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Full Length Research Paper

Seed storage protein components are associated with curled cotyledon phenotype in soybean

Suoyi Han[1,2], Rui Fan[1], Tuanjie Zhao[1], Jingjing Yu[1] and Deyue Yu[1]*

[1]Nanjing Agricultural University, National Center for Soybean Improvement, Nanjing, 210095 P.R. China.
[2]Economic Crops Research Institute, Henan Academy of Agricultural Science, Zhengzhou, 450002 P.R. China.

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Local soybean cultivar "Nannong 94-16" was treated with NaN_3-^{60}Coγ ray and EMS separately, and two curled cotyledon mutants were obtained. Local soybean cultivar "TSB" was treated with ^{60}Coγ ray and one curled cotyledon mutant was obtained. Two-dimension electrophoresis analysis was employed to determine the differential proteins between three mutants and their wild types. The results showed that there were three common differential protein spots between the mutants and their wild types. Further analysis suggests that the two increased protein spots in mutants were both globulin subunit G3/A1aB1b, while the decreased protein spot in mutants was lectin. These results indicate that there may be an association between protein components (glycinin subunits G3/A1aB1b, lectin) and the curled cotyledon phenotype in soybean.

Key words: Soybean, cotyledon mutant, seed storage protein, glycinin subunits, lectin.

INTRODUCTION

Soybean [*Glycine max* (L.) Merri.] is one of the most economically important crops in the world (Wilcox, 2004). Accumulation of specific proteins and other compounds for nutrient storage to high levels is one of the characteristic events during seed development. Improvement of storage protein in seed is being given more and more attention all over the world (Kim et al., 1990).

Protein is the performer of life activity, the change of plant morphology corresponds with the change of relative proteins. Cotyledon is one of the most important organs of growth and metabolism for seeds germinating. Mutants are good materials to carry out the molecular biology studies. Many important discoveries have been obtained from mutant research. Some research about the characters of cotyledon mutant in many plants is developing. For example, there was cup-shaped cotyledon mutant in *Arabidopsis* (Aida et al., 1999; Kwon et al., 2006) and single cotyledon mutant in pea (Liu et al., 1999). However, there have been a few reports related to cotyledon mutant in soybean. The curled cotyledon soybeans used in this research are novel soybean discovered through physical and chemical mutagenesis. For a better understanding of the consequences of genetic phenomena, elucidation of the protein composition is necessary because of its direct relationship to phenotype (Skylas et al., 2000).

In recent years, the applications of proteomic tools have become popular, and the tools are powerful methodologies for detecting and examining changes in protein composition accurately. The tools have been extensively used in examining the composition of both natural and transgenic soybean storage protein profiles and determining seed qualities of soybeans, and have been used on development research (Kerim et al., 2003) and inducible expression of resistance (Imin et al., 2004).

The main objective of this work was to gain further understanding of the influence of curled cotyledon on the seed storage protein components in soybean by comparative proteomics research on curled cotyledon mutant obtained by three different physical and chemical mutagenesis events.

MATERIALS AND METHODS

Plant materials

Local soybean cultivars "Nannong 94-16" and "TSB" were provided

*Corresponding author. E-mail: dyyu@njau.edu.cn. Tel/Fax: +86 25 84396410.

Figure 1. Appearance of wild types and curled cotyledon mutants. The upper row showed mature seeds. The under row showed seeds without seed coats 1 h after imbibitions. (A) and (F): cv. "Nannong 94-16". (B) and (G): MU1, obtained by treating "Nannong 94-16" with NaN_3-^{60}Coγ ray and detected from M_3 generation. The seeds of this mutant have their cotyledons curled outwards, and it also showed curled cotyledons in seedling period, until the cotyledons abscise. (C) and (H): MU2, obtained by treating "Nannong 94-16" with EMS, and detected from M_2 generation. The phenotype of the MU2 is similar to MU1. (D) and (I): cv. "TSB". (E) and (J): MU3, obtained by treating "TSB" with ^{60}Coγ ray, and detected from M_7 generation. The seeds of this mutant have their cotyledons curled outwards, and its germ only link with one of the cotyledons.

by National center for soybean improvement in Nanjing Agricultural University in China. The curled cotyledon mutant was obtained by treating "Nannong 94-16" with NaN_3-^{60}Coγ ray and detected from M_3 generation, named as MU1 (Han et al., 2008). The seeds of this mutant have their cotyledons curled outwards, and it also showed curled cotyledons in seedling period, until the cotyledons abscise. Another curled cotyledon mutant was obtained by treating "Nannong 94-16" with EMS, and detected from M_2 generation, named as MU2. The phenotype of the MU2 is similar to MU1. The third one was obtained by treating "TSB" with ^{60}Coγ ray, and detected from M_7 generation, named as MU3. The seeds of this mutant have their cotyledons curled outwards, and its germ only link with one of the cotyledons (Figure 1).

Chemicals

Chemicals for electrophoresis included acrylamide, bis-acrylamide, sodium dodecyl sulfate (SDS), TEMED, ammonium persulfate, thiourea, CHAPS, urea, ampholytes, Tris-HCl (pH 8.8), β-mercaptoethanol (β-ME) and trichloroacetic acid (TCA). IPG strips were purchased from Bio-Rad Laboratories. Water from a Millipore Milli-RO6 reverse osmosis system was used for making all solutions.

Isolation of soybean seed protein

Total protein was isolated from soybean seeds. Dry seeds (1 g) were pulverized into a fine powder by a mortar and pestle in the presence of liquid nitrogen and then were incubated with 10% (w/v) TCA and 20 mM DTT in acetone at -20°C for 1 h. The precipitated proteins were resuspended and washed with ice-cold acetone containing 20 mM DTT to remove pigments and lipids until the supernatant was colorless. The protein pellet was dried under vacuum, suspended in buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.2% (v/v) carrier ampholyte (pH 4.0-7.0), and cocktail protease inhibitor. Samples were mixed in a vortex mixer for 30 s and supersonic treated using VCX600 for 3 min. The insoluble tissue was removed by centrifugation at 15000 g for 15 min. The supernatant was stored at -80°C. Protein concentration was determined according to Bradford (Bradford, 1976), with bovine serum albumin (BSA) as a standard.

2-D electrophoresis and image analysis

pH gradient (IPG) strips (7 cm, pH 4.0-7.0, linear gradient) were 50 v rehydrated at 20°C for 12 h with 150 µl rehydration buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.2% (v/v) carrier ampholyte, 1% (w/v) DTT] containing 0.7 mg proteins in every sample. Focusing was carried out in a Bio-Rad Protein IEF Cell. The voltage setting was 250 V for 30 min, 500 V for 30 min, 4000 V for 3 h, and 4000 V for 20,000 volt h. After IEF, strips were equilibrated for 2 × 10 min in 6 M urea, 30% (v/v) glycerol, 5% (w/v) SDS in 0.05 M Tris-HCl (pH 6.8) containing 1% (w/v) DTT for the first equilibration step and 2.5% iodoacetamide for the second equilibration step, and then were transferred onto a 12% polyacrylamide gel. Electrophoresis was performed in Tris/glycine/SDS buffer on Mutiphor system (Amersham Pharmacia Biotech) according to the manufacturer's recommendations. For calibration, low-molecular weight marker proteins (Amersham Biosciences) were applied on the gel via a small piece of filter paper. Gels were stained overnight with Coomassie brilliant blue G-250 (Neuhoff et al., 1988) and scanned

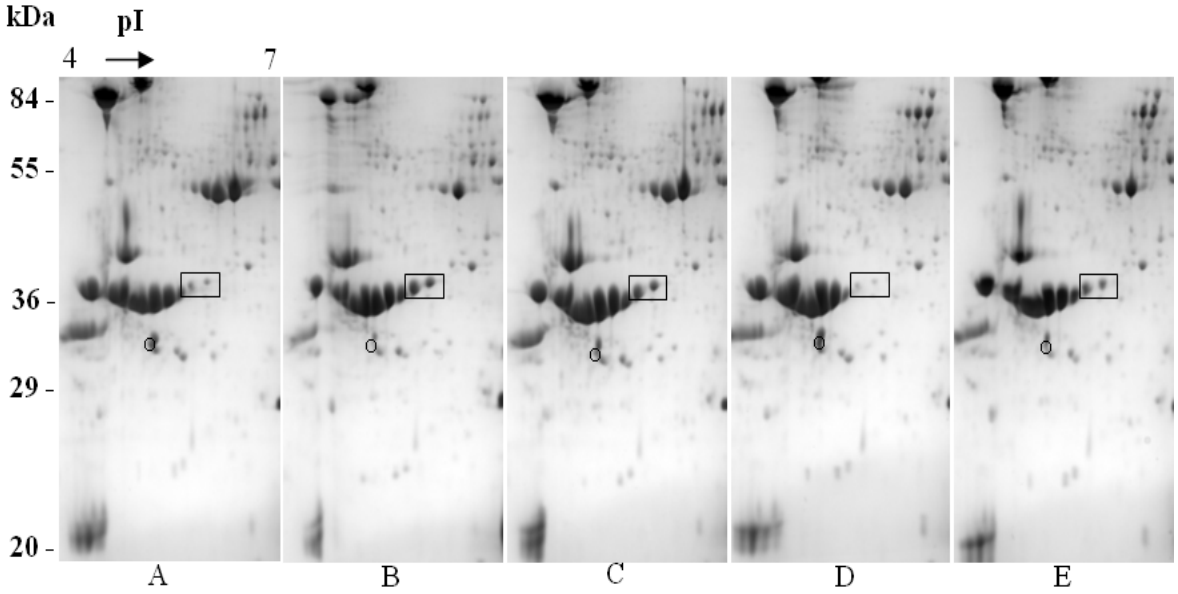

Figure 2. Proteomic comparison of the seed proteins of mutants and wild types with Coomassie Brilliant Blue G250 staining. Isoelectric points (pI) and molecular mass (in kDa) are noted. The two squared protein spots are up-regulated protein in mutants. The circled protein spots are down-regulated proteins in mutants. (A) cv. "Nannong 94-16". (B) MU1, obtained by treating "Nannong 94-16" with NaN_3-^{60}Coγ ray and detected from M_3 generation. (C) MU2, obtained by treating "Nannong 94-16" with EMS, and detected from M_2 generation. (D) cv. "TSB". (E) MU3, obtained by treating "TSB" with ^{60}Coγ ray, and detected from M_7 generation.

using VersaDoc image system (Bio-Rad). Of all samples, three replicate gels were analyzed using PDQuest software (v730, Bio-Rad). The gel with most amounts of protein spots was selected as reference gel.

Image analysis was carried out with PDQuest software (v730, Bio-Rad), which allows spot detection, quantification, and spot matching among multiple gels. To compensate for subtle differences in sample loading, gel staining, and destaining, the volume of each spot was normalized as relative volume. This normalization method provided by PDQuest software divides each spot volume value by the sum of total spot volume values to obtain individual relative spot volumes.

Identification of differential proteins with bioinformatics resources

2D-PAGE image was compared to the existing soybean gel for pI 4-7. (http://oilseedproteomics.missouri.edu/soybeandata_thelen/soybeangelpi47/soybean47_modified.html) (Natarajan et al., 2006). Results can be viewed through list of identified 2-D proteins spots by peptide mass fingerprint (MALDI-TOF-MS).

RESULTS

In this study, we applied 2D-PAGE with pH gradient (IPG) strips (pH4.0-7.0) to study and compare the protein compositions between curled cotyledon mutants and their wild types. We compared and analyzed one hundred and forty nine distinguishable protein spots appeared in 2D-PAGE. In condition of the same loading quantity of protein samples, we chose proteins which color intensities differ for over three times and considered them different. We divided five images into three groups and carried out comparative analysis: "Nannong 94-16" (Figure 2A) and MU1 (Figure 2B); "Nannong 94-16" and MU2 (Figure 2C); "TSB" (Figure 2D) and MU3 (Figure 2E).

MU1 has fourteen spots down-regulated and sixteen spots up-regulated than "Nannong 94-16"; MU2 has eight spots down-regulated and fourteen spots up-regulated than "Nannong 94-16"; MU3 has twenty spots down regulated and eight spots up-regulated than "TSB".

We also compared the increased spots of the three groups above, as well as the decreased spots, and search for all the protein spots that increased or decreased in mutant compared to wild types. These three mutants shared cotyledon curled characters. Besides, there may be other mutation characters and further research is ongoing. The discoveries of all the protein spots that increased or decreased in mutant compared to their wild indicate that these spots are correlated with the curled cotyledon character. The results indicated that there were two increased protein spots of all mutants compared to their wild types; these spots were marked by squares; and there was one decreased protein spots of all mutants comparing to their wild types, marked by a circle (Figure 2). The result of identification indicated that the increased two protein spots in mutants are both glycinin protein subunits G3/A1aB1b, and the decreased protein spots is lectin.

DISCUSSION

The three protein spots that were discovered from 2-D gels may be correlated with curled cotyledon mutants. The result preliminarily illustrates correlations between curled cotyledon and the seed storage proteins. Curled cotyledon mutants have more glycinin subunit G3/A1aB1b and less lectin than their wild type.

Before this, there was some other research about the correlation between seed trait and seed reserve deposition. The shriveled seed trait is associated with seed reserve deposition. SDS-PAGE analysis of severely shriveled seed of a mutant showing a reduced level of a polypeptide of 48 kD molecular weight has been identified as the β-subunit of the 7S storage protein (Honeycutt et al., 1989). Here, the result showed that curled cotyledon mutants have more glycinin subunit G3/A1aB1b and less lectin than their wild type in this work. Our work also demonstrated that proteomic analysis in general could help to define specific changes in protein composition, which can occur in the mutants. The comparative studies of the storage proteins of mutant and their wild type would help us to understand the relationship between morphology of cotyledon and the storage proteins. The result showed that there is close relationship between seed trait and seed storage protein.

Storage protein accounts for approximately 40% of seed dry weight in soybean and it serves as an important protein source for human food (Krober et al., 1962; Nielsen et al., 1996). During seed germination and early growth, the young plant is nutritionally dependent on storage compounds in the cotyledons. The storage proteins of the soybean cotyledon are degraded and mobilized during germination and seedling growth (Wilson et al., 1986), and they serve as sources of both nitrogen and carbon.

The soybean globulin and the agglutinin are the important seed protein. Glycinin, the most abundant storage protein of soybean, may represent as much as 10 to 15% of the dry weight of mature seed (Hill et al., 1974; Derbyshire et al., 1976). In addition, lectins are carbohydrate-binding proteins found at moderately high levels in the seeds of many plants, including soybean. Lectins are also soybeans' significant anti-nutritional factors (Friedman et al., 2001). Apart from also being significant storage proteins, lectins take part in the extension and growth regulation of cell walls, transporting carbohydrate, partake in infection nodulation of legumenous plants, act as significant factors of plant cells, and possess enzymatic functions.

Some of the genes affecting seed morphology or seed composition that have been described in *Arabidopsis* and pea. A wrinkled-seeded mutant of *Arabidopsis* is caused by a splicing mutation in a gene that encodes an APETALA2/ethylene-responsive element binding (EREB) transcription factor (Cernac et al., 2004). However, seed developmental processes in pea are affected by the single cotyledon (sic) and cytokinesis-defective (cyd) mutations that produce enlarged cells, mainly in the cotyledons, and by mutations that do not allow the seed to complete a normal developmental program (Johnson et al., 1994). Related genes of curled cotyledon need further research on this basis.

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REFERENCES

Aida M, Ishida T, Tasaka M (1999). Shoot apical meristem and cotyledon formation during Arabidopsis embryogenesis: interaction among the cup-shaped cotyledon and shoot meristemless genes. Development. 126: 1563-1570.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Cernac A, Benning C (2004). Wrinkled1 encodes an AP2/EREB domain protein involved in the control of storage compound biosynthesis in Arabidopsis. Plant J. 40: 575-585.

Derbyshire E, Wright DJ, Boulter D (1976). storage proteins of legume seeds. Phytochem. 15: 3-24.

Friedman M, Brandon DL (2001). Nutritional and health benefits of soy proteins. J. Agric. Food Chem. 49: 1069-1086.

Han SY, Yang ML, Chen YD, Yu JJ, Zhao TJ, Gai JY, Yu DY (2008). Construction of mutant library for soybean "Nannong94-16" and analysis of some characters. J. Nucl. Agric. Sci. 21: 131-135.

Hill JE, Breidenbach RW (1974). Proteins of soybean seeds II. Accumulation of major protein components during seed development and maturation. Plant Physiol. 53: 747-751.

Honeycutt RJ, Burton JW, Palmer RG, Shoemaker RC (1989). Association of major seed components with a shriveled seed trait in soybean. Crop Sci. 29: 804-809

Imin N, Kerim T, Rolfe BG, Weinman JJ (2004). Effect of early cold stress on the maturation of rice anthers. Proteomics 4: 1873-1882.

Johnson S, Liu CM, Wang TL (1994). An analysis of seed development in Pisum sativum. XVIII. The isolation of mutants defective in embryo development. J. Exp. Bot. 45: 1503-1511.

Kerim T, Imin N, Weinman JJ, Rolfe BG (2003). Proteome analysis of male gametophyte development in rice anthers. Proteomics 3: 738-751.

Kim CS, Kamiya S, Sato T, Utsumi S, Kito M (1990). Improvement of nutritional value and functional properties of soybean glycinin by protein engineering. Protein Eng. 3: 725-731.

Krober OA, Cartter JL (1962). Quantitative interrelations of protein and nonprotein constituents of soybeans. Crop Sci. 2: 171-172.

Kwon CS, Hibara K, Pfluger J, Bezhani S, Metha H, Aida M, Tasaka M, Wagner D (2006). A role for chromatin remodeling in regulation of CUC gene expression in the Arabidopsis cotyledon boundary. Dev. 133: 3223-3230.

Liu CM, Johnson S, Gregorio SD (1999). Single cotyledon (sic) mutants of pea and their significance in understanding plant embryo development. Dev. Genet. 25: 11-22.

Natarajan SS, Xu CP, Bae HH, Caperna TJ, Garrett WM (2006). Characterization of Storage Proteins in Wild (*Glycine soja*) and Cultivated (*Glycine max*) Soybean Seeds Using Proteomic Analysis. J. Agric. Food Chem. 54: 3114-3120.

Neuhoff V, Arold N, Taube D, Ehrhardt W (1988). Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis 9: 255-262.

Nielsen SS, Belury MS, Nichel KP (1996). Plant nutrient composition under wild typed environment life-support systems. In: Janick J (eds) Progress in New Crops, ASHS Press, pp. 624-632.
Skylas DJ, Mackintosh JA, Cordwell SJ (2000). Proteome approach to the characterization of protein composition in the developing and mature wheat-grain endosperm. J. Cereal Sci. 32: 169-188.
Wilcox JR (2004). World distribution and trade of soybean. In: Boerma RH, Specht JE(eds) Soybeans: Improvement, Production, and Uses, Ed 3. American Society of Agronomy. Madison. WI, pp. 1-14.
Wilson RF, Kwanyuen P (1986). Triacylglycerol synthesis and metabolism in germinating soybean cotyledons. BBA- Lipids and Lipid Metabolism 877: 231-237.