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Improvement of protein quality in transgenic soybean plants

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Abstract

Glycinin is one of the abundant storage proteins in soybean seeds. A modified *Gy1* (A1aB1b) proglycinin gene with a synthetic DNA encoding four continuous methionines (*V3-1*) was connected between the *hpt* gene and the modified green fluorescent protein sGFP(S65T) gene, and a resultant plasmid was introduced into soybean by particle bombardment in order to improve nutritional value of its seeds. After the selection with hygromycin, the efficiency of gene introduction was evaluated. More than 60 % of the regenerated plants tolerant to hygromycin yielded the *hpt* and *V3-1* fragment by polymerase chain reaction (PCR) analysis, and the expression of *sGFP* was detected in about 50 % of putative transgenic soybeans. Southern hybridization confirmed the presence of transgenes in T_0 plants and the transgenic soybeans hybridized with the *hpt* and *V3-1* genes were analyzed showed different banding patterns. Most of the transgenic plants were growing, flowering normally and produced seeds. Analysis of seed obtained from transgenic soybean plants expressing *hpt* and *V3-1* genes showed higher accumulation of glycinin compared with non-transgenic plants. In addition, protein expression in transgenic soybean plants was observed by using 2D-electrophoresis.

Additional key words: genetic improvement, *Glycine max*, glycinin, particle bombardment, PCR, seed storage proteins.

Introduction

There have been many attempts to manipulate the balance of essential amino acids in important crops by conventional plant breeding, but these improvements in nutritional quality have often come at the expense of yield. Soybean is one of the most important vegetable protein sources, since proteins account for 40 % of all components of soybean seeds. However, the protein quality is also limited by the low content of sulfur-containing amino acids. Glycinin (11S globulin) and β-conglycinin (7S globulin) are predominant components of seed storage proteins in soybean and glycinin contains three to four times more sulfur amino acids than β-conglycinin (Koshiyama 1968). Five different glycinin subunits have been purified and grouped into two subfamilies (Group I and Group II) based on the similarity of their amino acid sequences (Nielsen 1985). Glycinin is composed of six non-identical subunits, each of which consists of an acidic and a basic polypeptide that are linked by a disulfide bridge. The subunits are synthesized as a single polypeptides precursor (preproglycinin) consisting of the covalently-linked two polypeptides together with signal sequence, and mature after post-translational cleavage (Staswick *et al*. 1984). Glycinin assembles as oligomers comprised of six pairs (subunits) of acidic and basic polypeptides into protein bodies. Several natural and induced mutants affecting accumulation of glycinin and β-conglycinin have been isolated in soybean (Harada *et al*. 1983, Kitamura *et al*. 1984, Odanaka and Kaizuma 1989, Takahashi *et al*. 1994, Hajika *et al*. 1996, Yagasaki *et al*. 1996). A negative correlation was observes between the contents of glycinin and β-conglycinin, suggesting that glycinin may be overproduced to compensatefor the reduction of β-conglycinin (Ogawa *et al*. 1989). Actually, the content of the sulfur-containing amino acids

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Abbreviations: GFP - green fluorescent protein; PCR - polymerase chain reaction.
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in breeding lines with a low content of β-conglycinin was on the average 20 % higher than that of the ordinary soybean cultivars. The 2S albumin from the seeds of the Brazil nut (*Bertholletia excelsa* H.B.K) has abundance of sulfur-containing amino acids, and is composed of 18 and 8 % of the sulfur amino acids, methionine and cysteine, respectively (Altenbach *et al*. 1987). The 2S albumin gene has been introduced into soybean and other grain legumes, and the transgenic seeds showed high content of the sulfur-containing amino acids compared with original seeds (Altenbach *et al*. 1989, Saalbach *et al*. 1994, 1995). The target of our research is improvement of protein quality in soybean seeds by introducing and expressing the modified Gy1 (A1aB1b) proglycinin gene with a synthetic DNA encoding four continuous methionines (*V3-1*) into soybean embryos via particle bombardment.

Materials and methods

Construction of chimeric genes: The construct containing the *hpt,* modified *Gy1* cDNA, and sGFP(S65T) genes was prepared into pUC19 vector (Fig. 1). These genes encoded for hygromycin phosphotransferase, a modified *Gy1* (A1aB1b) proglycinin with a synthetic DNA encoding four continuous methionines, and a modified green-fluorescent protein sGFP(S65T), respectively. The *hpt* and sGFP(S65T) genes were under regulatory control of the cauliflower mosaic virus (CaMV) 35S promoter. The modified *Gy2* cDNA had a seed specific promoter from a soybean glycinin (A1aB1b) gene (El-Shemy *et al*. 2004).

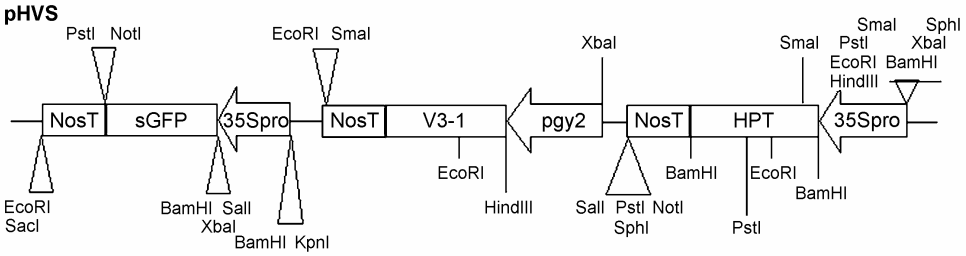

Fig. 1. Structures of plasmid construct for soybean transformation. pHVS contains a modified glycinin gene, *V3-1*, between *hpt* as a selectable gene and *sGFP(S65T)* as a reporter gene. Restriction sites are indicated.

The promoter sequence of a *Gy2* (A2B1a) proglycinin gene was isolated by PCR reaction from the genomic DNA of a soybean cultivar, Bonminori, according to the sequence data (Kitamura *et al*. 1990). The resultant 1.0 kb *Gy2* promoter was digested with *Xba*I and *Hind*III, and then ligated with the modified *Gy1* cDNA, pUG1V, (Kim *et al*. 1990). The chimeric gene was recovered after digestion with *Hind*III and *Sac*I, and then inserted into corresponding sites of the pBI221 (*Clontech*, Franklin Lakes, NJ, USA). After the digestion with *Not*I, a 2.7kb fragment containing the *Gy2* promoter, the modified *Gy1* cDNA and the nopaline synthase terminator (*nos*T) was repaired with T4 DNA polymerase (*Takara*, Shiga, Japan) and dNTPs and digested with *Xba*I. The fragment was inserted into the sites of *Xba*I and *Sma*I of pUC19 to create pV3-1. A 2.1kb fragment containing CaMV 35S-*hpt* gene, which confer hygromycin tolerance, was digested with *Not*I from pSKHyg, filled in with T4 DNA polymerase, and digested with *Sal*I. The fragment was inserted into the sites of *Pst*I and *Sal*I of pV3-1. The sGFP(S65T) gene was isolated by PCR reaction from with a primer set of 5'-AAGGTACCGGATCCC CCCTCAGAA-3' and 5'-AAGAGCTCCGATCTAGTA ACATAGATGACACC-3'. The amplified fragment was digested with *Sac*I and *Kpn*I and inserted into the corresponding sites to create pHVS (Fig. 1).

Initiation and proliferation of embryogenic cultures: Transformation and regeneration systems for soybean was optimized and described previously (El-Shemy *et al*. 2004). Soybean (*Glycine max* (L.) Merr.) cv. Jack was grown in soil in the glasshouse at 25 °C. After sterilization of the pod surface with 70 % ethanol followed with 3 sterile water rinses, the immature embryos about 4 - 5 mm long were extracted, removed the end with the embryonic axis, and placed with flat side up on MSD40 medium consisting of Murashige and Skoog (1962; MS) salts and B5 vitamins (Gamborg *et al*. 1968) supplemented with 3 % sucrose, 40 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.2 % *Gelrite* (pH 7.0). Embryogenic tissues were initiated at 25 °C under cool white fluorescent dim light (23 h, 5 - 10 μmol m^{-2} s^{-1}) for 3 to 4 weeks. Soybean embryogenic tissues were suspended and maintained in the FN Lite liquid medium (Finer and Nagasawa 1988) consisting of FN Lite macro salts, MS micro salts and B5 vitamins supplemented with 1 g dm^{-3} asparagine, 5 mg dm^{-3} 2,4-D, and 1 % sucrose (pH 5.8), and maintained by sub-culturing every week into 25 cm^{-3} of fresh FN Lite medium in a 100 cm^{-3} flask.

Transformation by particle bombardment: Approximately 1 g of embryogenic suspension tissue was transferred on the center of MSD20 medium consisting of MS salts and B5 vitamins supplemented with 3 % sucrose,

1 g dm^{-3} asparagine 20 mg dm^{-3} 2,4-D, and 0.2 % *Gelrite* (pH 5.8) in a 9-cm Petri dish, and then excess liquid medium was wiped away with sterile filter paper. Bombardments were performed using a *Biolistic PDS-1000/He Particle Delivery System* (*Bio-Rad*, Richmond, CA, USA) according to the instruction manual. Each sample of embryogenic tissue was bombarded twice.

Selection for transgenic embryogenic tissues: Bombarded tissues were resuspended in the FN Lite medium. One week after bombardment the embryogenic tissues were transferred in fresh FN Lite medium containing 15 mg dm^{-3} hygromycin B (*Roche Diagnostics*, Mannheim, Germany). The bombarded tissues were transferred in fresh antibiotic-containing FN Lite medium weekly for 3 additional weeks. After then the white lumps of tissues that contained blight green lobes of embryogenic tissues were selected and transferred in fresh FN Lite medium containing 30 mg dm^{-3} hygromycin B. The hygromycin tolerant tissues were selected and resuspended in fresh antibiotic-containing FN Lite medium weekly for 3 additional weeks.

Embryo development and germination: Hygromycin tolerant embryos were resuspended in FNL0S3S3GM liquid medium, which contained FN Lite macro salts, MS micro salts and B5 vitamins supplemented with 30 mM glutamine, 2 mM methionine, 3 % sucrose, and 3 % sorbitol (pH 5.8) (Samoylov *et al*. 1998). Three weeks after suspension, excess liquid of the developing embryos was withdrawn with sterile filter paper, and the embryos were placed in dry Petri dishes for 3 to 5 d. After the desiccation treatment, the embryos were placed on MS0 medium containing MS salts, B5 vitamins, 3 % sucrose, and 0.2 % *Gelrite* (pH 5.8). The germinating plantlets were transferred to half-strength B5 medium. After root and shoot elongation, plantlets were transferred to pots containing soil, and maintained under high humidity. Plantlets were gradually adapted to ambient humidity and placed in the glasshouse.

GFP detection: The presence of sGFP(S65T) was detected by blue light excitation (Chiu *et al*. 1996). Embryos, cotyledone, leaves of regenerated plants were observed with a stereomicroscope (*Leica Microsystems*, Wetzlar, Germany) equipped with a filter set providing 455 - 490 nm excitation and emission above 515 nm.

PCR and Southern blot analysis: Total DNA was isolated from soybean leaves by the method of (Draper and Scott 1988), and used to investigate the presence of the transgene. PCR analysis was conducted to screen transformed plants in a 0.02 cm^3 reaction mixture containing 10 ng of genomic DNA, 200 μM of each of dNTP, 0.2 μM of each primer, and 2.5 units of *Ampli-taq Gold* polymerase (*Applied Biosystems*, Foster City, CA, USA) in the corresponding buffer. Reaction were hot-started (9 min at 94 °C) and subjected to 30 cycles as fellows: 30 s at 94 °C; 1 min at 55 °C; and 1 min at 72 °C. The last extension phase was prolonged 7 min at 72 °C. The primer set for *hpt* was designed for amplification of the 560 bp fragment; sequences are 5'-ATCCTTCGC AAGACCCTTCCT-3' and 5'-GGTGTCGTCCATCA CAGTTTG-3'.

The primer set for *V3-1* was designed for amplification of a 1403 bp fragment; sequences are 5'-TTCAGTTCCAGAGAGCAGCAGCCT -3' and 5'CTGATGCATCATCATCTGAGG -3'.

Southern blot analysis was conducted to confirm stable integration of transgenes soybean, 10 μg of total DNA was digested with the restriction enzymes, *Hind*III and *Sac*I, and digested DNA was separated by electrophoresis in a 1 % agarose gel and transferred onto *Hybond N+* membrane (*Amersham Biosciences*, Little Chalfont, UK). Labeling and detection were conducted following the protocol of ECL direct nucleic acid labeling and detection (*Amersham Biosciences*). DNA fragment containing the *hpt* and *V3-1* genes were amplified from the plasmid by same primer sets for the PCR analysis, and used as hybridization probe on Southern blot membrane.

Analysis of seed proteins in transgenic soybean: Subunit composition of seeds from individual transgenic soybean was analyzed by SDS-PAGE (Laemmli 1970). A total globulin fraction was extracted from soybean seed meal by grinding with 50 mM Tris-HCl, pH 8.0, and standing for 60 min at room temperature. The supernatant was obtained after centrifugation at 15 000 *g* for 10 min. Protein concentration was determined by BCA protein assay (*Pierce*, Rockford, IL, USA) kit using bovine serum albumin as a standard. Protein solution was diluted with the same amount of a twice-concentrated SDS sample buffer containing 2-mercaptoethnol. The proteins (20 μg) were separated by a gel containing 12 % (m/v) acrylamide and 0.2 % (m/v) bis-acrylamide, and were stained with *Bio-Safe CBB G-250* stain (*Bio-Rad*).

2D-gel electophoresis: The globulin fraction was precipitated with 80 % acetone and the resulting pellets were dissolved in 0.25 cm^{-3} of IEF sample buffer for very basic proteins (Gorg *et al*. 1997, Ikeda *et al*. 2005) containing 16 % isopropanol, 8.5 M urea, 4 % CHAPS, 25 mM DTT and 0.5 IEF buffer pH 3~10 (*Amersham Bioscience*). After incubation for 30 min at room temperature, samples were applied to *Immobiline DryStrip* pH 3~10 (*Amersham Bioscience*) and fit to *Protean IEF Cell* (*Bio-Rad*). After in-gel hydration for about 12 h, IEF was carried out for a total of 18 kVh. The gels were equilibrated with 6 M urea, 2 % SDS, 20 % glycerol, 10 mM DDT and 50 mM Tris-Cl pH 8.8 and stored at -25 °C. For the second dimension, SDS-PAGE was performed using 10 % gel. The gels were stained with Coomassie Brilliant Blue G-250 according to the method of Neuhoff *et al*. (1988).

Results and discussion

Although it has been recognized for many years that grain legumes in general are deficient in the sulfur-containing amino acids, methionine and cysteine, conventional plant breeding methods have not been successful in increasing the content of these amino acids without deterioration of agronomic traits and food-processing properties. The recent development of tissue culture procedures for the stable transformation of a limited range of grain legumes such as soybean (Hinchee *et al*. 1988, El-Shemy *et al*. 2004), common bean (Russel *et al*. 1993), pea (Schroeder *et al*. 1993), and azuki bean (Yamada *et al*. 2001) has made possible a new phase in the efforts to improve the sulfur amino acid content of these important crops. In a number of laboratories including ours, methods to improve the nutritional quality of seed proteins by genetic engineering are being evaluated.

The transformation and regeneration system was optimized for soybean according to the described method (Finer and Nagasawa 1988, Finer and McMullen 1991, Sato *et al*. 1993, Hadi *et al*. 1996, Li *et al*. 2004, El-Shemy *et al*. 2004). Embryogenic tissues was obtained from jack cultivar and a line on MSD40 media within 3 weeks, although were easily proliferated on FN Lite liquid media, and used for particle bombardment transformation.

To optimize the condition of particle delivery, transient expression of the sGFP(S65T) gene was observed after bombardment with particles coated with pHVS (Fig. 2). Expression of sGFP(S65T) in soybean was also monitored during selection with hygromycin and development of plants (Fig. 2).

The efficiency of multi gene transformation was evaluated by the selection with hygromycin and the expression of sGFP(S65T). The embryogenic tissues introduced pHVS by particle bombardment were selected on the FN Lite liquid medium containing hygromycin (15 - 30 mg dm^{-3}), and after 8 weeks the green embryos were matured on the FNL0S3S3 liquid medium. After the desiccation for 5 d, embryos germinated on the MS0 medium, and the plants were transferred to pots with soil in a glasshouse. More than 50 % of the regenerated plants tolerant to hygromycin yielded the *hpt* and *V3-1* fragments by PCR analysis (Fig. 3), and the expression of sGFP(S65T) was detected in about 70 % of the positive *hpt* soybeans (data not shown). Out of a total of 122 regenerated plants obtained from the introduction of pHVS, 82 plants produced an expected band with a 0.5 kbp of PCR product within the *hpt* gene and 49 were produced *V3-1* gene band (Fig. 3).

Southern blot analysis was conducted to confirm the

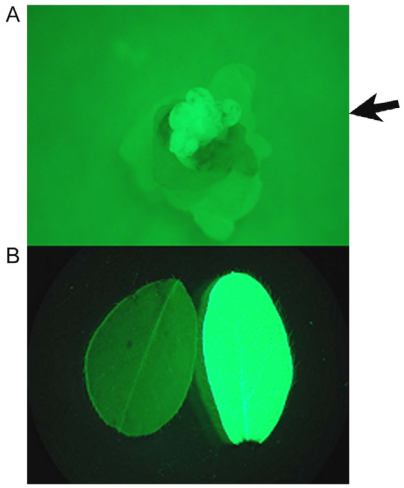

Fig. 2. Stable sGFP(S65T) expression in embryos and plantlets. *A* - sGFP(S65T) expressed in transgenic embryos (*arrow*). *B* - Leaves from transgenic plant (*right*) and non-transgenic plant (*left*).

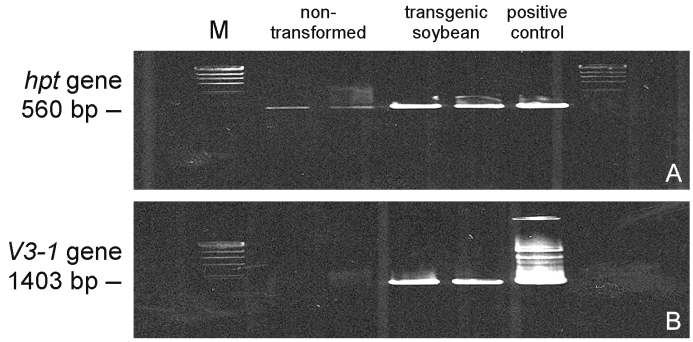

Fig. 3. PCR analysis of transformed soybean plants with *hpt* (*A*) and *V3-1* (*B*) genes. *Lane M* - molecular standard of DNA digested with *HindIII DNA*. *Lanes 1 - 2*: non transformed soybean, *lanes 3 - 4*: transformed soybean (*pHVS* construct), *lane 5*: plasmid pUC 19 contining *pHVS* construct). Expected 560 bp and 1403 bp fragments of *hpt* and *V3-1* genes, respectively.

presence of transgene. Total DNA was digested with a restriction enzyme, *Hind*III and *Sac*I and hybridized with the probe for *hpt* and *V3-1* gene. All transgenic soybeans yielded several bands hybridized with the *hpt* and *V3-1* genes and showed different banding patterns (Fig. 4). The insertion of the gene construct into soybean genome by

the selection of hygromycin and expression of sGFP(S65T) showed high efficiency and contained the modified glycinin gene.

All the transformants were analyzed yielded 2 to 5 bands hybridized with the *hpt* probe (Fig. 4*B*). The *V3-1* gene was altered genetically from a proglycinin

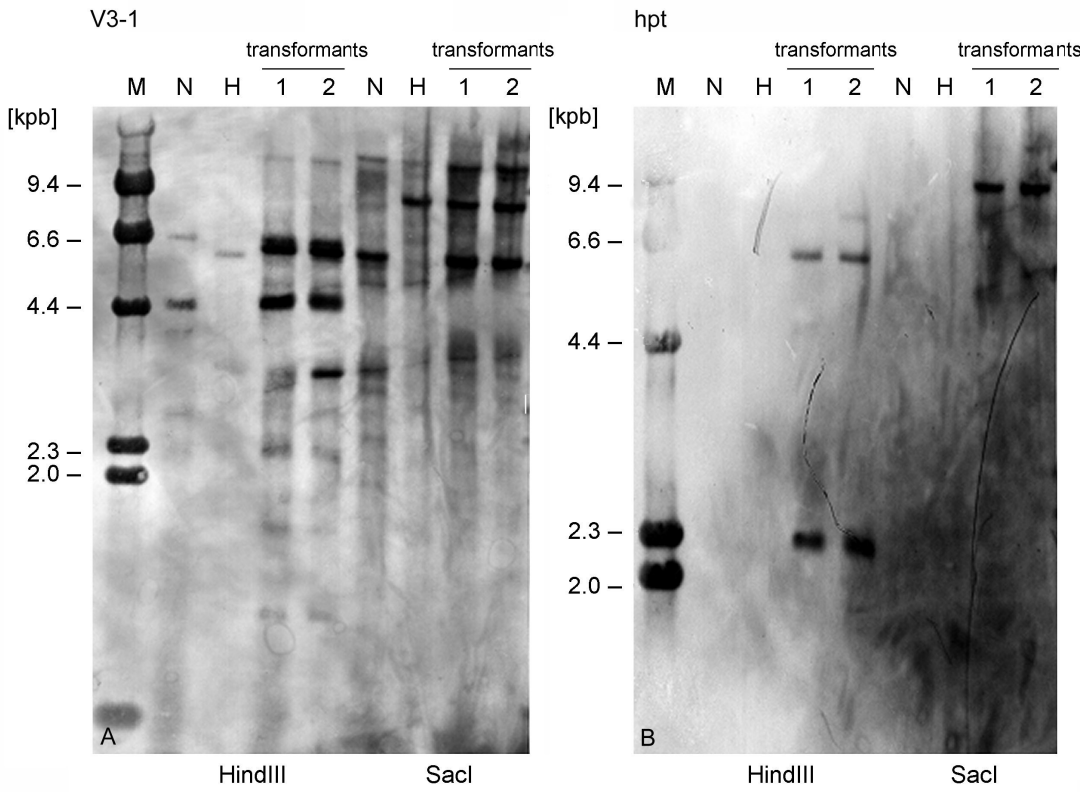

Fig. 4. Southern blot analysis of soybeans transformed with *pHVS*. Total DNA was digested with *HindIII* and *Sac*I and loaded in *lane M* (DNA size marker), *lanes N, H* (untransformed soybean), *lanes 1 - 2* (transformed soybeans with *pHVS*). Separated DNA was transferred to a nylon membrane and hybridized with each probe containing the coding region of *V3-1* (*A*) and *hpt* (*B*).

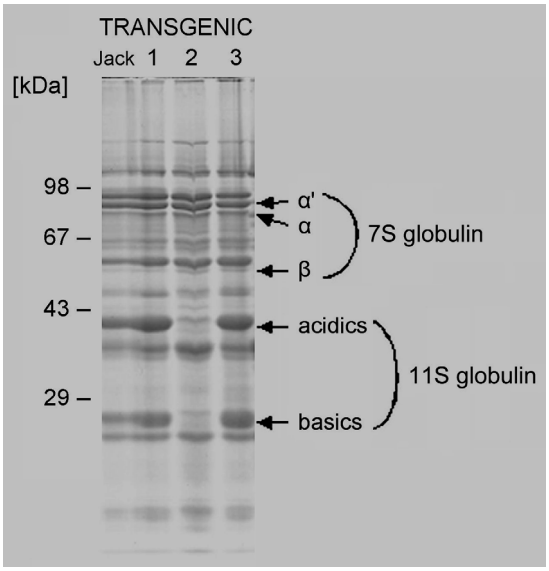

Fig. 5. SDS-PAGE analysis of components of seed storage proteins in transformed soybean. Globulin fractions were isolated from Jack (untransformed control) and transformants, and 20 μg of each fraction was fractionated by SDS-PAGE and then stained with CBB.

(A1aB1b) cDNA, which ordinarily exists in soybean. Therefore, untransformed plants also gave multiple bands, indicating that these bands would correspond to the endogenous glycinin genes. The transgenic plants gave additional bands resulting from the integration of the *V3-1* gene (Fig. 4*A*). Most of the transgenic plants are growing and flowering normally.

The accumulation of glycinin was confirmed by SDS-PAGE analysis of the globulin fraction extracted from transgenic seeds (Fig. 5). The modified glycinin *V3-1* could not be distinguished from endogenous glycinin subunits by the SDS-PAGE, because the modified glycinin contained only six additional amino acids in the basic subunit. The glycinin subunit polypeptides in some transformants were more intensely stained with CBB compared to non-transformants (Fig. 5). This may be due to the accumulation of the modified glycinin *V3-1* in transgenic seeds. On the other hand, some transgenic soybeans lack all subunits of glycinin, suggesting the transgene may cause the suppression of endogenous glycinin genes by the effect of gene co-suppression (Fig. 5). Two approaches have been used for genetic engineering of grain legumes for increased sulfur amino acid content. One approach has been to use a

modified gene derived from a legume seed storage protein to increase its methionine. A second approach involves the identification of seed proteins with unusually high methionine/cysteine contents from nonlegumes, and the expression of genes coding for these proteins at a high level in the seeds of the target grain legume species. Saalbach *et al.* (1995) introduced the gene for the Brazil nut 2S albumin protein into *Vicia narbonensis* and found the 2S albumin accumulated up to 4 % of extractable protein. However, work of transgenic soybeans accumulating the 2S albumin had cast doubt on the usefulness of this protein as a means of improving

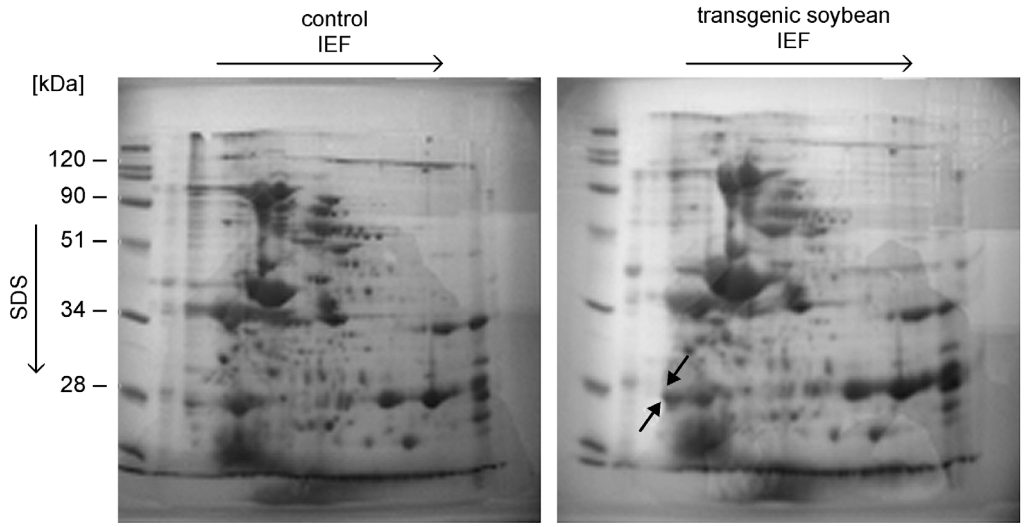

Fig. 6. Two-dimensional PAGE analysis of globulin storage proteins extracted from untransformed soybean and transgenic soybean with pHVS construct.

nutritional quality. The Brazil nut 2S albumin protein was found highly allergenic for some humans (Nordlee *et al*. 1996). To identify proteins corresponding to glycinin groups, the globulin fraction for both transgenic and non-transgenic were separated by 2D-gel electrophoresis (Fig. 6). The results indicated that in transgenic soybean one spot was observed compared with non-transgenic and this due to the expression of new proteins led to the accumulation of glycinin (Fig. 6). Together with 2D-PAGE, these tools can be used both to visualize and compare complex mixtures of proteins and to gain a large amount of information about the individual proteins involved in specific biological responses (Finnie *et al*. 2002, Adessi *et al*. 1997). Although several methods for 2D analysis of plant and seed proteins have been reported in a variety of crops (Hu and Esen 1982, Hajduch *et al*. 2005), only a limited number of methods have been reported for soybean seed protein analysis (Herman *et al*. 2003, Mooney and Thelen 2004, Natarajan *et al*. 2005). Although the high 11S lines represent a major advance in the genetic manipulation of soybean protein functionality, the potential applications of products derived from these lines are still limited by the intrinsic functional properties of 11S proteins. Protein engineering techniques have become increasingly sophisticated, and the molecular structures for these storage proteins are now known (Maruyama *et al*. 2001, Adachi *et al*. 2003).

In conclusion, the transgenic soybean were produced via pHVS construct showed the high elevation of glycinin subunits (acidic and basic) and that was improved the nutritional quality of soybean crops.

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Lal, R., Cerri, C.C., Bernoux, M., Etchevers, J., Cerri, E. (ed.): **Carbon Sequestration in Soils of Latin America.** - Food Product Press. An Imprint of The Haworth Press, New York - London - Oxford 2006. Pp. 554. USD 49.95. ISBN 1-56022-137-1.

This voluminous book deals with two important problems of general importance: *1*) Global warming could be substantially slowed down by an increased carbon sequestration in soils. But it could also be considerably enhanced, if soils will continue to be degraded at the present rate. *2*) Latin America represents an important part of the world not only because of its area and population, but also because of its tropical rainforests.

In 2004 The Ohio State University, University of Sao Paulo, and the French Institut de Recherche pour le Développement organized a workshop in Brazil. Its main topics aimed at evaluating the recent soil management and its impact on carbon content in the soils and carbon fluxes in Latin America. This book offers both analyses of the present state as well as recommendations for future rational management of soils in the Latin America countries. The 24 contributions written by 61 authors have been divided into 4 main chapters.

The book starts with an introduction about the editors, list of contributors and a brief preface. The contributions are ordered into four parts. Part I deals with physiography and general back-ground. Its four contributions describe soil ecoregions in Latin America and their carbon sequestration characteristics. The largest Part II with its 14 contributions deals with soil carbon sequestration in the individual biomes in Latin America. They are devoted either to individual states (Argentina, Mexico, Costa Rica, Colombia, Venezuela) or geographic and ecological biomes (Amazonian tropical rainforests, tropical Andean hillsides, pampas, *etc*.). Part III offers an overview of methods suitable for the assessment of the carbon soil pools. The last Part IV contains one contribution summarising recommendations for future research and development. A detailed index containing also references to tables and figures terminates the book.

I very much appreciate that this book offers valuable and up-to-date evaluation of the soil carbon sequestration from both the general point of view and special features of the appropriate regions.

The term Latin America embraces individual countries from North America (Mexico), Central America (7 countries), Caribbean (20 countries) and South America (13 countries). Anybody interested in carbon fluxes between the soils and atmosphere in any of the mentioned countries should consult this book. However, I would strongly recommend it also to those scholars and researchers who are interested in general aspects of carbon sequestration in the plant biomass and soil, carbon fluxes, crop management, land-use changes, and analytical methods of carbon assessment in the soil. They will find much valuable information and important stimuli in this book.

The editors are well aware of the fact that no considerable progress in increasing the soil carbon sequestration could be achieved if no support is obtained from the governmental bodies and policy makers. Hence, the last chapter also contains summaries on "How governments can encourage soil carbon sequestration" and "Communication among scientists, policymakers, and land managers". No doubts, their recommendations are important by far not only for Latin America.

Briefly, this book represents a valuable contribution in summarizing recent knowledge on soil carbon sequestration. Because of its detailed analyses of Latin America as well as much information of general importance, I recommend it to all those interested or engaged in research or teaching of global carbon cycle, soil carbon sequestration, and crop management.

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