Genetics and Molecular Research

Agrobacterium-mediated transformation of the *β-subunit* gene in 7S globulin protein in soybean using RNAi technology

J. Qu[1], S.Y. Liu[2], P.W. Wang[1], S.Y. Guan[2], Y.G. Fan[3], D. Yao[2], L. Zhang[4] and J.L. Dai[4]

[1]College of Agronomy, Jilin Agricultural University, Changchun, China
[2]College of Life Sciences, Jilin Agricultural University, Changchun, China
[3]Changchun Boao Bio-Chemical Pharmaceutical Co. Ltd., Changchun, China
[4]Biotechnology Center of Jilin Agricultural University, Changchun, China

Corresponding author: P.W. Wang
E-mail: peiwuw@163.com

Genet. Mol. Res. 15 (2): gmr.15027561
Received September 1, 2015
Accepted November 19, 2015
Published April 26, 2016
DOI http://dx.doi.org/10.4238/gmr.15027561

ABSTRACT. The objective of this study was to use RNA interference (RNAi) to improve protein quality and decrease anti-nutritional effects in soybean. *Agrobacterium tumefaciens*-mediated transformation was conducted using RNAi and an expression vector containing the 7S globulin *β-subunit* gene. The *BAR* gene was used as the selective marker and cotyledonary nodes of soybean genotype Jinong 27 were chosen as explant material. Regenerated plants were detected by molecular biology techniques. Transformation of the *β-subunit* gene in the 7S protein was detected by PCR, Southern blot, and q-PCR. Positive plants (10 T_0, and 6 T_1, and 13 T_2) were tested by PCR. Hybridization bands were detected by Southern blot analysis in two of the T_1 transgenic plants. RNAi expression vectors containing the soybean 7S protein *β-subunit* gene were successfully integrated into the genome of transgenic plants. qRT-PCR analysis in soybean seeds showed a clear decrease in expression of the soybean *β-subunit* gene. The level of 7S

protein *β-subunit* expression in transgenic plants decreased by 77.5% as compared to that of the wild-type plants. This study has established a basis for the application of RNAi to improve the anti-nutritional effects of soybean.

Key words: β-subunit gene; RNAi expression vector; Soybean; *Agrobacterium* mediated

INTRODUCTION

Soybean is an important source of protein and oil in human diets, and plays a significant role in human and animal nutrition. In China, the annual consumption of food grade soybean is over 8 million tons (Wang, 2010). In addition, the consumption of soybean oil is over 9 million tons, accounting for over 40% of the total amount of vegetable oil (Wang, 2009). Soybean antigens are large molecular proteins and glycoproteins that can cause hypersensitive reactions in both humans and animals and are responsible for 1/8 major food allergies (Taylor, 1992). Following Duke's discovery that soybean protein may cause diarrhea, collapse, and intestinal inflammation in infants in 1934, a large number of studies have been carried out worldwide investigating soybean antigens. Because they have high heat stability, antigens in soybean cannot be effectively inactivated by traditional thermo process (Li et al., 1991; Friesen et al., 1993). Therefore, the existence of allergens in soybean has greatly limited the application of soybean and related products.

Glycinin, which accounts for 10-20% of the total soybean protein, and β-conglycinin, which accounts for 1-2%, are the two allergens in soybean that can cause hypersensitive reactions (Krishnan et al., 2009). Although the amount of β-conglycinin in soybean is much lower than the amount of glycinin, it has an important role in allergic reactions (Zheng et al., 2009). β-conglycinin is a major component of 7S globulin in soybean, and is a trimer with a molecular weight of 150 kDa (Hill and Breidenbach, 1974) and is composed of three subunits: α' (76 kDa), α (72 kDa), and β (52-54 kDa) (Fontes et al., 1984). It shows high similarity in the domain area of each domain, being 90.4% between subunits α and α', 76.2% between α and β, and 75.5% between subunits α' and β (Maruyama et al., 1998).

To eliminate antigens from soybean, current strategies include thermal processing, enzyme treatment, the use of chemical agents, and traditional breading techniques. However, these methods often have limited effects. With the development of molecular biology and genetic engineering, RNA interference (RNAi) has become a widely used technology, and provides a new way of improving soybean quality.

The aim of the present study was to obtain *β-subunit* gene suppressed soybean products with no or low soybean allergens. To achieve this, RNAi technology was used; an expression vector containing the *β-subunit* interference gene was constructed and used to transform soybean cotyledonary nodes via *Agrobacterium*.

MATERIAL AND METHODS

Materials and reagents

Soybean seeds, Jinong 27, were obtained from the Biotechnology Centre of Jilin

Agriculture University. The vector pCAMBIA3301-PFNZ-*BAR* was used for RNAi expression of the *β subunit* and is shown in Figure 1. *Agrobacterium* (strain EHA101) was provided by the Biotechnology Centre of Jilin Agriculture University. Taq polymerase and dNTPs were purchased from MBI Corporation. The DIG High Prime DNA Labeling and Detection Starter Kit I was purchased from Roche. ETC811 gradient PCR machine was from Eastwin Corporation, Suzhou, China; and an MX3000 Real-time PCR machine was from Agilent Corporation.

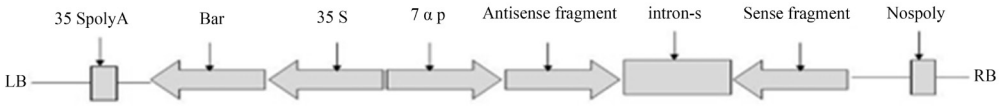

Figure 1. Schematic of pCAMBIA 3301-PFNZ expression vector.

Agrobacterium-mediated soybean cotyledonary node transformation

Disease-free, non-damaged soybean seeds were selected for use in *Agrobacterium*-mediated soybean cotyledonary node transformation (Zhu et al., 2006; Zhu et al., 2010). These seeds were sterilized by overnight exposure to chlorine gas. Sterilized seeds were placed in seed germinating medium. After a 3-day incubation period, three-to-five scratches were made in the cotyledonary node region of each plant using a blade. Next, the wounded plants were soaked in *Agrobacterium* medium for 20 min. Extra medium was passed through sterilized filter paper before the plants were cultivated in co-cultivation medium and incubated in the light for 3 days. Induction medium containing 1 mg/L PPT was used to induce adventitious shoot growth. The shoot induction medium was refreshed after two-week cultivation, followed by another two-week selection. When the shoots reached 2-3 cm in length, shoot elongation medium was used. Elongated shoots were transferred to rooting medium. With the appearance of 3-5 thick roots, the plants were transferred to a greenhouse for further 3-5 days to allow environmental adaptation. Finally, the surviving plants were cultivated in a greenhouse. Composition of the media used in these experiments is listed in Table 1.

Table 1. Composition of media used during *Agrobacterium*-mediated soybean cotyledonary node transformation.

Medium	Composition
Seed germination	MSB + sucrose 20 g/L + agar 6.0 g/L, pH 5.8
Pre-differentiation	MSB + sucrose 30 g/L + agar 6.0 g/L + MES 0.59 g/L + 6-BA2 mg/L + IBA 0.2 mg/L, pH 5.8
Co-cultivation	MSB + sucrose 30 g/L + agar 6.0 g/L + MES 0.59 g/L + 6-BA2 mg/L + IBA 0.2 mg/L + AS 100 μM, pH 5.6
Selection	MSB + sucrose 30 g/L + agar 6.0 g/L + MES 0.59 g/L + 6-BA 2 mg/L + IBA 0.2 mg/L + cef 250 mg/L+ Carb 100 mg/L, pH 5.8
Elongation	MSB + sucrose 30 g/L + agar 6.0 g/L + MES 0.59 g/L + GA_3 0.58 mg/L + IBA 0.2 mg/L + cef 250 mg/L+ Carb 100 mg/L, pH 5.8
Rooting medium	MSB + sucrose 30 g/L + agar 6.0 g/L + MES 0.59 g/L + IBA 2 mg/L, pH 5.8

MSB is MS macroelements + MS microelements + iron salts + organic B_5.

Detection of transgenic plants by molecular biology

Transgenic plants were identified using PCR. The CTAB method was used to extract genomic DNA from transgenic and untransformed plants. The RNAi vector pCAMBIA3301-PFNZ-*BAR* was preserved in our lab. Primers designed for the *BAR* gene in the vector were as follows: P1: 5'-TCAAATCTCGGTGACGGG-3'; P2: 5'- ATGAGCCCAGAACGACGC-3'. PCR was conducted using genomic DNA extracted from leaves as a template. The plasmid

pCAMBIA3301-PFNZ-*BAR* gene was used as a positive control, and the same type of gene from untransformed plants was used as a negative control, and sterilized water was used as blank. Each 25-μL PCR mixture contained 2.5 μL 10X PCR buffer (Mg free), 2.5 μL $MgCl_2$ (25 mM), 0.8 μL dNTP mixture (50 mM), 0.5 μL P1 (25 pM), 0.5 μL P2 (25 pM), 0.2 μL template DNA l μL, Taq (5 U/μL), 17.0 μL ddH_2O. The reaction program consisted of 5 min at 94°C for pre-denaturation, 30 cycles of 94°C for 40 s for denaturation, 58°C for 40 s for annealing, 72°C for 40 s for elongation, and elongation at 72°C for 10 min.

Southern blot analysis

Genomic DNA was extracted from leaves of transgenic plants (tested positive by PCR) using the CTAB method. Purified *BAR* gene was used as a probe template. A sample of 10-15 μg genomic DNA from positive plants was digested overnight using *Bam*HI, after which Southern blot analysis was conducted using a Roche DIG High Prime DNA Labeling and Detection Starter Kit I.

Detection of transgenic plants by RT-RCR

The immature seeds were collected 35 days after the podding stage. Total RNA was obtained using a total RNA Extraction kit. After reverse transcription, the cDNA was used as a template for RT-PCR. The primers designed were as follows: *β-subunit* primers were P3: 5'-CTCTTCTTCTACCACACTTCA-3'; P4: 5'-CTGCTTCTGTTGTTGTTCTT-3'; primers for the reference gene, *Tub*, were P5: 5'-GGCGTCCACATTCATTGGA-3'; P6: 5'-CCGGTGTACCAATGCAAGAA-3'.

This experiment was performed following the instructions provided with the SYBR Premix Ex Taq™ kit, which adopted a two-step method to amplify the *β-subunit* and *Tub* genes. The PCR program was as follows: 94°C for 10 min for pre-denaturation, followed by 40 cycles of 94°C for 10 s for denaturation, 60°C for 40 s for annealing. The $2^{-\Delta\Delta Ct}$ method was used to calculate the expression of the *β-subunit* gene relative to that of the *Tub* gene.

RESULTS

Transgenic plants with RNAi *β-subunit* gene of 7S globulin were obtained by *Agrobacterium*-mediated transformation

The RNAi expression vector containing the *β-subunit* was transformed into Soybean (Jinong 27), and transgenic plants were selected by PPT. The scheme of study is shown in Figure 2. After germination, pre-cultivation, co-cultivation, selection, elongation, rooting, and transplantation, it took ~90 days to produce regrowth shoots, from which regrowth seeds could be harvested by regular care.

Molecular detection of transgenic plants

Identification of T_0 transgenic plants by PCR

Genomic DNA extracted from young leaves of T_0 transgenic plants was used as a

template; the specific primers P1 and P2 were used for PCR detection. A plasmid containing the gene *BAR* was used as a positive control, untransformed plants were used as a negative control, and sterilized water was used as a blank. PCR products were tested on 1% agarose gel electrophoresis and 10 T_0 transgenic plants were found to contain the 441-bp target fragment, found in the positive control. On the other hand, untransformed plants did not express this specific fragment. This indicates that the gene encoding the β-subunit of 7S globulin was successfully transformed into the soybean genome by RNAi (Figure 3).

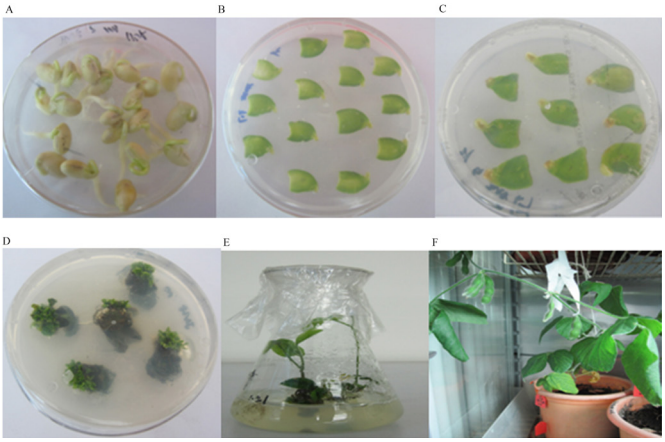

Figure 2. *Agrobacterium*-mediated soybean cotyledonary node transformation system. **A.** Germination. **B.** Pre-cultivation. **C.** Co-cultivation. **D.** Selection. **E.** Elongation. **F.** Transplantation.

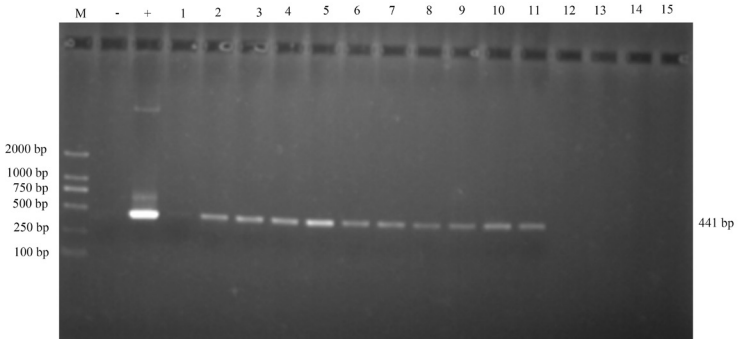

Figure 3. Identification of T_0 transgenic plants by PCR. *Lane M* = DNA marker DL2000; (-) = negative control; (+) = positive control; *lane 1* = blank; *lanes 2-11* = transgenic plants; *lanes 12-15* = untransformed plants.

Identification of T_1 transgenic plants by PCR

Overall, 21 soybean seeds were harvested from positive T_1 transgenic plants. These seeds were sown in a greenhouse and tended with regular care. Genomic DNA was extracted from the first trifoliolate leaves of the T_1 transgenic plants, and PCR amplification was conducted using the plasmid gene *BAR* as a positive control and untransformed plants as

a negative control. PCR products were tested on 1% agarose gel. As a result, 6 of 21 T_1 transgenic plants tested positive (expressed a specific fragment of 441 bp) (Figure 4).

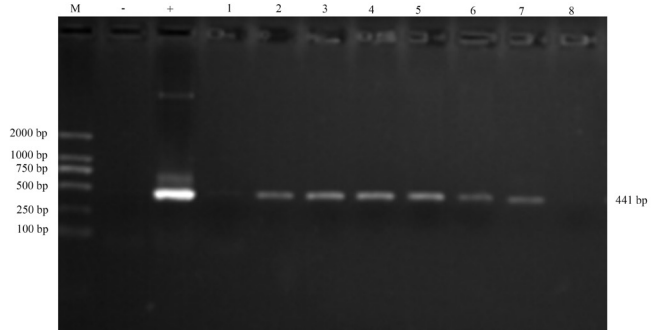

Figure 4. Identification of T_1 transgenic plants by PCR. *Lane M* = DNA marker DL2000; (-) negative control; (+) positive control. *Lane 1* = blank; *lanes 2-7* = transgenic plants; *lane 8* = untransformed plants.

Southern blot analysis of T_1 transgenic plants

The six positive T_1 transgenic plants were used for Southern blot hybridization. The restriction enzyme *Bam*HI was used to digest DNA, which was then loaded on agarose gel and transferred to a membrane. The digoxin-labeled gene *BAR* in the RNAi expression vector of the β-subunit was used as the probe; the plasmid containing the gene *BAR* was used as a positive control while untransformed plants were used as a negative control. The results are shown in Figure 5. Two of the positive transgenic plants showed a signal-specific hybridization signal, while untransformed plants showed no signal, implying that the expression vector was integrated into the genomic DNA of soybean. In conclusion, two transgenic plants with a single copy of a transgene were collected.

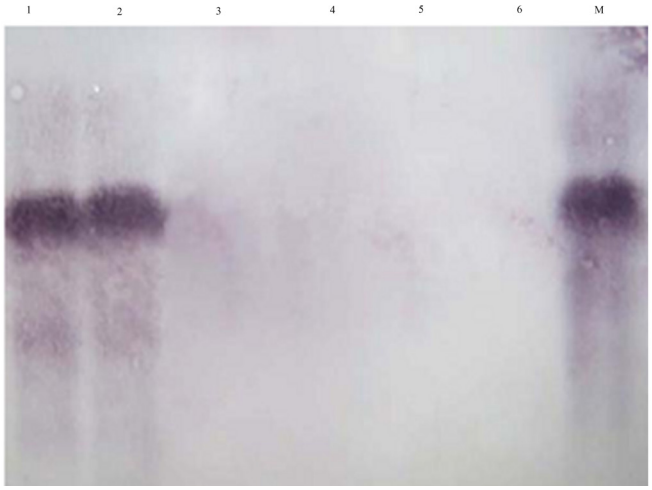

Figure 5. Southern blot analysis of T_1 transgenic plants. *Lane M* = positive control; *lanes 1-2* = transgenic plants; *lanes 3-6* = untransformed plant.

Determination of the expression level of β subunit in T_1 transgenic plants by q-PCR

Seeds from positive transgenic plants (tested by PCR and Southern blot) were selected for use in RT-PCR analysis. Immature seeds were harvested after the 35-day podding stage. Total RNA was isolated and reverse transcribed into cDNA. SYBR Green I was used as the fluorescent dye in the q-PCR. The level of *β-subunit* mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method, taking the expression in untransformed plants of Jinong 27 as 1. Figure 6 shows that the expression of β-subunit mRNA in No. 1-6 transgenic plants was significantly reduced. Moreover, the level of expression varied in each transgenic plant. The inference rate of transgenic plants from Nos. 1-6 was 33.0, 77.5, 27.0, 62.8, 55.7, and 52.5%, respectively. No. 2 transgenic plant showed the highest inference rate (Figure 6).

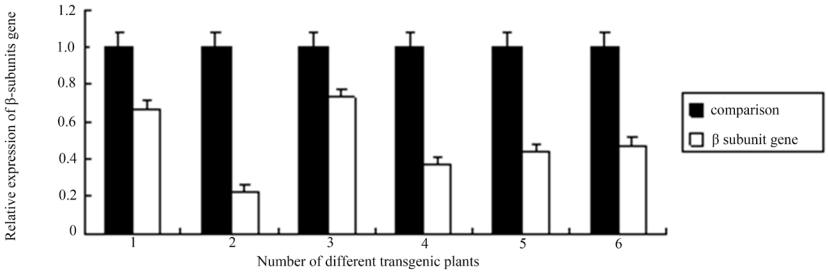

Figure 6. Fluorescent quantitative analysis of transgenic soybeans by q-PCR. *Columns 2-6* = transgenic plants; *column 1* = untransformed plant. In columns 1-6, black columns indicate untransformed plants and white columns indicate the transgenic plants.

Identification of T_2 transgenic plants by PCR

A total of 49 T_1 positive soybean seeds were collected. The seeds were sown in the field during spring and tended with regular care. When trifoliolate leaves appeared, genomic DNA was isolated from these leaves. The plasmid containing the gene *BAR* was used as the positive control and untransformed plants were used as a negative control. After PCR, the amplified products were subjected to 1% agarose gel electrophoresis and the results revealed 13 *BAR* gene-positive transgenic soybeans that carried specific fragments of 441 bp in size, which were identical to the size of the target fragment (Figure 7).

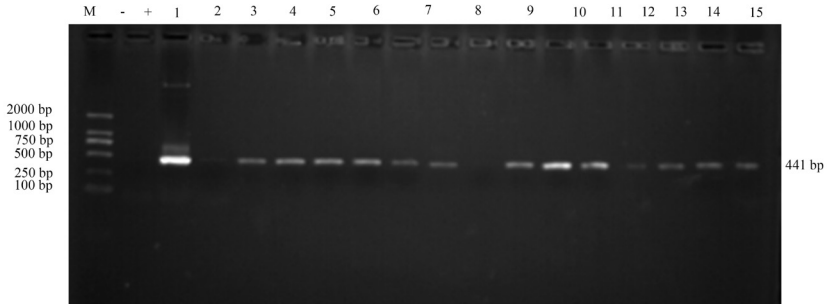

Figure 7. Identification of T2 transgenic plants by PCR. *Lane M* = DNA Marker DL2000; (-) = negative control; (+) positive control. *Lane 1* = blank; *lanes 2-7*, *lanes 9-15* = transgenic plants; *lane 8* = untransformed plants.

DISCUSSION

RNAi is a biological process in which endogenous or exogenous double stranded RNA interacts with specific cellular mRNAs, resulting in mRNA degradation and therefore, silenced expression of target genes and subsequent phenotypic changes. It is an ancient and highly conserved mechanism of gene regulation. Although many reports have shown that RNAi-based genetic engineering can improve the quality of crops such as wheat, rice, and corn (Kusaba et al., 2003; Li et al., 2005; Bai et al., 2010; Guan et al., 2011), few researchers have reported the use of RNAi to improve soybean quality.

In the current study, *Agrobacterium*-mediated transformation of soybean cotyledonary nodes using an RNAi expression vector containing the *β-subunit* gene of 7S globulin (Jinong, 27) was performed, and PCR and Southern blot analysis was used to confirm the successful integration of the vector into the soybean genome. Real-time PCR was performed to confirm that the β-subunit of 7S globulin was expressed at lower levels in transgenic plants than in control plants.

In this study, the transformation of soybean plants using an RNAi expression vector containing the β subunit in 7S globulin was successful. In total, 10 T_0 positive transgenic plants, 6 T_1 positive transgenic plants, and 13 T_2 positive transgenic plants, were obtained. The expression interference level of β-subunit in 7S varied in each transgenic plant and the highest interference level reached 77.5%. Therefore, a new strain of soybean containing low levels of allergen was obtained, which contained the RNAi expression vector. Therefore, we may conclude that this study has laid a foundation for the cultivation of new strains of soybean that lack the antigen in 7S globulin. Further qualitative and quantitative research on the β-subunit protein will be carried out in the near future.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the Country Cultivate New Varieties of GMO Major Projects (#2014ZX08004-004) and the National Natural Science Foundation of China (#31171568).

REFERENCES

Bai YQ, Kang J, Sun Y, Yang QC, et al. (2010). Construction of ihpRNA expression vector of MsLEA3-1 from *Medicago sativa* L. and genetic transformation in tobacco. *Acta Agron. Sin.* 36: 1484-1489. http://dx.doi.org/10.1016/S1875-2780(09)60073-0

Fontes EPB, Moreira MA, Davies CS and Nielsen NC (1984). Urea-elicited changes in relative electrophoretic mobility of certain glycinin and beta-conglycinin subunits. *Plant Physiol.* 76: 840-842. http://dx.doi.org/10.1104/pp.76.3.840

Friesen KG, Goodband RD, Nelssen JL, Blecha F, et al. (1993). The effect of pre- and postweaning exposure to soybean meal on growth performance and on the immune response in the early-weaned pig. *J. Anim. Sci.* 71: 2089-2098.

Guan SY, Zhao LN, Liu HJ, Liu GN, et al. (2011). Study on the Agrobacterium mediated genetic transformation of maize sbe2a gene by RNAi. *Crops* 2: 36-40.

Hill JE and Breidenbach RW (1974). Proteins of soybean seeds II accumulation of the major protein components during seed development and maturation. *Plant Physiol.* 53: 747-751. http://dx.doi.org/10.1104/pp.53.5.747

Krishnan HB, Kim WS, Jang S and Kerley MS (2009). All three subunits of soybean β-conglycinin are potential food allergens. *J. Agric. Food Chem.* 57: 938-943. http://dx.doi.org/10.1021/jf802451g

Kusaba M, Miyahara K, Iida S, Fukuoka H, et al. (2003). Low glutelin content1: a dominant mutation that suppresses the glutelin multigene family via RNA silencing in rice. *Plant Cell* 15: 1455-1467. http://dx.doi.org/10.1105/tpc.011452

Li DF, Nelssen JL, Reddy PG, Blecha F, et al. (1991). Measuring suitability of soybean products for early-weaned pigs with immunological criteria. *J. Anim. Sci.* 69: 3299-3307.

Li JR, Zhao W, Li QZ, Ye XG, et al. (2005). RNA silencing of Waxy gene results in low levels of amylose in the seeds of transgenic wheat (Triticum aestivum L.). *Yi Chuan Xue Bao* 32: 846-854.

Maruyama N, Katsube T, Wada Y, Oh MH, et al. (1998). The roles of the N-linked glycans and extension regions of soybean beta-conglycinin in folding, assembly and structural features. *Eur. J. Biochem.* 258: 854-862. http://dx.doi.org/10.1046/j.1432-1327.1998.2580854.x

Taylor SL (1992). Chemistry and detection of food allergens. *Food Technol.* 46: 146-1531.

Wang EH (2010). Current situation and Prospect of soybean consumption in China. *Agric. Outlook* 6: 33-36.

Wang YG (2009). The present situation and prospect of the supply and demand, trade and policy of Chinese oil. *Grain Sci. Technol. Econ.* 34: 11-13.

Zheng SG, Cao SY, Sun ZW, et al. (2009). Purification and characterization of the β-subunit of β-conglycinin. *Soybean Sci.* 28: 301-304.

Zhu C, Wu J and He C (2010). Induction of chromosomal inversion by integration of T-DNA in the rice genome. *J. Genet. Genomics* 37: 189-196. http://dx.doi.org/10.1016/S1673-8527(09)60037-0

Zhu QH, Ramm K, Eamens AL, Dennis ES, et al. (2006). Transgene structures suggest that multiple mechanisms are involved in T-DNA integration in plants. *Plant Sci.* 171: 308-322. http://dx.doi.org/10.1016/j.plantsci.2006.03.019

