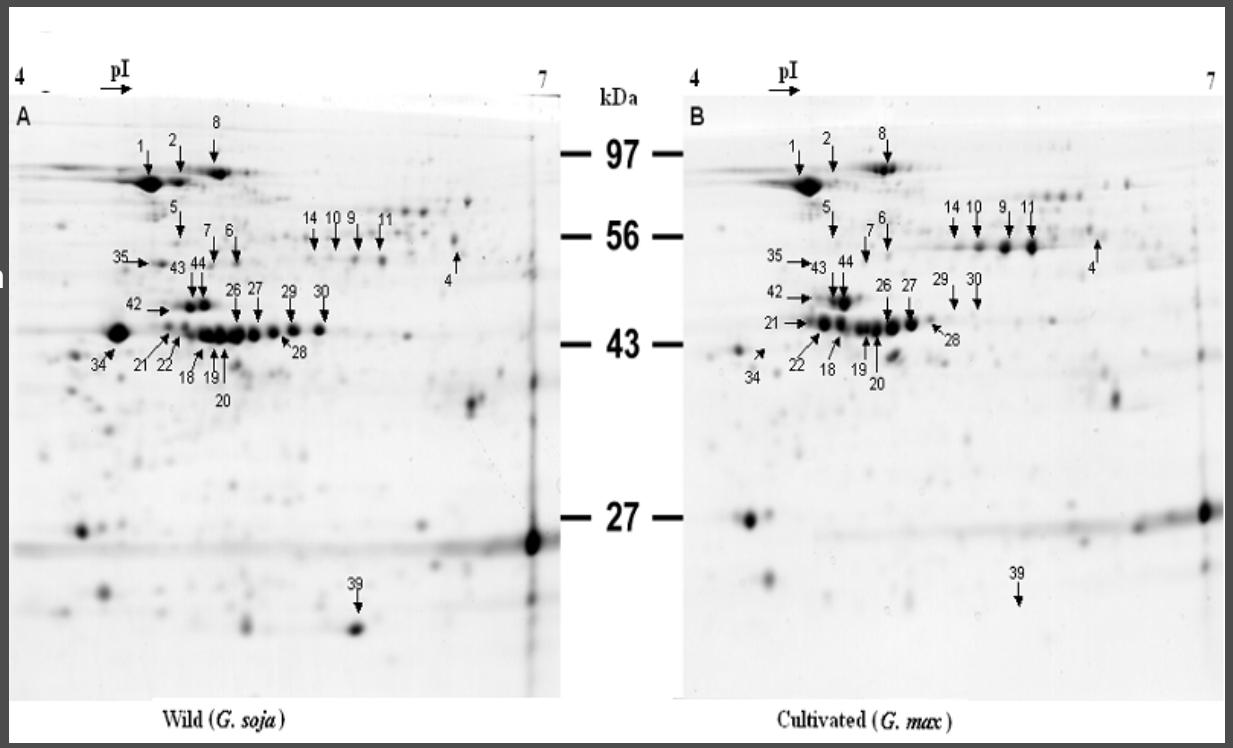
- **Glycinin**
  - Glycinin G1/A1aBx subunit
  - Glycinin G2/A2B1 subunit
  - Glycinin G3/A1ab1B subunit
  - Glycinin G4/A5A4B3 subunit
  - Glycinin G5/A3B4 subunit

- Identified and mapped an additional two genes in soybean variety Resnik, gy6 & Gy7

(*Beillinson et al. 2002. Physiol. Plant. 115: 585-597*)

Variation of Storage Proteins in Wild and Cultivated Soybeans





Natarajan et al. 2006. J. Agric & Food Chem, 54: 3114-3120
Krishnan, Natarajan, Mohmoud & Nelson. 2007. J. Agric. & Food Chem, 55(5):1839-1845
Mohmoud, Natarajan, Bennett, Mawhinney, Wiebold & Krishnan. 2006. J. Agric. Food Chem. 54: 3916-3922

Glycinin

- Important storage protein that lowers cholesterol levels in human serum
- Involved with structural properties of soybean products & Responsible for gel matrix structure
  - Hardness of tofu
  - Unfracturability of tofu
- Gel formed by glycinin is a turbid gel in contrast to transparent gel formed by β-conglycinin

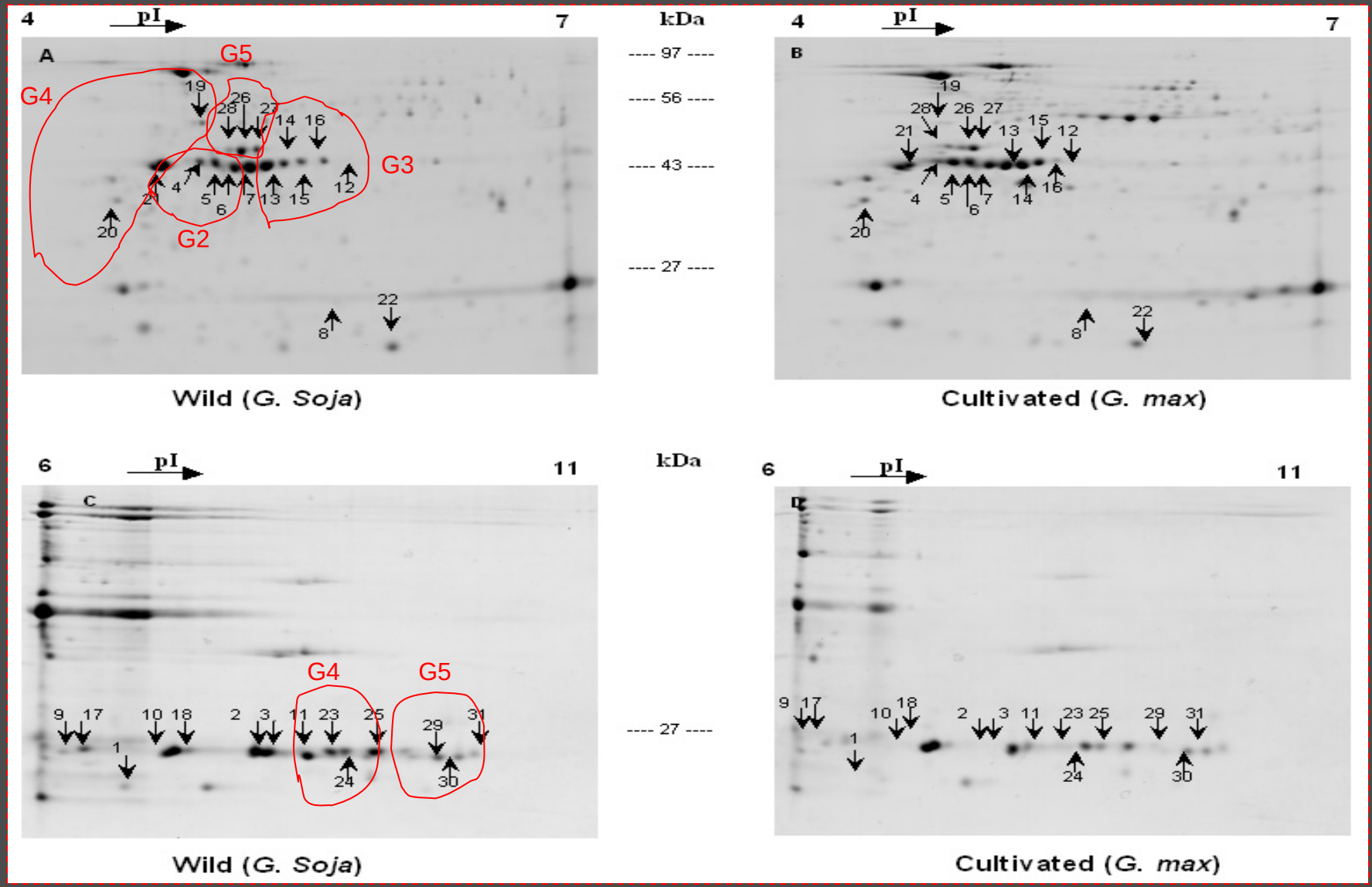
Glycinin Proteins

Two major groups

Group 1-	Glycinin G1 / A1aBx subunit
	Glycinin G2 / A2B1 subunit
	Glycinin G3 / A1ab1B subunit
Group 2-	Glycinin G4 / A5A4B3 subunit
	Glycinin G5 / A3B4 subunit

- 84% homology within the group
- 45-49% homology between the group
- Various subunits play an important role in tofu gel formation

2D-PAGE of Glycinin Proteins



Natarajan et al., 2007. Pl. Physiol. & Biochem. 45:436-444

Allergens & Anti-Nutrients of Soybeans

Allergens

- Gly m Bd 60K (α, α' and β subunit of β-conglycinin, acidic polypeptides of G1 and glycinin G2)
- Gly m Bd 30K (Soybean vacuolar protein)
- Gly m Bd 28K (closely related to peanut Ara h 1)
- Soybean hydrophobic proteins (Gly m 1.0101, Gly m 1.0102)
- Soybean hull protein (Gly m 2)
- Soybean profilin (Gly m 3, homologous to Bet v 2, a birch pollen allergen with sequence identity of 73%)

Anti-nutrients

- Lectins
- Saponins
- Protease inhibitors
- Phytic Acid
- Lipoxygenase

Natarajan et al. 2006. J. pl. Biochem & Biotech. 15: 103-108

Cordle 2004. J. Nutr. 134: 1213S-1219S

Beardslee et al. 2000. Int. Arch. Allergy. Immunol. 123: 299

Krishnan et al. 2009, 57: 938-943

Variation of Allergen Proteins in Sixteen Soybean Genotypes

Soybean allergen composition in sixteen genotypes

Soybean genotypes	Accession	Protein Spot Number of Allergenic Proteins			
		Gly m Bd 60 K		Gly m Bd 30 K	Gly m Bd 28 K
		α subunit of β conglycinin	Glycinin G2		
Wild (*G. soja*)	PI366120	1, 3, 4, 5, 6, 7	8, 9, 10, 11, 12,13, 14, 15	16	18, 19
	PI393551	1, 2, 3, 4, 5, 6, 7	8, 9, 10, 11, 12,13, 14, 15	16	18, 19
	PI407027	1, 2, 3, 4, 6, 7	8, 9, 10, 11, 12,13, 14, 15	16, 17	18, 19
	PI407282	1, 3, 4, 5, 6, 7	8, 9, 10, 11, 12,13, 14, 15	16, 17	18, 19
Landrace (*G. max*)	PI423954	1, 3, 4, 6, 7	8, 9, 10, 11, 12,13, 14, 15	16, 17	18, 19
	PI89138	1, 2, 3, 4, 5, 6, 7	8, 9, 10, 11, 12,13, 14, 15	16, 17	18, 19
	PI594777	1, 3, 4, 6, 7	8, 9, 10, 11, 12,13, 14, 15	16, 17	18, 19
	PI59845	1, 3, 4, 6, 7	8, 9, 10, 11, 12,13, 14, 15	16, 17	18, 19
Ancestor (old)	PI548445	1, 3, 4	8, 9, 10, 11, 12, 13, 14	16, 17	18, 19
	PI548298	1, 3, 4	8, 9, 10, 11, 12,13, 14, 15	16, 17	18, 19
	PI548318	1, 3, 4, 6, 7	8, 9, 10, 11, 12,13, 14, 15	16, 17	18, 19
	PI548362	1, 3, 4, 6, 7	8, 9, 10, 11, 12,13, 14, 15	16, 17	18, 19
Modern (elite)	PI536635	1, 3, 4	8, 9, 10, 11, 12,13, 14, 15	16, 17	18, 19
	PI533655	1, 3, 4, 6, 7	8, 9, 10, 11, 12,13, 14, 15	16, 17	18, 19
	PI525453	1, 3, 4, 6, 7	8, 9, 10, 11, 12,13, 14, 15	16, 17	18, 19
	PI513382	1, 3, 4	8, 9, 10, 11, 12,13, 14, 15	16, 17	18, 19

Characterized allergen proteins in sixteen soybean genotypes from four groups

Major proteomic variation was observed between wild and cultivated soybean genotypes rather than among genotypes in the same group

Xu, Caperna, Garrett, Cregan, Bae, Luthria & Natarajan. 2007. J. of the Sci. of Food and Agric. 87: 2511-2518

Natarajan et al. 2006. J. Pl. Biochem & Biotech. 15:103-108

Soybean Anti-nutritional Proteins

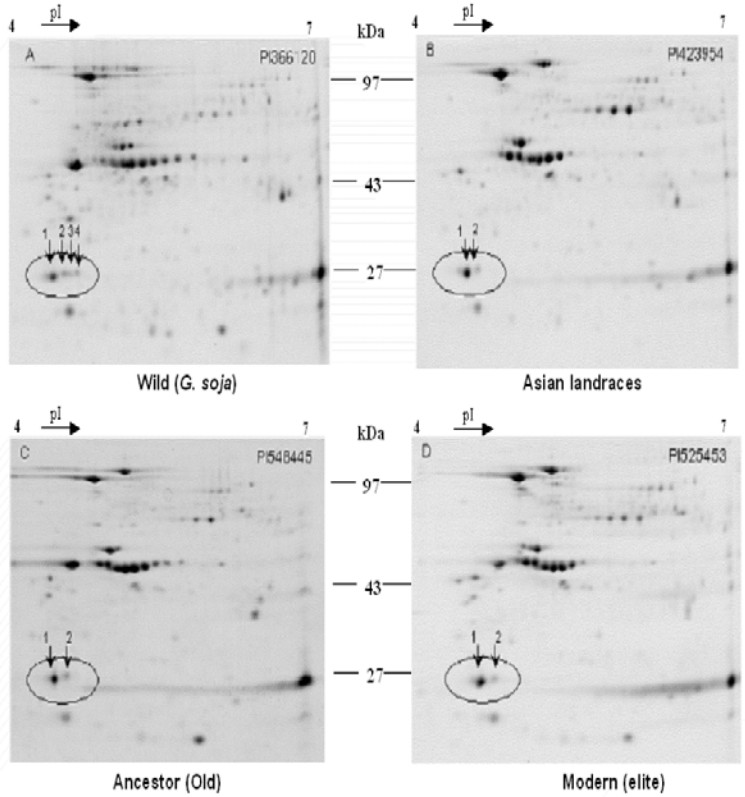
- Kunitz Trypsin Inhibitor (KTI) is an anti-nutritional protein found in soybean that acts as a proteinase inhibitor
- Upon consumption, they induce pancreatic enzymes resulting in pancreatic hypertrophy and hyperplasia
- The total seed protein of soybean contains about 6 % proteinase inhibitor

Wang et al., 2004, Genome 47(1), 9-14

Liener et al., 1995, Journal of Nutrition 125, 744-750

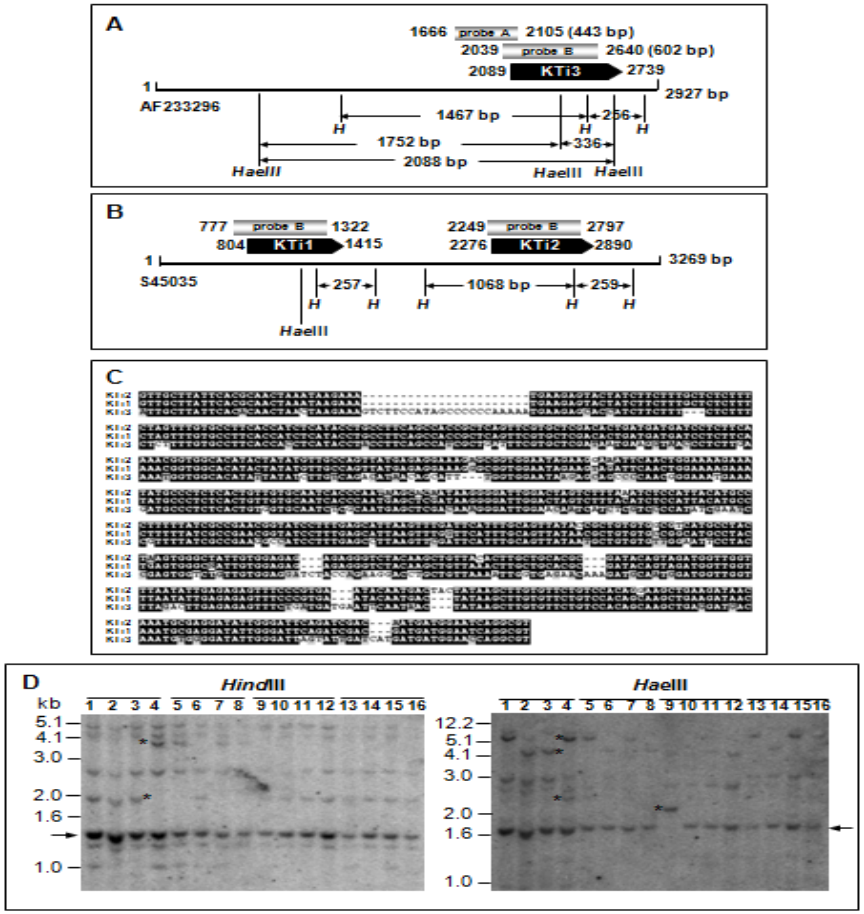
Variation of KTI in Sixteen Soybean Genotypes

Figure1



2D-PAGE

Figure 2



DNA blot analyses of soybean KTI genes

Natarajan et al., 2007. J. Pl. Physiol. 164:756-763

Protein Variation in Soybean Genotypes

- Generation of baseline data
  - Natural protein variation exists in different cultivars of soybeans and maturity groups.
  - SoyProDB was established containing comprehensive details of soybean proteins (storage, allergen, and anti-nutritional). http://bioinformatics.towson.edu/Soybean_Seed_Proteins_2D_Gel_DB/Home.aspx
  - Examined the variability of proteins produced in a wide range of soybean genotypes including wild and cultivated soybeans.
  - This data will be used to determine if the protein variability in soybeans resulting from transgenic soybean falls outside the range occurring in wild soybean and soybean landraces.

- Use proteomics to examine protein variation in GMO soybean
  - High methionine GMO soybean
  - Glyphosate herbicide tolerance GMO soybean

Tavakolan, Alkharouf, Khan, and Natarajan 2013, Bioinformation.9(3): 165–167

Summary

- Allergens and anti-nutritional proteins are major concerns.
- Advanced technologies are useful for identifying unintended protein expression in new crops.
- Multiple isoforms of β-conglycinin and glycinin were identified.
- Soy allergen & anti-nutritional (KTI) proteins were characterized.
- A database, SoyProDB, was developed containing comprehensive details of soybean proteins.
- Natural variation was observed in different classes of soybean seed proteins.
- Natural protein variation data will be used to determine if the protein abundance in transgenic soybean are within the limits of natural variation.

Research Team & Collaborators

- USDA, ARS, Soybean Genomics and Improvement Laboratory: Dr. Sukla Lakshman, Dr. Chenping Xu, Dr. Xi Chen, Dr. Bary Prince, Mr. Farooq Khan, Dr. Perry Cregan, & Dr. Benjamin Matthews
- USDA, ARS, Food Composition & Method Development Lab. Dr. Devanand Luthria
- Clemson University, Clemson, SC, Dr. Emerson R. Shipe
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- University of Maryland, College Park, MD, Dr. Joe H. Sullivan
- USDA, ARS, Biotechnology and Germplasm Laboratory, Dr. Wesley Garrett & Dr. Thomas Caperna
- Yeungnam University, Republic of Korea, Drs. Sang Kang and Rino Bae

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