Stability of PAT Protein Expression of Multiple Generations of Genetically Modified Soybean Developed in Korea

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***Abstract*—Genetically modified (GM) soybeans (*Glycine max* (L.) Merrill) expressing the γ-tocopherol methyltransferase gene (γ-TMT) and the selectable marker phosphinothricin acetyltransferase (PAT) were developed by the Rural Development Administration in Korea. In this study, we used Southern blot analysis to examine the stability of inserted genes in GM soybeans, and enzyme-linked immunosorbent assay (ELISA) was conducted to analyze inherent PAT protein levels. PAT expression levels varied among different plant generations and plant organs isolated from GM soybean. PAT expression was the highest in the leaves of plants at the beginning of the pod stage (R3) (12.36 μg/g) and the lowest in pods collected from plants that were at the fully mature stage (R8) (1.43 μg/g). The PAT protein showed a decreasing pattern of expression during plant growth. As expected, the expression of PAT proteins was not detected in control soybean plants.**

***Index Terms*—genetically modified soybean , γ-*TMT*, phosphinothricin acetyltransferase, ELISA**

I. Introduction

The development of genetically modified (GM) crops has continuously increased in recent years, expanding to approximately 170 million hectares in 28 countries by 2012. According to the International Service for the Acquisition of Agri-biotech Applications (ISAAA), the most widely grown GM soybean expresses the 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) gene, which confers glyphosate herbicide tolerance [1]. The herbicide-tolerant soybean accounts for 81% of the entire soybean cultivation area worldwide, and this is based on the financial benefits of cultivating this plant, mainly curtailing the labor costs for weed management and use of agricultural machinery. Moreover, the environment-related advantages of no-till farming such as soil conservation and fossil fuel energy savings serve as countermeasures against climate change. Nevertheless, the dispute over the use of GM soybeans continues, especially regarding the possibility of creating of herbicide-resistant super weed through the transfer of inserted genes, as well as its impact on ecosystems. No GM crops are currently cultivated in Korea, but a number of GM crops are being developed through investment expansions in the biotechnology, as well as through focused research and development (R&D) activities.

The use of biotechnology to enhance the productivity and nutritional quality of crops has been investigated around the world. In Korea, the Rural Development Administration (RDA) developed a GM soybean that expresses a γ-tocopherol methyltransferase gene (γ-TMT) and the phosphinothricin acetyltransferase (PAT) gene, which serves as a selectable marker. Soybean oil is also a natural source of vitamin E, which includes a group of structurally related compounds, namely α-, β-, γ-, and δ-tocopherol. Each form of vitamin E has a slightly different biological activity. α-tocopherol is considered the most important form of vitamin E for human health, harboring a 10-fold higher antioxidant activity than that by other tocopherols [2]. The γ-TMT gene from Perilla frutescens converts γ-tocopherol to α-tocopherol, which improves the nutritional value of the seeds by increasing its α-tocopherol content and enhancing the vitamin E activity. The PAT gene has been widely used as a dominant selectable marker gene in plant transformation and as a glufosinate herbicide tolerance gene for weed control in agronomically important crops such as canola, cotton, maize, and soybean. To cultivate GM crops, environmental and food risk assessments must be conducted pursuant to the laws and regulations governing LMOs, and the safety evaluation data must be submitted. Protein expression data is essential for assessing and monitoring the biosafety of GM crops in Korea [3].

In the present study, multiple generations of GM soybean plants were used for the quantitative analysis of PAT expression across plant organs and during plant development. Several immunoassay methods are currently available for the detection and quantification of proteins expressed by most commercially produced GM crops. Among these, the enzyme-linked immunosorbent assay (ELISA) has emerged as the most widely used technique for the assessment of food authenticity, primarily because of its specificity, simplicity, and sensitivity [4]. This study involved the quantitative ELISA analysis of PAT protein expression and Southern blot hybridization of the inserted genes in GM soybeans grown in an isolated field. Analyses were conducted to study the effects of plant generation, growth stage, and

Manscript received May 4, 2013; revised July 4, 2013.

doi: 10.12720/jomb.2.3.182-185

organ-type on PAT concentrations. The objectives of this study were to assess the stability of exogenous protein expression across various generations and to estimate the variability in protein expression among different organs during plant growth.

II. Material and Methods

A. Collection of Organs from Field-Grown Plants

GM soybean (cv. Jack) was generated using *Agrobacterium tumefaciens*-mediated transformation [2]. Both GM soybean and its parent cultivar were obtained from the National Academy of Agricultural Science (NAAS) of RDA in Korea. The plants were grown from the seeds in a NAAS greenhouse for 3 weeks before transplanting the GM soybean (T3, T4, and T5) and its parent cultivar in isolated fields approved for the growth of GM crops in Suwon, Korea. The GM soybean and its parent cultivar (Jack) were grown in adjacent fields under the same environmental conditions and standard field management. Soybean plants were cultivated in accordance with common local agricultural practices. Leaves, stems, roots, pods, and seeds were collected from the GM soybean and parent cultivar plants at 2 stages of development. The samples were collected between the beginning pod (R3) and full maturity (R8) stages after sowing. Considering that the major edible portion of the soybean plant is the seed, they were collected at the R8 stage and used for analysis of the protein content. The plant tissues were transported to the laboratory in a closed box and were then quickly frozen in liquid nitrogen. The samples were then sealed in airtight plastic bags (10 × 10 cm) and stored at −80°C until analysis.

To determine protein expression levels from multiple generations, leaf tissues were collected from the GM soybean (T3, T4, and T5) and its parent cultivar plants at the R3 stage. Fresh leaves were collected from each plant and pooled to form a combined leaf sample for subsequent analysis. Stems were separated into main stems and branches. To remove soil particles, the roots were gently washed in running water and then blotted on tissue paper. Pods and seeds were collected directly from the field, air-dried overnight, and stored at –80 ℃. After weighing the samples, the individual parts were lyophilized in a freeze drier (Ilshin Lab Co., Ltd., Yangju, Korea) for 48 h, cut into small pieces (5 mm), mixed well, frozen in liquid nitrogen, and then reduced to a fine powder by using a mortar and pestle. Each powdered sample was stored at –80 ℃ until analysis by using ELISA.

B. Preparation of Tissue Extracts

To extract the PAT protein, 100 mg of each powdered lyophilized tissue sample was suspended in 1 mL of phosphate buffered saline containing Tween-20 (PBST buffer), which was supplied as part of the ELISA kit. The samples were incubated on ice for 10 min, vortexed briefly, and then again incubated on ice for 10 min. After centrifugation for 10 min at 12,000 ×g at 4 ℃, the supernatant was used for the quantification of PAT. All supernatants were transferred into the wells of a microtiter plate, and the concentration of PAT was determined using ELISA.

C. Quantification of PAT

Quantitative estimation of PAT proteins in GM and non-GM soybeans was conducted using commercially available ELISA 96-well plate kits (EnviroLogix LibertyLink pat/bar ELISA, USA). All ELISAs were performed according to the manufacturer's protocols [3]. All procedures related to the standard curves, dilution factors, positive and negative controls, and calculations were conducted as suggested in the kit protocol. All samples were incubated in the reaction wells for 2 h at room temperature, and sample absorbances were then measured at 450 nm by using an ELISA reader (Multiskan EX; Thermo Scientific). All samples were measured in triplicate. Protein quantification was conducted by plotting test sample absorbance values on standard curves generated from a purified PAT protein standard that was provided by the respective ELISA kit. The results were expressed as μg/g (PAT protein/tissue dry weight), taking into consideration the dilution factor. For all assessments, comparable GM soybean and its parent cultivar plants were grown and analyzed in parallel to identify any potential background effects of the plant matrix on the ELISA results.

D. DNA Isolation and Southern Blot Hybridization

Genomic DNA was isolated from fresh leaf tissues by using a DNeasy plant mini kit (Qiagen). Aliquots of DNA were digested using the restriction enzyme NcoI, and the digested DNA was separated on a 0.7% (w/v) agarose gel and blotted onto a Hybond N+ nylon membrane (Amersham Pharmacia), following the manufacturer's instructions. The probe bar gene was labeled using the RediPrimeII random prime labeling system (Amersham Pharmacia), and the hybrid DNA molecules were detected using a Molecular Imager FX (BioRad).

E. Statistical Analysis

The mean values and standard deviations (SD) of the triplicate samples were calculated using Microsoft Excel (Microsoft, USA). All statistical tests were conducted at a 0.05 significance level by using Fisher's least significant difference (LSD) method and SAS 9.1 software (SAS Institute, USA).

III. Results

A. Expression of PAT Proteins in Various Organs from Multiple Generations

Differences in protein expression over multiple generations of the GM soybean were evaluated using ELISA. Plant leaf tissues derived from 3 generations (T3, T4, and T5) grown in the field were collected at the R3 stage. Samples from all generations of the GM soybean expressed PAT proteins. As expected, PAT was not detected in the parental cultivar (Jack). As shown in Table I, the mean PAT levels from each generation were 10.96, 12.36, and 11.06 μg/g, respectively. The mean

PAT levels were significantly different among the plant organs. The results clearly show that PAT levels were the highest in the leaves (11.06 μg/g) and the lowest in the roots (1.83 μg/g). No significant differences among plant generations were observed. No significant inter-tissue differences in PAT protein levels across various plant generations were observed, suggesting that the genes inserted into the GM soybeans were stably maintained and expressed.

TABLE I. PAT PROTEIN LEVEL IN GM SOYBEAN ORGANS

Generations	Organs		
	Leaves	Stems	Roots
T3	10.96±0.41	9.73±0.66	2.30±0.10
T4	12.36±0.77	10.90±0.50	2.63±0.11
T5	11.06±0.20	9.43±0.35	1.83±0.05

B. *Expression of PAT Proteins in Seeds and Pods from Multiple Generations*

The expression patterns of PAT proteins were analyzed in seeds, which are the edible portion of soybean plants. The seeds were collected from 3 generations grown in the field. As expected, PAT expression were not detected in the Jack cultivar seeds and pods. However, PAT proteins were present in all generations of GM soybean seed and pod samples. The PAT protein level in the seeds remained stable regardless of the plant's growth stage, whereas that in the pods was reduced by more than half during the maturity stage of the plant. This reduction may be attributed to the aging process of the GM soybean. However, the PAT protein levels were maintained at a constant level in the seeds and pods of GM soybean across the 3 generations, similar to the results shown in the other plant tissues.

TABLE II. PAT PROTEIN LEVEL IN GM SOYBEAN SEEDS

Generations	Growth stages			
	Beginning pod (R3)		Full maturity (R8)	
	Seed	Pods	Seeds	Pods
T3	3.76±0.20	4.93±0.49	3.80±0.10	2.23±0.23
T4	3.70±0.17	4.90±0.51	3.70±0.17	1.43±0.11
T5	3.30±0.26	5.26±0.11	3.40±0.20	2.33±0.05

C. *Inheritance of the PAT Gene*

The stability maintenance of introduced genes is an important factor when assessing the health risks of GM crops. The protein expression of introduced genes was constant across plant generations; however, we sought additional verification whether the genes introduced into GM soybeans were actually being maintained. To study bar gene stability, we analyzed multiple generations of GM soybean and its parent cultivar by using the Southern blot hybridization method (Fig. 1). The results revealed identical banding patterns across multiple generations, indicating no loss or rearrangement of the genes during successive generations from T3 to T5.

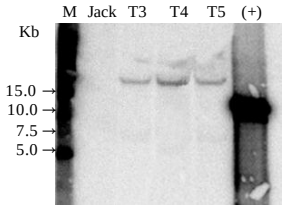

Figure 1. Southern blot analysis of GM soybean

IV. DISSCUSSIONS

Unlike other countries where GM soybeans are mainly used as animal feed, in Korea, soybeans will most probably be used for human consumption. The Korean consumers' low acceptance level is expected to raise concerns about GM soybeans. To overcome continuing controversies around the issue, it is crucial to conduct detailed scientific analyses of the various factors related to the effects of GM crops on the environment. Before commercialization of GM crops, the safety of GM crops on human health and environment must be assessed. The increasing use of GM crops has led to a greater number of animal and human consumables, resulting in a huge demand for appropriate risk assessment regarding the safety of GM crops [5]. Under the regulation issued by the Korea Food and Drug Administration (KFDA), data related to the characterization of the genetic modification should be provided for any substances expressed in GM crops, including the total levels of expression of gene products and tissue-specific patterns of expression [6]. The safety assessment information should also show that the genetic modification has achieved its intended effects and that all expressed traits are expressed and inherited in a stable manner across multiple generations of GM crops.

The results of our Southern blot analysis showed that the introduced genes in GM soybeans are stably maintained across multiple generations. Our results also confirmed that the levels of PAT expression were significantly different among the developmental stages and plant tissue types. For instance, the level of PAT expression in soybean plants slightly decreased as the plants reached maturity. Although the mechanism behind this decline in protein expression is not clear, it might be related to late-season plant phenology that was specific to the region [7]. Analysis of the expression levels of transgenic products is a common safety assessment feature for GM crops. It facilitates estimation of the consumers' level of exposure to transgenic proteins. Therefore, food safety assessments are generally conducted to assess the potential human and animal risks of consuming GM crops based on the exposure to the PAT proteins. Our results were combined with the food balance sheet from the Korea Rural Economic Institute to determine the amount of soybean-derived food consumed by Koreans that could potentially contain these proteins from GM soybean [8].

According to the 2010 Food Balance Sheet, the average daily soybean consumption per person, per day is 23.12 g, and the average daily total protein intake is 97.5 g. As a result, the estimated daily intake of PAT would be

approximately 84 μg, making the daily intake of this protein only 0.000086% of the daily intake of total proteins (97.5 g). The protein content of GM soybeans was confirmed as similar to that shown in other studies, such as that in GM rice [9]. This observation suggests that the consumption of GM soybeans poses no risks to human health, specifically in terms of PAT exposure. The safety assessment of foods derived from GM plants involves methods for identifying and detecting unintended effects and procedures to evaluate their biological relevance and potential impact on food safety [3]. Therefore, our results provide meaningful information on GM plants exhibiting such unintended traits. Extensive data, including the levels and potential food toxins and allergens in GM soybean [10], are required for a comprehensive safety assessment of this crop.

ACKNOWLEDGMENT

This work was supported by grants from the National Academy of Agricultural Science (Code PJ009609022013), Rural Development Administration, Republic of Korea.

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